Investigating the effect of *Staphylococcus epidermidis* on the growth dynamics of *Staphylococcus aureus* in atopic dermatitis

Rasalika Tamika Moonsamy (MNSRAS001)

Department of Molecular & Cell Biology

Supervisor: Dr Felix Dube

Co-supervisors: Dr Gillian Ndhlovu and Dr Siobhan Brigg

Thesis presented for:

Master of Science in Molecular and Cell biology

Faculty of Science

University of Cape Town

Plagiarism Declaration

I, Rasalika Tamika Moonsamy, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I authorise the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signature: Signed by candidate Date: 16 January 2023

Personal Acknowledgements

I would like to thank the participants included in this study and provided specimens and data.

I would also like to express my gratitude to the University of Cape Town for providing financial assistance throughout this degree.

To my lab- a massive thank you to my PI, Dr Felix Dube, and my supervisors, Dr Siobhan Brigg, and Gillian Ndhlovu. This degree would simply not be possible without your guidance, mentorship, encouragement, and understanding. My lab mates, Thabo and Celine, thank you for the friendship we have fostered throughout the years. It is truly appreciated.

I would like thank God for bringing me through this degree. My parents, Mum, and Dad, thank you for your unwavering support. Your encouragement, prayers and love are truly appreciated. No amount of gratitude expressed will be enough. I am because you are.

My amazing family, thank you for all for your support, love, and encouragement throughout this journey. You are my pillars of strength that I have relied upon and continue to do so. You mean the world to me.

To my friends, Zo, Shenice, Ansuya, Zahra, Mlibo, Neha and Kyllen, thank you for being my home away from home and constants. I appreciate the help, encouragement, and venting sessions.

To my Akili family- thank you for being my 'happy place' during some of the hardest moments. You have shown me what teamwork and compassionate leadership looks like. I am so grateful to have been a part of your family.

A special word of appreciation to my uncle V- thank you for always being there when I needed you. Thank you for being one of my best friends. And thank you for helping me see my world differently.

To my best friend and all-rounder, Jav. You have been the most incredible support to me during this time. Thank you for all the calls, listening to me rant about failed experiments, the great advice about using a multichannel pipette, making sure I had my dose of junk food, and of course, the jokes. The love and compassion you have shown me is beyond what I ever imagined.

Lastly, and a bit unconventional, I would like to thank Bangtan Sonyeondan, or BTS, whose music and lyrics have helped me get through some hard days and the last push of this degree. Thank you for being a funny distraction, a great study partner, and a soulful 'hug' when things got a bit overwhelming. Borahae! Apobangpo!

Sincerely,
Rasalika Tamika Moonsamy

Abstract

Background: In atopic dermatitis (AD), studies have demonstrated inconsistencies in the interactions between *Staphylococcus epidermidis* and *Staphylococcus aureus* concerning growth and biofilm formation outcomes. This suggests that there might be a strain-level role played by the bacteria that affect the outcomes of their interactions, and consequently, the pathophysiology of AD. Furthermore, the factors that mediate these effects by *S. epidermidis,* are currently poorly characterised.

Objective: To evaluate the effect of skin and nasal *S. epidermidis* on the growth dynamics and biofilm formation of co-colonising *S. aureus* in toddlers with and without AD.

Methods: Twenty-eight (28) previously identified co-colonising pairs of *S. aureus* and *S. epidermidis* isolates were selected based on different combinations of biofilm-forming strengths. Filtered *S. epidermidis*-derived supernatant was incubated with *S. aureus* cultures to determine its effect on biofilm formation and growth. Biofilm formation was evaluated using crystal violets assays and Alamar Blue assays were used to determine cell viability. *S. aureus* biofilms were treated with Proteinase K (PK) or sodium metaperiodate (SM) to determine their composition. The expression of extracellular serine protease (*esp*) was determined by Real Time Polymerise Chain Reaction (RT-PCR). *S. epidermidis*-derived supernatants subjected to heat, PK and SM to characterise other potential effector molecules. Lastly, sequence types (ST) and clonal complexes (CC) of *S. epidermidis* and *S. aureus* isolates were inferred from multi-locus sequence typing profiling.

Results: Pairs of *S. aureus* and *S. epidermidis* from 75% (6/8) of controls and 65% (13/20) of cases demonstrated a decrease in biofilm formation compared with *S. aureus* alone. No significant differences in biofilm formation were noted between pairs isolated from cases and controls. From the viability assays, we observed that pairs of *S. epidermidis* and *S. aureus* from 25% of cases compared with 80% of pairs from controls had an increase in cell viability. The majority (70%) of established biofilms were composed of a proteinaceous matrix. Expression of *esp* was detected in 63% of *S. epidermidis* isolates. Heat-treated supernatant resulted in 50% of controls and 35% of case pairs displaying an increase in biofilm biomass. Incubation with SM-treated *S. epidermidis* supernatants resulted in 50% of isolates from control and case pairs showing decreases in biofilm

biomass. Pairs from control (88%) and case (65%) participants treated with PK showed decreases in biofilm formation. Only five *S. epidermidis* isolates matched to exact STs, while several partially matched to several possible STs. The clonal lineages of the *S. aureus* control isolates were made up of CC1, CC5, and CC121, whereas the case isolates also included CC15 and CC8.

Conclusions: The variation displayed in the biofilm formation results indicates *S. epidermidis* strain specificity plays a role in the outcome of interactions. Results from treated supernatant suggest the possibility of multiple effector molecules with different constituents. Lastly, *S. aureus* strains exhibit monoclonality whereas *S. epidermidis* strains present diverse strain compositions, which could be the basis for the differential outcomes observed. Future research should apply a holistic approach to microbiome studies to understand the different factors influencing the outcome of interactions leading to biofilm production.

Table of Contents

List of Tables

List of Figures

Abbreviations

AD	Atopic dermatitis
Agr	Accessory gene regulator
AIPs	Autoinducing peptide(s)
AMP	Antimicrobial peptide
BA	Blood agar (Sheep)
ATCC	American Type Culture Collection
CER	Ceramides
CC	Clonal complex
CoNS	Coagulase-negative *Staphylococcus*
DNase	Deoxyribonuclease
Esp	*Staphylococcus epidermidis*-derived serine protease
EPS	Extracellular polymeric substances
FFA	Free fatty acids
FLG	Filaggrin
hBD	Human β-defensin
ISAAC	International Study of Asthma and Allergies in Children
MHA	Mueller-Hinton agar
Min	Minute(s)
MLST	Multilocus sequencing typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
MSCRAMMS	Microbial surface components recognising adhesive matrix molecules
oSCORAD	Objective SCORing of Atopic Dermatitis
PK	Proteinase K
PIA	Polysaccharide intercellular adhesin

PSMs	Phenol-soluble modulins
QS	Quorum sensing
SC	Stratum corneum
S	Second(s)
SCORAD	Scoring of atopic dermatitis
Spa	*Staphylococcus aureus* protein A
ST	Sequence type
STGG	Skimmed milk-tryptone-glucose-glycerol
TEWL	Transepidermal water loss
Th2	T-helper type 2
TLR	Toll-like Receptor
TSB	Tryptic soy broth
µL	Microlitres
°C	Degrees Celsius

Chapter 1: Introduction

1.1. Global Epidemiology of Atopic Dermatitis

Atopic dermatitis (AD), also known as atopic eczema, is a chronic, inflammatory skin disease affecting both children (prevalence: 15–20%) and adults (prevalence: 1–3%) worldwide [1-3], with a reported incidence of 24.4 million cases in 2019 [4]. The best estimates for the prevalence of childhood AD, according to the International Study of Asthma and Allergies in Children (ISAAC), show an overall increase in AD prevalence globally between 1995 and 2002 [5, 6]. The prevalence of AD varies across different countries and geographical regions (Figure 1. The global age-standardised disability-adjusted life year AD in both sexes per 100,000 persons in 2019. Blue areas indicate low prevalence and red areas indicate high prevalence. Figure); however, limited data exists for Southern Africa [7, 8]. Between 2008 and 2012, disease trends indicated an increased prevalence in Africa (English-speaking countries, 12.7% to 14.0%; French-speaking countries, 8.6% to 12.3%), and the Asia–Pacific region (4.7% to 6.3%) [6, 9, 10]. Conversely, countries such as New Zealand (12.9% to 8.8%) and the United Kingdom (14.7% to 10.6%), in which prevalence of AD was previously high, showed a decrease or levelling off of AD cases [6]. In Africa, increases in the prevalence and incidence of AD have been observed in adolescents and children, as obtained from questionnaire data on thirteen- to fourteen-year-olds in Kenya and South Africa, and six- to seven-year-olds in Nigeria [11-13]. In South Africa, there was an increase in the lifetime prevalence of AD between the years 1995 and 2002 (9.6% to 16.7%) [12]. That being said, it should be highlighted that many studies investigating the global burden of AD may not accurately reflect the burden of disease. This could be due to a lack of prevalence data in certain areas, particularly in developing countries; or due to the risk misclassification of the disease when using questionnaires to gather data [8]. Hence, better surveillance systems and larger population-based studies are required in order to better understand the AD disease burden.

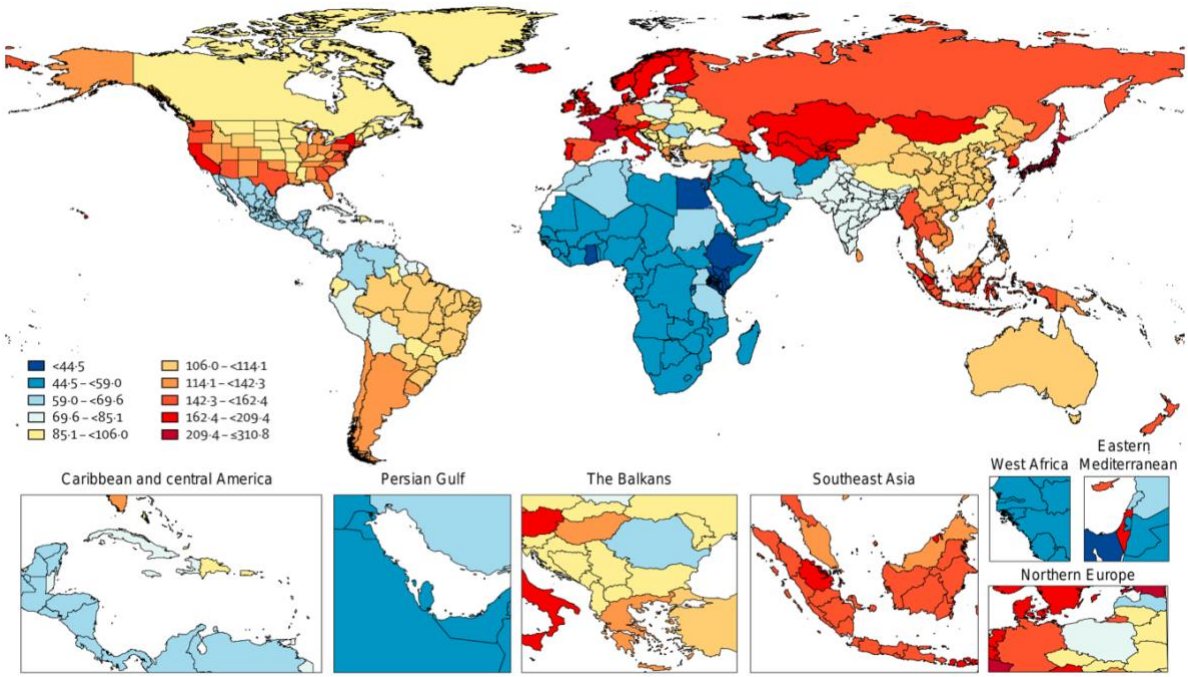


Figure 1. The global age-standardised disability-adjusted life year AD in both sexes per 100,000 persons in 2019. Blue areas indicate low prevalence and red areas indicate high prevalence. Figure sourced from the Global Health Metrics [4]

1.2. Atopic Dermatitis Pathogenesis and Pathophysiology

AD typically manifests in early childhood. It is viewed as the first step in the atopic march, the progression of atopic manifestation. This progression begins with AD in infancy and is followed by other allergic diseases, such as allergic rhinitis and asthma later in childhood [1]. AD is biphasic, displaying distinct acute and chronic stages [1]. The condition is marked by acute flare-ups of eczematic itchy lesions over dry skin [1]. These lesions are usually red and flat in the acute phase, and turn dull red, dry, and thick in the chronic phase [14]. Non-lesional skin in chronic AD patients was shown to also be distinct from healthy patients in terms of keratinocyte terminal differentiation and certain inflammatory pathways [15]. The scoring of atopic dermatitis (SCORAD) index is one of the best measures for the clinical signs of AD. It considers the intensity and extent of clinical signs of AD and the severity of the AD symptoms to give a consistent and interpretable composite score [16]. Other scoring indices, such as the Eczema Area and Severity Index (EASI), which integrates body surface and intensity of lesional skin into generating a composite score, has also been recommended for AD clinical trials, due to its interrater reliability and supported use for people of colour [17]. However, all scoring systems are inherently subjective to a degree and require adequate training and guides to ensure consistency and reliability. AD is multifactorial, and can result from genetic deficiencies in the host which are

related to skin barrier integrity, bacterial symbioses, cutaneous immune dysregulation – which results in a dominant T-helper type 2 (Th2) environment – and environmental influences [1].

1.2.1. Role of the Skin Barrier in AD

The outermost layer of the epidermal barrier, known as the stratum corneum (SC), functions to lessen the absorption of chemicals from the environment, limit passive water loss, , and prevent microbial infection [18]. The 'brick and mortar model' (Figure 2) suggests a lipid matrix (mortar) surrounds the corneocytes (bricks). Ensuring proper development and maintenance of the SC is vital for fulfilling its protective functions [19]. In AD, skin barrier defects can stem from multiple components to promote pathogenesis.

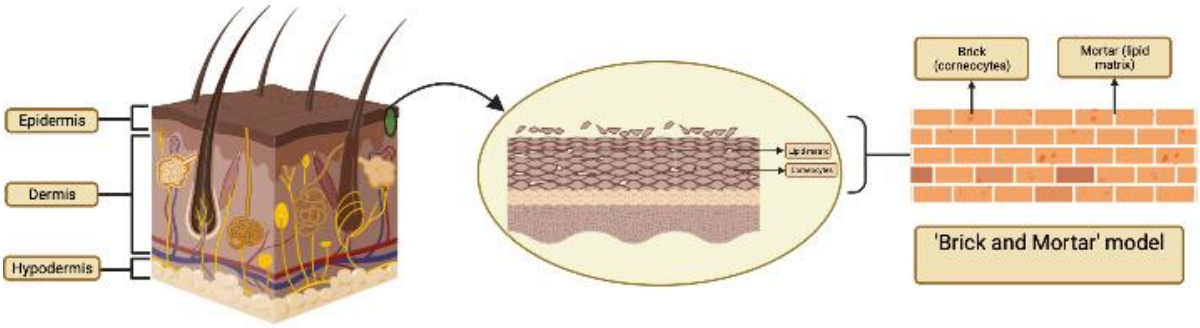


Figure 2. Brick and Mortar model of the stratum corneum of the skin epidermis

1.2.1.1. The Role of Skin Lipids in AD

In AD, the water retention and permeability function of the SC becomes defective. This has been associated with AD skin being more vulnerable to irritants that trigger inflammatory mediators in the epidermis, resulting in clinically visible atopic lesions [20]. AD lesional skin also has reduced levels of SC lipid subclasses, such as ceramides (CER) [21]. Furthermore, reduction in the chain lengths of free fatty acids (FFAs) and an increase in unsaturated FFAs are noted in the skin of both lesional and non-lesional AD patients, when compared to the skin of healthy participants [22]. This is thought to contribute to skin barrier impairment in AD [22]. Enzymes participating in the biosynthesis of CERs and FFAs showed altered expression in AD lesional skin, thereby playin a role in changes in lipid composition [23]. Taken together, these changes are associated with reduced lipid organisation in

the SC, leading to an impaired skin barrier, which in turn becomes more susceptible to bacterial infections [22].

1.2.1.2. Skin Mutations and AD

A hallmark of AD is a disrupted skin barrier. Mutations in the filaggrin gene (*FLG*), which encodes for structural proteins that aid in the formation of the SC [24], are commonly associated with AD. However, 40% of individuals who carry filaggrin mutations do not develop AD [25, 26]. A review indicated numerous studies have demonstrated an association between reduced filaggrin expression in AD skin and increased skin permeability [27]. Uncommon *FLG* loss-of-function variants were found in African American children, and were shown to be associated with more persistent AD [28]. Ethopian AD patients and healthy controls were found to not carry *FLG* mutations commonly found in European populations [29]. Instead, a novel mutation (2 bp deletion, 632del2) was found in the Ethopian populations [29]. In South Africa, a study indicated that previously known *FLG* loss-of-function mutations do not significantly contribute to AD in the AmaXhosa child population [30]. This was further evidenced by significantly lower filaggrin breakdown products on the SC of AD children who were wildtype for these *FLG* mutations [30]. Hence, given these differences, the contribution of *FLG* mutations in African populations show conflicting results and requires more investigation in the context of AD.

Alterations in the skin's natural pH in atopic dermatitis are due to diminished release of amino acids, lactic acid, and urocanic acid, as well as impaired fatty acids formation [31]. Filaggrin breakdown products and their derivatives make up a significant proportion of the SC. For example, pyrrolidone-5-carboxylic acid is an important natural moisturising factor of the skin which plays a role in water retention of the SC. Another example is urocanic acid, which plays a crucial role in maintaining the pH of the skin. Acidic skin plays a significant role in the maintenance of a healthy skin microbiome, by supporting the growth of resident microflora, encouraging their adherence [32] and creating a hostile environment for the colonisation of pathogenic bacteria [31]. Furthermore, a recent review has indicated repression of various genetic factors implicated in the virulence and pathogenesis of certain bacteria such as *S. aureus* by an acidic pH [33]. However, reduced expression of filaggrin leads to decreased production of urocanic acid, and pyrrolidone carboxylic acid. This increases the pH of the skin, thereby promoting colonisation of *S. aureus,* whose enterotoxin secretions can induce eczema [32, 34]. Studies have noted that increased colonisation of lesional skin by *S. aureus* is also

associated with decreased or no filaggrin expression, thereby creating an impaired skin barrier [35, 36]. However, other factors contributing to the microenvironment of skin, such as hydration and osmolarity, may also influence bacterial colonisation [37].

1.2.2. Role of Immune Dysregulation in AD

Immune dysfunction in AD is characterised by Th2 mediated immune responses. This results in higher serum IgE levels, which can trigger an allergic-autoimmune response, thereby perpetuating inflammation [25, 38]. Immune responses in AD develop in both acute and chronic AD [39]. During the acute phase of AD, Th2 cells and Th22-mediated immune responses are predominant, while in chronic AD, Th1-mediated responses are induced [40-42]. In lesional AD skin, other T-cell subpopulations (Th1, Th17 and Th22), eosinophils and inflammatory dendritic cells are increased, encouraging *S. aureus* colonisation [40].

1.3. Role of microbial Interactions in AD

Bacteria within an ecological niche can have positive (mutualism, synergism, or commensalism) or negative (predation, parasitism, competition, or antagonism) interactions [43]. In different environmental settings, these microbial interactions can lead to various outcomes to other microbes or the host. These interactions can be co-operative, whereby microorganisms exist by maintaining homeostasis with the host and other host-related microorganisms. Or, they can be competitive, where microorganisms adapt to the host and related microbe stressors [44]. The skin and nasal microbiomes are made up of different microorganisms that occupy and maintain a complex relationship with their niches [45]**.** Various studies have cited the contribution of skin microbes in host immune augmentation, colonisation resistance to pathogenic bacteria and skin disorders [46-48]. Furthermore, a recent study utilising a transcriptomics approach revealed that a diverse microbiome influences a distinctly altered expression of host human skin genes, when compared to that of any individual microorganism [49]. Most of these skin microorganisms act as commensals and function in the cutaneous immune function of the host [50] and homeostasis of the skin [51]. Although the gut microbiome has been extensively researched, the influence of the skin and nasal microbiomes on skin physiology and function has been limited.

1.4. Healthy Skin and Nasal Microbiome

In a healthy skin microbiome, the major bacterial phyla consist of Actinobacteria, Firmicutes, Proteobacteria and Bacteroidetes. The dominant genera include *Streptococcus* (Firmicutes), *Staphylococcus* (Firmicutes), *Propionibacterium* (Actinobacteria)*, Corynebacterium* (Actinobacteria) and *Lactobacillus* (Firmicutes) [52-54]. The genus *Staphylococcus* includes many common skin commensals, such as the coagulase-negative staphylococci (CoNS) species [55]. However, CoNS dominance is not as robust in the anterior nares of individuals compared to the skin [56, 57]. Instead, distinct microbial communities are found in the nose and skin of children [56], although similar phyla are found in both niches [52, 56]. The anterior nares are the preferred colonisation site for *S. aureus*, where nasal carriage of *S. aureus* is a major risk factor for *S. aureus* infections. Nasal *S. aureus* isolates often belong to a specific clonal complex, whereas other co-inhabiting staphylococcal species have multiple strain types [58].

1.4.1. Role of Staphylococcal Commensals

CoNS species play an essential role in the defence system of the skin. Antimicrobials produced by CoNS strains, from healthy skin and the nasal cavity, have been linked to inhibition of growth of *S. aureus* [59]. Many commensal CoNS regulate *S. aureus* infections by secreting various factors, such as proteases [60], antimicrobial peptides (AMPs), including lantibiotics, phenol-soluble modulins (PSMs) [61], and auto-inducing peptides (AIPs) during quorum sensing (QS) [62]. Furthermore, CoNS have been found to suppress the production of *S. aureus* virulence factors [59]. In addition, they play a role in amplifying the host immune system, thereby inhibiting the growth of pathogenic bacteria on the skin [46, 63]. *S. lugdunensis* produces a novel peptide antibiotic called lugdunin [64]. This thiazolidine-containing cyclic peptide can work synergistically with host-derived AMPs dermicidin-1(L) and LL-37 to eliminate methicillin-resistant *S. aureus* (MRSA), thus, enhancing the commensal-induced immune response [65]. Lantibiotics synthesised by *S. hominis* selectively suppresses *S. aureus* growth, without affecting the growth of commensal microbiota. Moreover, the activity of these lantibiotics against *S. aureus* was shown to be enhanced when used in conjunction with host endogenous AMPs [59].

QS is a form of bacterial intercellular signalling, modulated by increasing bacterial density. The QS systems are involved in the regulation of *S. aureus* by CoNS. AIPs derived from commensal CoNS

species exhibit strong inhibitory effects on the S. *aureus agr* QS activity. This leads to a decrease in *S. aureus* protease activity, thereby preventing damage to AD skin [62]. Despite the potent antimicrobial activity exhibited against *S. aureus* by commensal CoNS species, *via* the production of AMPs, AMP-producing CoNS strains are deficient in AD subjects, thereby underscoring the importance of AMPs in maintaining a normal skin microbiome [59].

S. epidermidis is the most dominant *Staphylococcus* species on healthy skin. It is commonly regarded as a skin commensal as it provides various protective roles for the skin [66, 67]. *S. epidermidis* has a significant function in the skin's defence system, particularly in the prevention of cutaneous bacterial infections, by secreting AMPs [60, 68], PSMs [69] and bacteriocins [70], which inhibit *S. aureus* colonisation (Figure 3). Furthermore, *S. epidermidis* has been shown to augment the host immune response by activating Toll-like Receptor 2 (TLR2), inducing human β-defensins, and thereby preventing *S. aureus* skin colonisation [48, 71]. *S. epidermidis* also enhances the production of AMPs, by sensitising host human keratinocytes towards *S. aureus*, aiding the host immune response towards clearing *S. aureus* infections [71]. In addition, *S. epidermidis* produces two PSMs, namely PSMδ and PSMγ, which exhibit similar antimicrobial properties to that of human AMPs [69]. Both PSMs selectively inhibit the growth of pathogenic *S. aureus* through the disruption of lipid membranes, without inhibiting *S. epidermidis* [72].

To determine the extracellular protease production in *S. epidermidis* isolates sourced from biofilms on endotracheal tubes, a study assessed the effect of supernatants of *S. epidermidis* isolates against *S. aureus* biofilms using crystal violet and viability staining, and confocal laser scanning microscopy [73]. Firstly, they noted lower protease production in *S. epidermidis'* supernatant isolated from biofilms, where *S. aureus* was also present, in the same patient, suggesting a connection between protease production and the absence of *S. aureus.* Further, *S. aureus* biofilms treated with the supernatant of protease-producing *S. epidermidis* showed significantly reduced biofilm mass, resulting in thinner biofilms, with little extracellular matrix [73]. The effect was suggested to be *S. aureus* strain-specific, due to variations in their biomass reductions. However, this was found to not kill bacterial cells within biofilms treated with *S. epidermidis* supernatant [73]. Biofilm cells grown in the presence of commensal *S. epidermidis* supernatant showed an increase in *icaR* expression, which is a negative regulator of the *ica* operon. This leads to decreased biofilm production, without affecting bacterial growth [74]. Biofilm production by *S. aureus* can be influenced by *S. epidermidis*-

derived serine protease (Esp) [60, 68, 73, 75]. As a result, pre-existing biofilms are degraded [68] and further *S. aureus* biofilm production is inhibited [60]. This inhibitory effect was also observed in biofilms formed by MRSA and vancomycin-intermediate *S. aureus* (VISA) [68], and was most likely achieved by the degradation of essential proteins, specific for biofilm formation and host interaction by Esp. This is supported by proteomic analyses, which showed that Esp degrades proteins such as extracellular adherence protein, extracellular matrix protein-binding protein, fibronectin-binding protein A and B, and host matrix receptors for *S. aureus* such as vitronectin fibronectin, and fibrinogen; which are associated with biofilm formation and colonisation (Figure 3) [60]. Additionally, Chen *et. al.* (2018) showed that Esp cleaves autolysin (Atl)-derived murein hydrolases and prevents the release of staphylococcal DNA, which is a component of the extracellular matrix [75]. Furthermore, the susceptibility of *S. aureus* to human β-defensin 2 (hBD2) [68] in biofilms was enhanced when Esp was tested in combination with hBD2. Although Esp has been shown to be a potent inhibitor of *S. aureus* biofilms, its expression is not ubiquitous. *S. epidermidis* strains are therefore divided into strains that inhibit biofilm formation and growth (inhibitory type), or strains that do not inhibit biofilm formation and growth (non-inhibitory type) [68, 76]. However, the mechanisms regulating this expression require further characterisation to explain the differences between inhibitory strains, and their contribution to the outcome of these bacterial interactions. This could provide valuable insight for effective drug development approaches for *S. aureus* infections.

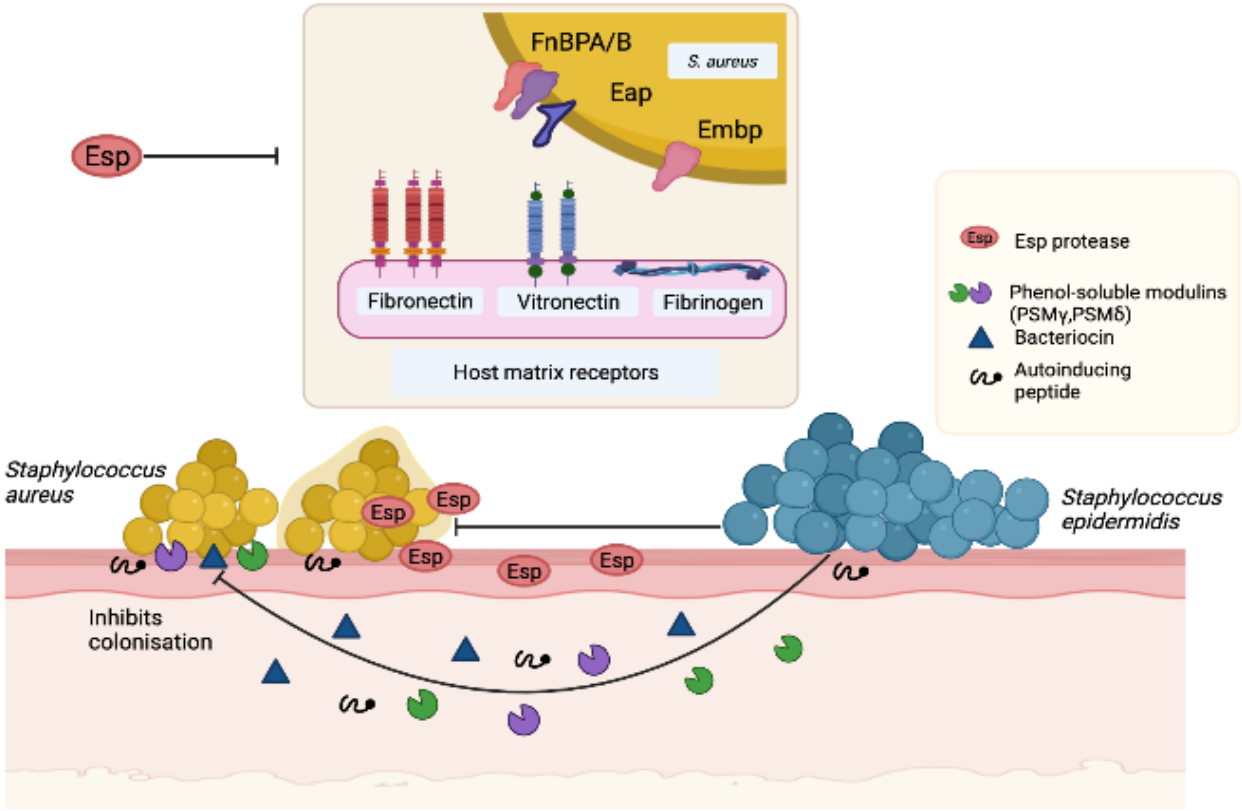


Figure 3.The commensal role of *S. epidermidis* against *S. aureus* colonisation and biofilm formation. Image created on BioRender.com.

1.5. Role of *S. aureus* in Atopic Dermatitis

Another hallmark of AD is *S. aureus* colonisation of the skin and nasal cavity. Multiple predisposing factors contribute to successful *S. aureus* colonisation, with one of the main factors being the changes in the biochemical composition of AD skin. In particular, the reduced levels of ceramides, sphingosine and filaggrin – and the downstream effects of these reduced levels – encourage *S. aureus* colonisation [77]. Furthermore, the levels of AMPs and host defence peptides, such as dermcidin, human β-defensins and cathelicidin-LL37 are reduced significantly, making the skin more conducive to colonisation [47, 77].

S. aureus-derived protease, staphopain, which is also detected on AD skin, decreases *S. aureus* growth inhibition by cleaving the endogenous AMP LL-37. This disrupts the host defence system and favours colonisation by *S. aureus* [78]. Furthermore, Di Domenico *et al. (*2018) have shown that overexpression of proinflammatory cytokines in AD induced growth of *S. aureus* on lesional skin but failed to induce growth of CoNS [79].

During AD flares, the microbiome of skin lesions shows an increased abundance of *S. aureus* and low microbial diversity; a phenomenon strongly associated with disease severity [67, 80, 81]. Although the nasal cavity is less frequently studied in the context of AD, research has shown an association between *S. aureus* colonisation of the nasal cavity and increased severity of AD in children [82]. There is also a decrease in the relative abundance of commensals such as *Streptococcus, Propionibacterium*, *Corynebacterium* and commensal *Staphylococcus* species on AD lesions, compared to non-lesional skin, thereby diminishing their protective effect against pathogens on the skin [80, 83, 84].

S. aureus proliferation in AD lesions usually coincides with a reduction in *S. epidermidis* abundance. However, Kong *et al*. (2012) reported a concurrent increase of both species during AD flares. This suggests that *S. epidermidis* has a compensatory mechanism, which allows it to grow in correlation with the abundance of *S. aureus* on AD lesions, to limit over-proliferation of *S. aureus* [67]. Whilst *S. aureus* was associated with more severe AD, the predominant presence of *S. epidermidis* was still found to be associated with AD, albeit less severe cases [81], suggesting that *S. epidermidis* can also play a pathogenic role in AD pathophysiology. Conversely, another study has reported *S. epidermidis* to augment *S. aureus* growth [76] . These conflicting reports highlight possible strain-level differences playing a role in the outcome of these interactions. Therefore, more studies are required in order to characterise this relationship that determines the outcome of interaction between these two species.

S. aureus virulence factor profiles from African American patients with AD were associated with more severe AD when compared to European American and Mexican American populations, which indicates a possible ethnic link d. Given bacterial colonisation may be affected by different geographic locations and distinct forms of environmental exposure, a study determined the molecular epidermiology of *S. aureus* from skin and nasal samples in AmaXhosa toddlers from rural and urban settings in South Africa with or atopic dermatitis [85]. *S. aureus* colonisation was found to be higher in cases independent of geographic location, with severe AD cases being associated with a higher colonisation compared to moderate AD in urban cases. Distinct dominant *spa* types were noted among cases, as well as specific *spa* types being more frequently found in different AD phenotypes, indicating a possible role for genetic background of the colonising *S. aureus* strains in determining AD severity [85]. Expanding on the staphylococcal colonisation patterns of this cohort, co-colonisation with CoNS and *S. aureus* on lesional skin was found to be more prevalent in cases from both geographic locations, when compared to the controls [86]. When exploring their association

with AD, cases co-colonised by *S. aureus* and CoNS on lesional skin, non-lesional skin or anterior nares showed higher AD severity scores, however, this associations between different body sites were dependent on geographic location [86]. Interestingly, non-lesional skin colonisation with *S. capitis* and *S. haemolyticus* was more widespread in urban and rural cases, respectively. Furthermore, a significant positive association was found between *S. capitis* colonisation and higher objective SCORAD scores for non-lesional skin of rural cases [86], potentially indicating that these species may play a role in AD pathogenesis. Although the colonisation of *S. hominis* on non-lesional skin was similar between cases and controls regardless of locations, lesional skin was less frequently colonised with this commensal compared to non-lesional skin of urban toddlers, supporting this species' protective role on the skin [86]. Taken together, these findings highlight the complexity between microbial interactions on the skin and the potential influence of geographic location. However, further investigations based on African populations are necessary to better understand and identify the significant contributors to AD pathogenesis in these populations, and the mechanisms that govern these varying outcomes.

S. aureus expresses various virulence factors that contribute to AD pathophysiology and severity. The evidence supporting the contribution of *S. aureus* in AD include: (a) production of proteases which degrade epidermal barrier proteins [87, 88]; (b) release of toxins that trigger cutaneous pro-inflammatory responses [89]; (c) *S. aureus*-induced decline in the ability of keratinocytes to produce antimicrobial peptides, which are associated with reduced regulation of *S. aureus* colonisation [90, 91]; and, (d) the production of biofilms to encourage recurrent and recalcitrant colonisation [79, 92].

1.6. Role of Biofilms

A biofilm is defined as a microbially-derived sessile community that is identified by aggregates of cells that attach to a surface or to each other and are embedded in a self-produced matrix of extracellular polymeric substances (EPS), such as polysaccharides, proteins, nucleic acids, and lipids [93, 94]. This offers multiple benefits including: 1) protection of microorganisms from ultraviolet radiation, extreme temperatures, extreme pH, high salinity, poor nutrient availability and antibiotics; 2) the ability to evade host clearance mechanisms; and 3) the potential for dispersal of the initial biofilm community to unoccupied sites of the host, encouraging bacterial spread and colonisation [95, 96].

1.6.1. Staphylococcal Biofilm Development

Biofilm-related infections have recently gained more interest due to an increase in the number of patients receiving implanted medical devices. *S. aureus* and *S. epidermidis* biofilm production has more frequently been associated with infection of indwelling medical devices, such central venous catheters, mechanical heart valves or urinary catheters [97]. Competition between host tissue cells and pathogenic bacteria occurs for occupancy on the surface of the implanted device. And, if bacteria adhere successfully to the surface, they initiate biofilm formation; resulting in a phenotype that is more resistant to common antibiotics [98]. Biofilm-associated resistance can be attributed to restricted penetration of antibiotics [99], altered growth rates [98] and changes in gene expression in response to stressful environments [100].

Biofilm development is an involved process that comprises three broad phases: (i) initial attachment; (ii) production of extracellular matrix and cell proliferation; and (iii) biofilm structuring and cell detachment (Figure 4) [101]. Evaluation of biofilm development using a microfluidic flow cell device, with an integrated fluorescence microscope, revealed that *S. aureus* exhibits a five-stage developmental process, namely: 1) attachment; 2) multiplication; 3) exodus; 4) maturations and 5) dispersal [102]. In this model, the exodus step refers to the stage in which a subset of cells are expelled from the biofilm so as to allow for characteristic structural development [102].

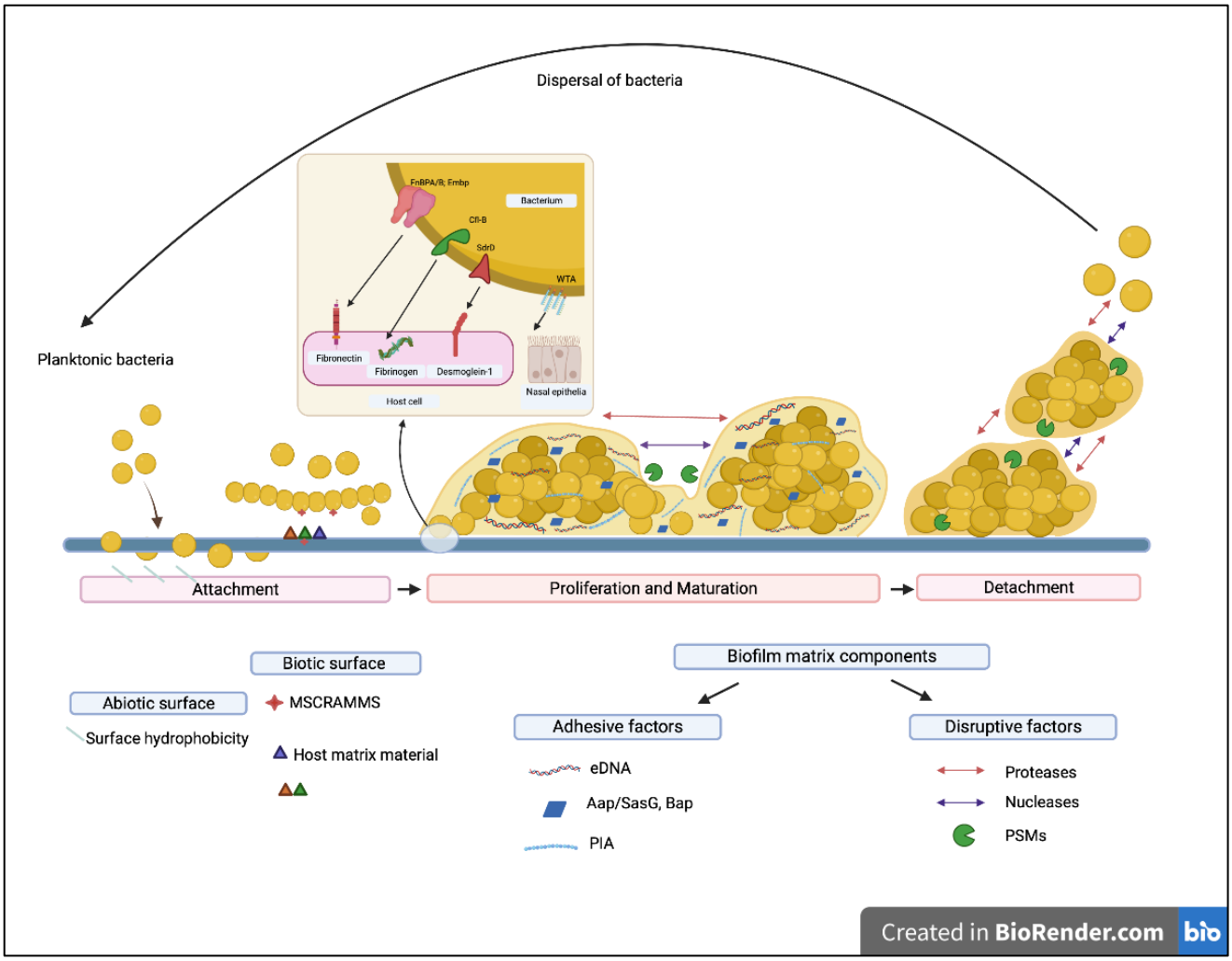


Figure 4. Staphylococcal biofilm production. Cell attachment to a surface takes place by hydrophobic interactions with an abiotic surface, or by certain surface proteins binding in a specific manner to host proteins. Proliferation and maturation of the biofilms occurs concurrently with the generation of cell–cell adhesive matrix factors and disruptive factors. In the detachment phase, disruptive factors lead to detachment of the biofilms, facilitating their dispersal. MSCRAMMS: microbial surface components recognising adhesive matrix molecules; PIA: polysaccharide intercellular adhesin; eDNA: extracellular DNA; Aap: accumulation-associated protein; SasG: *S. aureus* surface protein G; Bap: Biofilm-associated protein; FnBP A/B: fibronectin-binding protein A/B; Embp: extracellular matrix binding protein; WTA: wall teichoic acids; SdrD: serine-aspartate repeat containing protein D; Clf-B: clumping factor B (Figure created in BioRender.com).

During attachment, staphylococci can directly adhere to abiotic or biotic surfaces, with a hydrophobic character enabling the attachment. However, with indwelling medical devices, the surfaces become covered in host matrix material – namely fibronectin, fibrinogen, and vitronectin – where adherence

to the surface is enabled by a family of staphylococcal surface proteins known as microbial surface components recognising adhesive matrix molecules (MSCRAMMS) [103].

Nasal colonisation by *S. aureus* relies on the essential interaction of cell wall teichoic acids (WTA), complex surface-exposed polymers [104], with nasal epithelial cells. Modification of the WTA with *N*-acetylglucosamine (GlcNAc), in α or β configuration, is necessary for effective binding to nasal epithelia [105]. It has been shown that scavenger receptor class-F member 1 (SREC-1), found on the nasal epithelial cell, interacts with WTA to initiate adhesion [106]. However, adherence is also facilitated by various cell wall-anchored proteins, such as MSCRAMM proteins, which promote long-term adherence to anterior nares, and include clumping factor B (Clf), iron-responsive surface determinant (IsdA), and serine–aspartate repeat protein (SdrD) (Figure 2) [58].

During the second phase of biofilm development, colonies undergo proliferation. Polymeric molecules secreted by the cells to form the biofilm matrix, which is composed of extracellular DNA (eDNA), exopolysaccharides, such as polysaccharide intercellular adhesin (PIA), or proteins such as accumulation associated protein (Aap) (Figure 4. Staphylococcal biofilm production. Cell attachment to a surface takes place by hydrophobic interactions with an abiotic surface, or by certain surface proteins binding in a specific manner to host proteins. Proliferation and maturation of the biofilms occurs concurrently with the generation of cell–cell adhesive matrix factors and disruptive factors. In the detachment phase, disruptive factors lead to detachment of the biofilms, facilitating their dispersal. MSCRAMMS: microbial surface components recognising adhesive matrix molecules; PIA: polysaccharide intercellular adhesin; eDNA: extracellular DNA; Aap: accumulation-associated protein; SasG: S. aureus surface protein G; Bap: Biofilm-associated protein; FnBP A/B: fibronectin-binding protein A/B; Embp: extracellular matrix binding protein; WTA: wall teichoic acids; SdrD: serine-aspartate repeat containing protein D; Clf-B: clumping factor B (Figure. The most well understood mechanism for biofilm formation by *S. aureus* and *S. epidermidis* is by PIA /polymeric *N*-acetyl-glucosamine (PNAG), which is synthesised by enzymes encoded by the *ica* operon, consists of four genes, viz.: *icaA*, *icaB*, *icaC,* and *icaD* [107-109].

Although PIA expression in *S. epidermidis* is detectable early in the course of foreign-body infection, PIA production is stimulated in *S. aureus* during the course of infection. Furthermore, it was observed that PIA expression and *ica*-specific transcripts were increased in all *S. epidermidis* strains under different environmental conditions [107]. Conversely, in *S. aureus* strain*s,* PIA expression proved to be dependent on genetic background and growth conditions, thereby indicating species-specific

differences in PIA expression activity between *S. epidermidis* and *S. aureus* [107]. However, the mechanisms regulating these differences between the staphylococcal species require further exploration, particularly in the context of diseases dominated by PIA-dependent biofilm formation.

Mutagenesis experiments of the *ica*-operon demonstrated PIA-independent biofilm formation in *S. aureus* [110, 111] and *S. epidermidis* [112]. Studies have shown that PIA-independent biofilms were in part mediated by Aap in *S. epidermidis* [113] and its homologue, SasG, in *S. aureus* [114]. This is because of their ability to self-polymerise and create fibrils which interlink cells [115, 116]. Although many biofilm studies have assessed the importance of PIA-dependent or protein-dependent biofilms in disease pathogenesis [117, 118], these have been limited to infection of medical devices and hospital-acquired infections. *S. epidermidis* strains isolated from AD patients showed the presence of both the *ica* operon and *aap* gene [119]. However, more extensive research is needed in order to assess the importance of the type of biofilm produced, and its association with disease phenotypes, particularly in AD.

Biofilms have a characteristic three-dimensional, “mushroom-like” structure, as well as fluid-filled channels. Biofilms acquire their characteristic shape from disruptive and adhesive factors, which result from the production of cell–cell adhesive matrix components, and PSMs and degradative secreted enzymes from bacterial cells [120]. The biofilm matrix component eDNA, which is DNA released from dying cells, is highly polymeric and anionic, allowing for interactions with surface molecules [121].

The last stage of biofilm development, viz. detachment, is mediated by the same nucleases and proteases that degrade biofilm polymers and PSMs, which disrupt non-valent interactions [120]. The eDNA component, may be degraded by nucleases secreted by *S. aureus* and *S. epidermidis*. In *S. aureus*, biofilm detachment involves the activity of the *agr* QS system. Furthermore, the *agr* QS is involved in biofilm structuring, *via* PSMs [120, 122]. Interestingly, it was suggested that the *agr* QS system plays a regulatory role in the switch between planktonic and biofilm lifestyles, as demonstrated by high levels of *agr* activity in bacterial cells, which are dispersed from biofilms. Conversely, cells within biofilms showed repressed *agr* activity [122]. This stage also encourages the dispersal of bacterial colonies.

Different methods have been developed to analyse biofilms for various measurements, such as number of viable cells, mass accumulation, and biofilm morphology (Table 1). Methods range from direct quantification methods, which rely on direct observation for quantification of the desired measurement, such as automated cell counting, or indirect quantification methods, whereby a proxy marker is used to determine biofilm quantity, such as the crystal violet assay. However, the lack of reproducibility among biofilm-related studies has proven to be a challenge, particularly due to the lack of standardised protocols for characterisation [123].

Table 1. Methods to Analyse and Quantify Biofilms

Method	Description	Application	Advantages	Disadvantages	Reference
Viable cell determination by plate count	Mature biofilms are homogenised in liquid media via sonicating or scraping, serial dilutions and then plating onto nutrient agar plates. After incubation, colonies are counted and number of cells per millilitre (cfu/mL) in the original culture are calculated.	Used to determine number of viable cells	Does not require specialised equipment or training	Time and labour-intensive; only live, colony-forming cells will be counted	[123]
Quantification of viable biofilm cells by resazurin-based viability assay	Non-fluorescent resazurin (blue colour) is reduced by metabolically active cells to a fluorescent compound, called resorufin (pink).	Tool for determining cell viability in biofilms	Fast, inexpensive, and non-toxic. It is useful for screening potential biofilm inhibitors.	Metabolically inactive but still viable cells will not be detected.	[124, 125]
Flow cytometry cell counting	Cells within liquid cultures pass through narrow openings and are counted by employing a laser that detects cells as they flow via scattering, absorbance, or fluorescence measurements.	Automated way to count cells	Speed, simplicity, and accuracy of measurements	Expensive equipment and training required.	[123]
Confocal scanning laser microscopy (CLSM)	Specialised type of microscopy that produces high resolution images of biofilms in 3D. Can use excitation lasers to view many fluorescent markers sequentially or simultaneously.	Tool for researching the biofilm matrix since it allows fully hydrated samples to be observed in real-time.	Reproducible; biofilms can be non-destructively images at various time points; obtain spatial information on cell and distribution can be obtained	Expensive equipment and media required; experienced and highly trained individuals required.	[126, 127]
Crystal Violet Assay	Indirect quantification approach involving the use of a gram staining dye solution, crystal violet. This dye stains negatively-charged surface molecules and the extracellular polysaccharide matrix of biofilms. Absorption of crystal violet relates to biofilm mass	Identification and visualisation of bacteria and useful for cell estimate for biofilm growth	Low cost, reproducible, advantageous for measuring amount of biofilm produced.	Cannot differentiate between live and dead cells.	[123, 128, 129]
Tetrazolium salt assay	Cells are incubated with tetrazolium salt that is reduced to a formazan derivative by mitochondrial enzymes, which allows direct reading of absorbance measurement	Metabolic activity is measured in proportion to production of formazan measured at A_{492}.	Low-cost technique that allows rapid imaging of metabolically active cells; shorter time to obtain results	Inaccuracy may arise when evaluating mature, bottom cell layers which tend to be more quiescent	[129-132]

1.6.2. Immune Responses to Staphylococcal Biofilms

Bacteria can produce protective biofilms, which allow recalcitrance to antibiotics and recurrent colonisation. Although studies have shown reduced efficacy of the host defence system in clearing various biofilm-producing bacterial species [133-135], recent studies have suggested that biofilms skew the immune response to favour anti-inflammatory and pro-fibrotic pathways, thus limiting bacterial clearance, particularly in *S. aureus* [136-138]. Microbial structural motifs are recognised by the innate immune system by toll-like receptors (TLR). TLR activation results in the recruitment and activation of additional immune cell populations by several pro-inflammatory mediators. TLR2 is expressed by different cell types such as macrophages and neutrophils and is involved in the recognition of *S. aureus* pathogen-associated molecular patterns (PAMPs), including lipoteichoic acids and lipoproteins [136]. Although few macrophages were shown to migrate into biofilms, biofilms elicited significant and swift cell death in the invading macrophages [136]. Furthermore, biofilms influenced macrophage gene expression leading to expression of a profile similar to a M2 phenotype, which is not optimal for bacterial clearance compared to the bactericidal M1 phenotype [136]. A study by Scherr *et. al.* (2015) demonstrated that conditioned media, from mature *S. aureus* biofilms inhibited macrophage phagocytic ability and induced cytotoxicity, indicating that secreted factors may play a role in macrophage dysfunction [138]. Alpha-toxin (Hla) and leukocidin AB (LukAB) were identified as potential candidates, and double mutant experiments suggested that they act synergistically to illicit macrophage dysfunction [139, 140]. Decreased macrophage activation was also shown for *S. epidermidis* biofilms [139, 140].

1.6.3. Staphylococcal biofilms in atopic dermatitis

Biofilm production by staphylococci is a necessary virulence mechanism allowing for adherence and persistent colonisation of the skin and anterior nares. Although the biofilm-forming capability of *S. aureus* and various CoNS (with *S. epidermidis* being the most frequently isolated) has often been associated with the infection of indwelling medical devices and disease (particularly in neonates) [97, 141, 142], the presence of biofilms in AD lesions has also been reported [92, 119, 143]. Using Congo red staining, Allen *et al.* (2014) highlighted the role of biofilm-producing staphylococci in the occlusion of eccrine (sweat) ducts, adjacent to AD lesional skin [119, 143]. Further immunopathologic results showed

activation of the innate immune system *via* TLR-2. Taken together, a 'double-hit hypothesis' was proposed. The first hit in the form of the genetic mutations observed in the disrupted SC of AD patients, combined with the second hit in the form of an environmental component causing biofilm-occluded sweat ducts, lead to activation of the innate immune system and subsequent activation of mediators known to produce itching, scratching, and a rash [144]. However, further research is needed to validate and characterise this potential pathway.

S. aureus strains with a higher propensity to form biofilms were associated with increased AD severity [79, 92, 119, 141, 145]. Furthermore, biofilm-producing strains isolated from anterior nares (the largest reservoir of *S. aureus*) were strong biofilm producers and were associated with a higher SCORAD value, extent of skin lesions, dryness, and extent of skin lesions during flares [141]. These reports suggest that biofilm production might contribute to the chronicity of bacterial colonisation *via* dispersal and maintenance of persistent *S. aureus* colonisation in AD [141]. However, this association is not always the case [146].

Although both staphylococcal species are often present on the skin, many studies highlight the inhibitory role of *S. epidermidis* against *S. aureus* on the skin, with very little being explored about the possible inhibitory or cooperative nature of multi-species staphylococcal biofilms, and how these interactions contribute to AD severity or pathogenesis. A study using crystal violet assays to assess the extent of biofilm growth of *S. aureus* and *S. epidermidis* isolates from children, reported on mixture of staphylococcal biofilm adherence strengths [92]. They further compared the mixed biofilm biomass from co-cultured *S. aureus* and *S. epidermidis* isolates to *S. epidermidis* mono-species biofilm biomass. These results showed that 32% co-cultured isolates exhibited antagonistic interactions, whilst 68% exhibited cooperative mixed biofilms, while 13% showed synergistic interactions by the mixed biofilm biomass being greater than the combined biomass of both mono-species biofilms. This suggests that *S. epidermidis* strains can either act synergistically, coexist, or inhibit during *S. aureus* biofilm formation. Furthermore, the presence of *S. aureus* on the child's skin was associated with a higher SCORAD, while S. *epidermidis* presence showed no such association. These results suggested that *S. aureus* colonisation shows significant association with increased AD severity and the relative abundance of biofilm-forming *S. aureus* and *S. epidermidis* strains could be involved in AD severity [92]. This emphasises the potential role

of strain-level differences in *S. epidermidis* and their interactions with *S. aureus* [92]. However, these strain-level differences remain under-investigated, particularly with regards to their contribution to AD pathophysiology.

1.7. Rationale

Research into *S. epidermidis* suggests potential pathogenesis in diseases, besides its already described protective role against *S. aureus* colonisation in health [147]. In AD, there are inconsistencies in the outcomes of *S. epidermidis* and *S. aureus* interactions, particularly colonisation and biofilm formation. In this regard, it is possible that the strain diversity of *S. epidermidis* may influence its behaviour and outcomes thereof in disease and health contexts. Furthermore, most studies investigating biofilm formation in patients with AD only considered *S. aureus* isolates from either healthy individuals or patients with mild AD. As such, the relationship between these bacterial interactions in the context of colonisation, biofilm formation and AD severity remains unclear. Geography is known to influence skin and nasal bacteria and strain diversity, and ultimately, how these bacteria contribute to health and disease [148]. Individuals of African descent are more likely to have AD and often present with severe disease compared with other populations [30, 149, 150]. Despite this, research primarily focus on European, American, or Asian populations [84, 151-153], while data from African populations are limited. In our parent study, we previously reported a higher likelihood of skin and nasal colonisation with *S. epidermidis* in cases compared with controls, independent of rural and urban residency. However, the rural environment had a strong relationship with *S. epidermidis* colonisation. These results highlight the influence of different environments on *S. epidermidis* colonisation and its potential pathological contribution in childhood AD, which is yet to be described.

1.8. Aims and Objectives

1.8.1. Aim

To evaluate the effect of skin and nasal *S. epidermidis* on the growth dynamics and biofilm formation of co-colonising *S. aureus* in AD toddlers.

1.8.2. Objectives

1. To evaluate the effect of *S. epidermidis*-derived supernatant on corresponding *S. aureus* growth dynamics and disease severity.
2. To determine whether the composition of *S. aureus* biofilms have an association with disease severity.
3. To assess the protease activity of extracellular *S. epidermidis,* and the presence and semi-quantitative expression of Esp, and correlate this with the effect of *S. epidermidis*-derived supernatant on corresponding *S. aureus* biofilms.
4. To characterise *S. epidermidis*-derived molecules that mediate effects on *S. aureus* growth and biofilm formation.
5. To determine the strain diversity of *S. epidermidis* isolates in selected pairs.

Chapter 2: Study design

2.1. Study design, setting and population

We utilised a nested case-control study within the South African Food Allergy (SAFFA) population-based study [154]. We recruited 220 children (aged 12–36 months [mean age: 22.49 ± 7.32 months]) of African ancestry (Xhosa) from urban (Cape Town) and rural (Umtata) South African settings, between February 2015 and May 2016 (Figure 5). Children with moderate-to-severe AD (cases) were recruited from an urban Paediatric Dermatology ward (n=56), Red Cross War Memorial Children's Hospital (Cape Town) and a rural Dermatology ward (n=60), Nelson Mandela Academic Hospital (Umtata). Non-AD participants (controls) were recruited from non-allergic, non-food sensitised individuals at randomly selected creches in the Cape Town metropole (n= 50). Due to the scarcity of creches in the rural district, rural control participants (n=54) were recruited from toddlers within an eligible age range from areas surrounding 10 district community health clinics in the rural Mqanduli district of Umtata [155].

2.2. Diagnosis of atopic dermatitis and determination of disease severity

AD was clinically diagnosed by a dermatologist using the validated UK Working Party diagnostic criteria for AD [156]. The severity of AD was determined using the Objective SCORing of Atopic Dermatitis (oSCORAD) index as moderate (15–40) and severe (> 40)

[157]. A study nurse recorded environmental exposure data in conjunction with the clinical data.

2.3. Ethics approval

The parent study (SAFFA) received approval from the University of Cape Town Human Research and Ethics Committee of the Faculty of Health Science (HREC/REF: 668/2020) and the Western Cape Provincial Child Health Research Committee. Written informed consent from parents or legal guardians of the participants was secured during recruitment. Participant enrolment and all associated procedures were conducted in compliance with all relevant regulations.

Chapter 3: Methodology

3.1. Skin and nasal sample collection

Skin samples were collected using nylon-tipped flocked swabs (Cat. No. 516C; Copan Italia, Brescia, Italy) from both lesional skin (most active area of eczematous skin) and non-lesional skin (unaffected/normal-appearing skin on the back). Lesional skin swabs were obtained exclusively from children with AD. The swabs were moistened beforehand with sterile distilled water, and a 4 cm^2 area of the skin (either lesional or non-lesional) was swabbed continuously for a minimum of at least 1 min in a non-overlapping manner. In addition, anterior nares (nasal) swabs were collected from all participants, as previously described [158]. The swabs were immediately transferred into 1 mL skimmed milk–tryptone–glucose–glycerol (STGG) medium (National Health Laboratory Services [NHLS], Green Point Media Laboratory, Cape Town, South Africa) and stored at –80 °C at the Division of Medical Microbiology, University of Cape Town, South Africa until further processing.

3.2. Selection of co-colonising pairs

Previously identified *S. epidermidis* and *S. aureus* isolates [159], originating from the same sample site, were confirmed using the VITEK® mass spectrometer based on MALDI-TOF (matrix-associated laser desorption ionisation-time of flight) technology (University of Pretoria). This isolate data was stratified according to case-control status, study site, and AD severity. Thereafter, a subset of 28 representative pairs of co-colonising *S. epidermidis* and *S.*

aureus isolates were chosen based on four combinations of biofilm-producing types as shown below in **Figure *5***. Briefly, these were moderate biofilm-producing S. *aureus* strains paired with strong biofilm-producing S. *epidermidis* strains (n = 6), or strong biofilm-producing S. *aureus* strains paired with either moderate (n = 12) or strong biofilm-producing *S. epidermidis* (n = 10) strains.

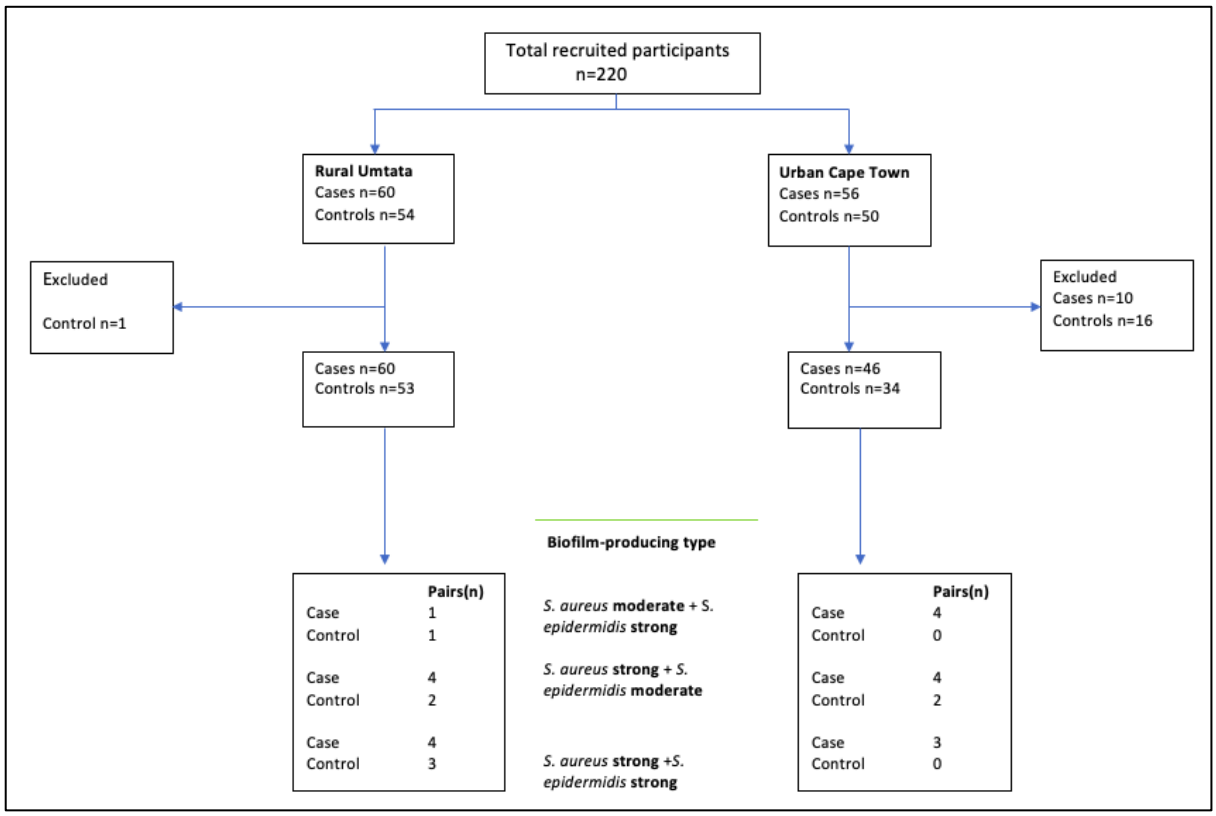


Figure 5. Participant recruitment and isolate selection strategy. From the confirmed *S. aureus* and *S. epidermidis* isolates from participants, we further stratified the data according to case-control status, study site and AD severity. Combinations of *S. aureus* or *S. epidermidis* were then chosen based on biofilm-producing strength (n=28).

3.3. Preparation of *S. epidermidis*-derived supernatant

The 28 chosen *S. epidermidis* isolates from skin and nasal samples were cultured on 5% Sheep Blood Agar plates (Blood agar base; 5% BA; NHLS, South Africa) and aerobically incubated at 37 °C for 48 hours. Subsequently, a single colony was grown in 5 mL of tryptic soy broth (TSB) at 37 °C on a shaking incubator at 120 rpm for 24 hours. After incubation, the *S. epidermidis* broth cultures were centrifuged at 4,500 ×*g* for 5 min then the resulting supernatants were filtered through a 0.2 µm syringe filter.

3.4. Determining the effect of *S. epidermidis* supernatant on corresponding *S. aureus* growth dynamics

Paired *S. aureus* and *S. epidermidis* isolates from corresponding skin and nasal samples were cultured on 5% BA overnight, then grown in 5 mL of TSB at 37 °C on a shaking incubator at 120 rpm for 24 hours. After incubation, the S. *aureus* culture was diluted to 6 x 10^6 CFU/mL in TSB (to an optical density of 0.05 measured at OD_{600}). Thereafter, a 1:1 (final volume: 200 µL) volume of *S. aureus* culture and supernatant from *S. epidermidis* (as prepared above) was added to tubes. Equal volumes of S. *aureus* culture and TSB, and TSB only were used as controls [68, 76]. Thereafter, 10-fold serial dilutions of each bacterial suspension were prepared and 10 µL was aliquoted onto brain heart infusion (BHI) agar plates and incubated aerobically at 37 °C for 24hr. The resulting colonies on each BHI plate were counted and the CFU determined. CFUs counts from suspensions of *S. aureus* culture mixed with *S. epidermidis* supernatants were compared to the corresponding S. *aureus* and TSB controls. Each experiment included three independent biological replicates.

3.5. Evaluating the effect of *S. epidermidis*-derived cell-free conditioned media on the growth dynamics of co-colonising *S. aureus*

Briefly, cultures of co-colonising *S. aureus* and *S. epidermidis* isolates from nasal and skin samples were grown as described above. The 24-hour cultures of *S. aureus* were diluted 1:100. Thereafter, a 1:1 ratio of the *S. aureus* cultures and paired *S. epidermidis* supernatants was added to a final volume of 200 µL in a 96-well clear, flat-bottomed microtiter plate (Greiner Bio-One, Austria). Wells containing *S. aureus* and TSB, and only TSB were used as controls. Thus, experiments were completed with three independent biological replicates. Plates were placed in a Tecan Infinite M200 Pro (Tecan, Austria) where growth curves were generated overnight. The i-control™-based protocol was set as follows: temperature: 37 °C; kinetic cycle (duration): 14 hours; kinetic interval: 30 mins; Shaking (10 sec): Orbital with amplitude of 3mm; absorbance: 600 nm, 25 flashes with 100 ms settle time; and lastly, incubation of two sets of 800 second orbital shaking at a 3 mm amplitude and a remaining wait (timer) step [160]. Data obtained from the readings was used to generate growth curves to compare the growth rate between control and experiment.

3.6. Determining the effect of *S. epidermidis* supernatant on corresponding *S. aureus* biofilm formation and growth in biofilm

For *S. aureus* biofilm formation, bacterial cultures were grown overnight as described above. To determine the effect of *S. epidermidis* supernatant on biofilm formation of *S. aureus*, aliquots of filtered *S. epidermidis* supernatants were added at equal volumes to the *S. aureus* culture (final volume: 200 µL) in sterile wells of flat-bottomed, 96-well plastic microtiter plates. Biofilms were allowed to form under aerobic conditions, during incubation at 37 °C for 24 hours [161]. Thereafter, the supernatants were aspirated, and the wells were washed 3 times with 300 µL reverse osmosis (RO) water. The residual attached biofilm was heat fixed at 60 °C for one hour. The heat-fixed biofilm was stained with 200 µL of 0.5% crystal violet for 10 mins. Thereafter, the crystal violet solution was discarded, and the excess stain was removed by washing each well 5 times with 300 µL sterile RO water. The stained biofilm was dissolved by incubation at room temperature for 30 mins with 30% glacial acetic acid. Biofilm mass was estimated by measuring the absorbance of each well at 492 nm using a Multiskan™ FC microplate photometer (Thermo Scientific, USA). The effect of *S. epidermidis* on biofilm formation was measured by comparing the amount of biofilm produced in the absence of *S. epidermidis* supernatant (control) to the amount of biofilm produced in the presence of *S. epidermidis* supernatant and reported as the fold change [60, 162].

To determine the effect of *S. epidermidis* supernatant on the cell viability of *S. aureus* in biofilms, a rezasurin assay was conducted. Biofilms were formed, as described above, and each well was washed thrice with 300 µL of sterile Phosphate-Buffered Saline. Thereafter, 200 µL of AlamarBlue™ Cell Viability Reagent (Invitrogen, USA) were added to each well and the plate was incubated for 4 hours at 37 °C. Results were recorded using an excitation wavelength of 560 nm and an emission wavelength of 590 nm in the Tecan Infinite M200 Pro (Tecan, Austria).

3.7. Determination of biofilm composition

To determine if *S. aureus* biofilms are proteinaceous or polysaccharide-based, biofilm inhibition assays were performed in the presence of proteinase K and sodium metaperiodate ($NaIO_4$) which act as protein-based and β-1,6-linked polysaccharide-based biofilm inhibitors, respectively [163, 164]. Established biofilms were treated with proteinase K (100 µg/ml) or

sodium periodate (40 nM) for 2 hours at 37 °C [74]. Thereafter, biofilm formation was assessed by means of a crystal violet assay, as detailed above. Controls did not include any treatment. The experiment was completed with three biological replicates and two technical replicates.

3.9. Semi-quantitative expression analyses of *esp*

The semi-quantitative expression of *esp* was evaluated by reverse transcription polymerase chain reaction (RT-PCR) [76] . Overnight cultures of *S. epidermidis* were grown at 37 °C in TSB. These cultures were diluted in fresh TSB at a ratio of 1:100. RNA extraction was carried out using the *Quick*-RNA™ Miniprep Plus Kit (Zymo Research Corp, USA), based on the manufacturers' instructions. RNA quantity and integrity was determined with the Nanodrop™ One (Thermo Scientific, USA). RT-PCR was performed under the following cycling conditions: 30 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 mins, using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), in accordance with the manufacturers' instructions. A non-template control (NTC) and no reverse transcriptase control (−RT) were included. PCR for both +RT and –RT samples was conducted using primers targeting *gseA* F157-177 (5′-ATGAAAAAGAGATTTTTATCT -3′) and *gseA* R660-642 (5′-GTTTGGTGACACTCTTAAG-3′) for *esp* [165]. The *gyrB* gene was used as the reference (Forward strand: *gyrB*- F 5′- AAGGCGGCTGAGCAATATAA-3′; Reverse strand: *gyrB* R 5′-CAGGTGAAGATACACGAGAAGG-3) [165]. PCR products were visualised by agarose gel electrophoresis. This experiment was performed with three independent biological repeats.

3.10. Characterisation of the *S. epidermidis*-derived molecules that mediate effects on the growth and biofilm formation in *S. aureus.*

For initial characterisation of *S. epidermidis*-derived active molecules, we investigated a group of *S. epidermidis* isolates which demonstrated significant effects on *S. aureus* biofilms. To do this, *S. epidermidis* supernatants were exposed to a variety of treatments, and thereafter, determination of the effect of supernatants on biofilm formation was conducted as described above. Supernatants were treated with proteinase K (20 mg/ml) at 37 °C for 1 hour, to determine if the inhibitory molecule is a protein. A separate treatment, to determine if the inhibitory molecule is deactivated by heat, was included by boiling supernatant at 95 °C for 5 mins first [74]. To determine the potential size of the effector molecules, supernatants were

passed through a 30 kDa, where the flow through was collected. The filters were washed through with TSB three times thereafter, the residue was resuspended to obtain filtrate containing proteins >30 kDa. Filtered supernatants were then incubated at 37 °C for 24 hours with corresponding *S. aureus* isolates.

3.11. Strain diversity of *S. epidermidis.*

To determine the sequence type of co-colonising *S. epidermidis*, isolates were analysed using multi-locus sequence typing (MLST) protocol as previously described [166]. Previously published primer sequences for each of the seven most discriminatory alleles were used [166]. Chromosomal DNA was isolated using the Maxwell® 16 Instrument (Promega Corporation, USA) and the Maxwell® 16 LEV DNA purification kit (Promega Corporation, USA), according to the manufacturers' instructions. DNA quantity and quality was determined by Nanodrop™ One (Thermo Scientific, USA). Total PCR reaction volumes of 25 µL were performed under the following conditions: initial denaturation of 95 °C for 3 mins; followed by 34 cycles of 95 °C for 30 sec, 50 °C for 1 min, and 72 °C for 1 min; and a final extension of 72 °C for 10 mins [166]. Amplicons were verified by gel electrophoresis, and thereafter, sequenced using the Sanger sequencing method (Inqaba Biotec, South Africa). The alleles at each locus were analysed using pubMLST (accessed by https://pubmlst.org). The allelic profiles formed by the allele numbers at each of the seven loci were assigned sequence types. Microreact (accessed https://microreact.org) was used to visualise clustering of sequence types or alleles to certain metadata.

3.12. Data analysis

Firstly, the raw data was normalised and log-transformed using Microsoft Excel (Version??). Data analysis was performed using GraphPad Prism for MacOS (Version 9.5.1 (528), GraphPad Software, Boston, Massachusetts, USA, www.graphpad.com) with a significance of an adjusted *P*- value at < 0.05.To evaluate the effects on biofilm formation and the growth of *S. aureus*, a Student's *t* test was conducted using GraphPad Prism. A two-way ANOVA was conducted using GraphPad Prism to evaluate the effect of the size of the effector molecules from *S. epidermidis* supernatant on *S. aureus* biofilm production.

Chapter 4: Results

4.1. Participant characteristics

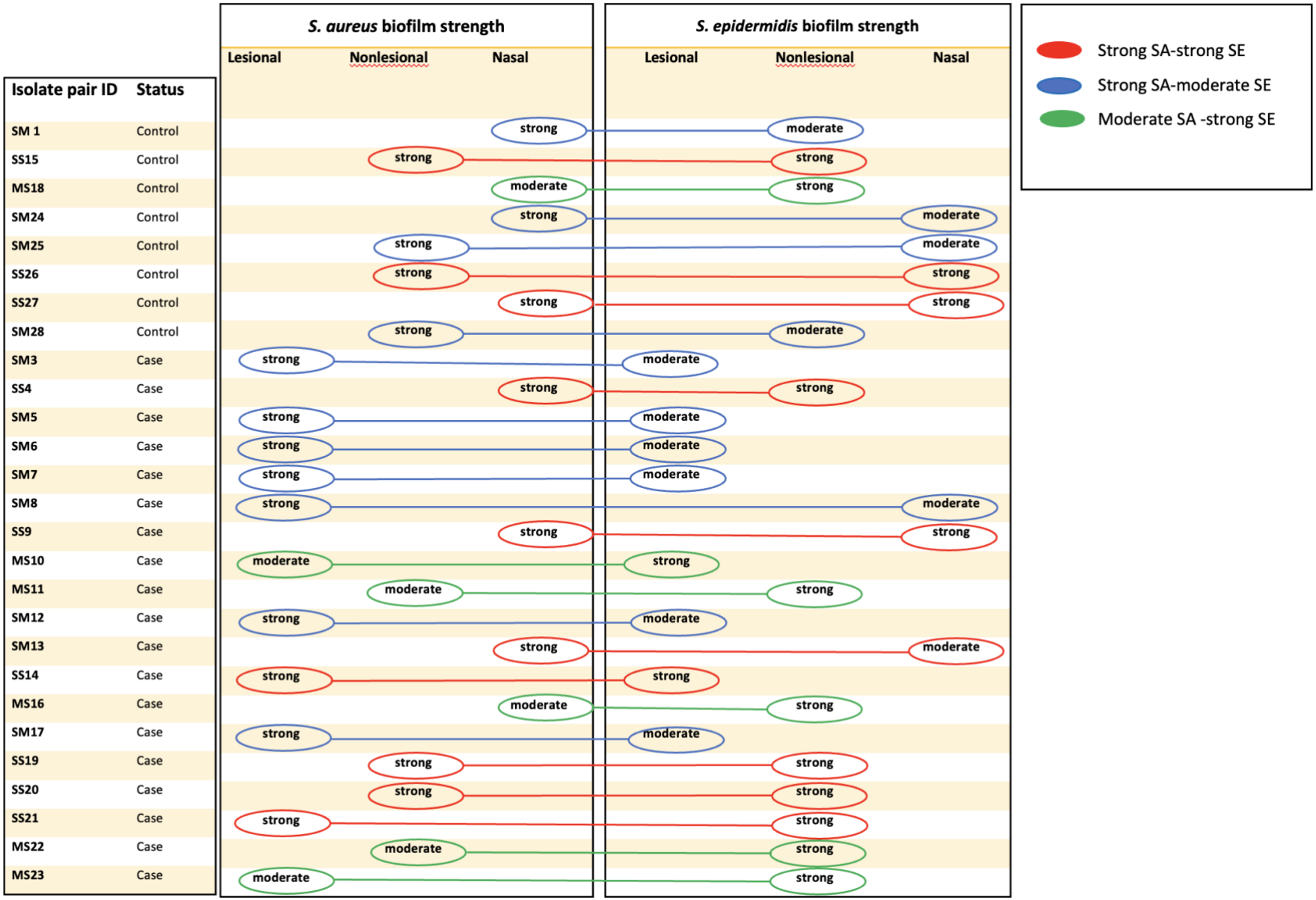
Of the recruited 220 participants, only 193 were included in the initial analysis (Figure 5). The mean age of the participants from urban and rural locations were 24.21 ± 7.34 months and 21.27 ± 7.08 months, respectively (Table 2). The rural cohort had more males than females, however, the urban cohort had equal numbers of both. More toddlers had severe AD than moderate AD in the rural cohort and compared to the urban cohort. Case participants had a higher frequency of *S. aureus* growth at the lesional site compared to the nasal and non-lesional site. Similarly, *S. epidermidis* growth was found on lesional skin of more frequently compared to the other sites. However, in urban participants, *S. epidermidis* growth was found in similar frequencies across body sites. From these participants, corresponding *S. epidermidis* and *S. aureus* isolate pairs were chosen from different body sites, depending on the biofilm-forming strength of either isolate making up the pair (Table 3).

Table 2. Participant characteristics of cohort.

Explanatory variable	**Umtata**				**Cape Town**			
	Total, *n* (%)	**Case, n (%)**	**Control, n (%)**	***p*-value**	**Total, *n* (%)**	**Case, n (%)**	**Control, n (%)**	***p*-value**
Total	113 (100)	60 (53.10)	53 (46.90)	0.560	80 (100)	46 (57.50)	34 (42.50)	
Age (months)								
Mean [standard deviation]	21.27 [7.08]	21.05[7.28]	21.53 [6.90]	0.722	24.21[7.34]	24.15[7.5]	24.29[7.16]	0.933
Sex								
Female	42 (37.84)	24 (41.38)	18 (33.96)	0.441	40 (50)	22 (47.83)	18 (52.94)	0.821
Male	69 (62.16)	34 (58.62)	35 (66.04)		40 (50)	24 (52.17)	16 (47)	
AD severity [a]								
Moderate	58 (100)	23 (39.66)	N/A	–	46 (100)	24 (52.17)	N/A	–
Severe		35 (60.34)	N/A			22 (47.83)	N/A	
***S. aureus* growth**								
Lesional skin	22 (19.47)	22 (19.47)	N/A	–	22 (19.47)	22 (19.47)	N/A	–
Nonlesional skin	23 (20.35)	17 (15.04)	6 (5.31)		21 (18.58)	16 (14.16)	5(4.42)	
Nasal site	15 (13.27)	10 (8.85)	5 (4.42)		27 (23.89)	19 (16.81)	8 (7.08)	
***S. epidermidis* growth**								
Lesional skin	24 (21.24)	24 (21.24)	N/A	–	10 (8.85)	10 (8.85)	N/A	–
Nonlesional skin	25 (22.12)	17 (15.04)	8 (7.08)		13 (11.50)	10 (8.85)	3 (2.65)	
Nasal site	21 (18.58)	16 (14.16)	5 (4.42)		11 (9.73)	8 (7.08)	3 (2.65)	

[a] Disease severity was measured using the Objective SCORAD. N/A: Not applicable. '–' were included in spaces where *p*-values could not be calculated. Participant data is as previously in described [159] as it is the same study cohort. Participant data was stratified according to study site and comparison of categorical variables and continuous variables were performed using the Fischer's exact test and a Student's t-test, respectively. Comparisons were drawn between cases and control participants from either site.

Table 3. Biofilm strength of *S. aureus* and *S. epidermidis* isolate pairs from the body site they were isolated.



Note: Isolate pair IDs were assigned depending on the biofilm-forming strength of *S. aureus* and *S. epidermidis*, respectively. For example, the pair SM1 would be made up of a **s**trong *S. aureus* isolate and a **m**oderate *S. epidermidis* isolate .SA: *S. aureus*; SE: *S. epidermidis.*

4.2.Effect of *S. epidermidis* supernatant on corresponding *S. aureus* biofilm formation and growth in biofilm

The interactions between *S. epidermidis* and *S. aureus* are unclear. We tested the effect of *S. epidermidis* on the biofilm formation of corresponding *S. aureus* isolates using a crystal violet assay. Our results showed strain-dependent increases and decreases in *S. aureus* biofilm formation when incubated with corresponding *S. epidermidis* supernatant. Most pairs isolated from healthy children (75%; 6/8) showed decreased biofilm formation, however, both decreases (65%; 13/20) and increases (35%; 7/20) in biofilm formation were observed in strains isolated from cases (Figure 6A). No significant results were observed comparing the decreases in biofilm formation between healthy participants and toddlers with AD (75% vs 65%; p = 0.6152). The pair which showed a significant result was isolated from a child with AD (SM17; adjusted p-value 0. 02), and was a decrease in biofilm formation compared to the control. We also note three pairs (SM5, SM6 and MS23), all of which were isolated from children with severe AD, showed the largest increases in biofilm formation. The four isolate pairs from children with AD showed the largest decreases and were from either moderate (SM17 and SS14) or severe (SM8 and SS21) AD cases. We further grouped the biofilm formation results according to sample site on the participant. Pairs where *S. aureus* was isolated from lesional skin showed the most variation, while having the largest decreases in biofilm formation (Figure 6B). Comparatively, the nonlesional skin site also showed variation in biofilm formation, however, to a lesser extent compared to lesional skin. The nasal site showed more decreases (78%; 7/9) in biofilm formation. When isolates were grouped according to the sample site where *S. epidermidis* was isolated (Figure 6C), both increases and decreases were observed for lesional and nonlesional skin sites. However, the nasal site only had pairs where biofilm formation decreased.

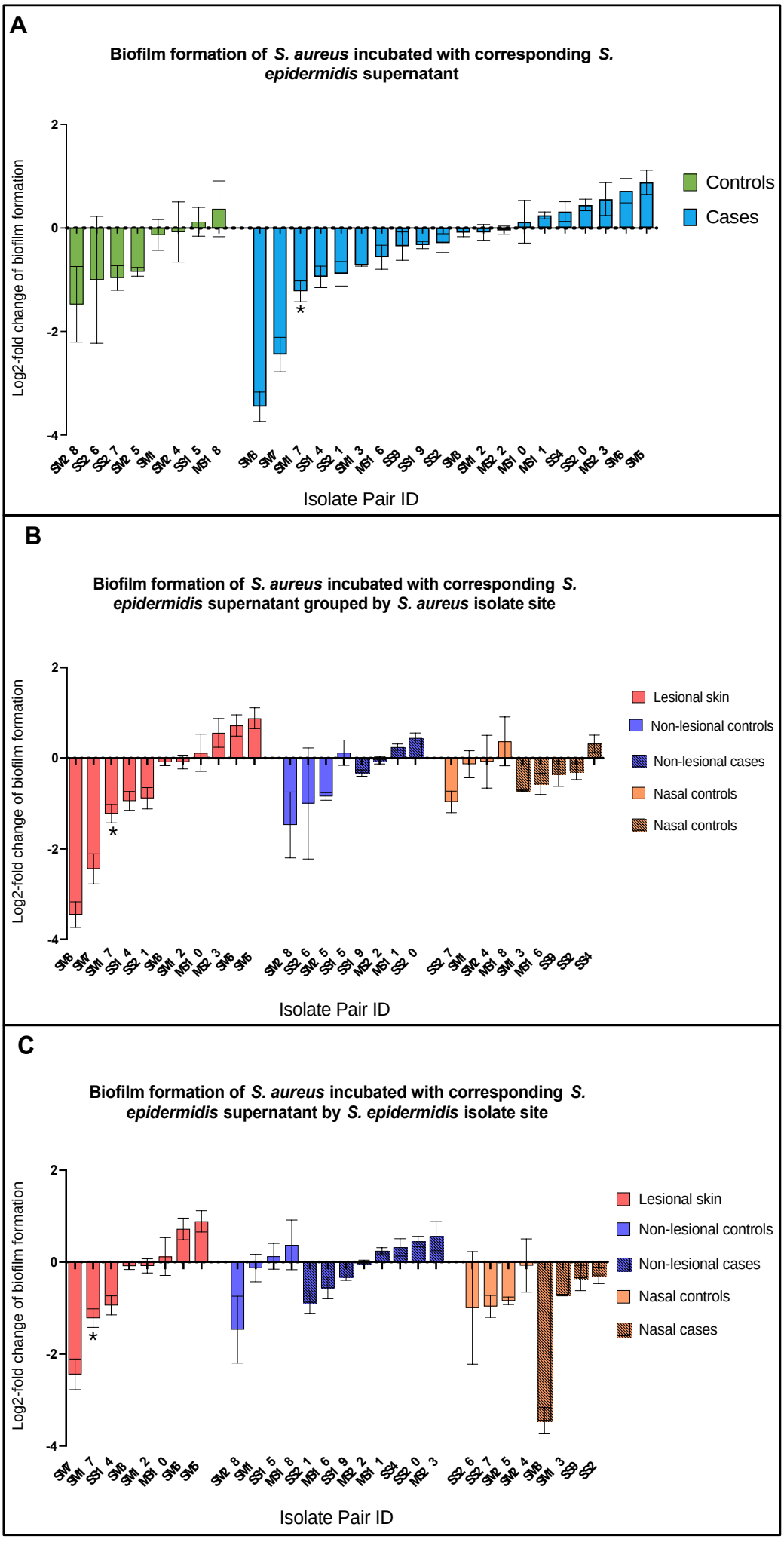


Figure 6. (A) Changes in biofilm formation of *S. aureus* isolates incubated with corresponding *S. epidermidis* supernatant compared to *S. aureus* incubated with media. Pairs were further grouped according to the sample sites from where *S. aureus* strains (B) and *S. epidermidis* strains were isolated (C).
Strains isolated from healthy and AD children are shown by green and purple bars, respectively. Isolate pairs marked with an '*' represent adjusted $p < 0.05$.

4.3. Effect of *S. epidermidis* supernatant on corresponding *S. aureus* growth in biofilm

An Alamar Blue Cell viability assay was used to assess the viability of *S. aureus* cells grown in biofilms after 24 hour incubation with corresponding *S. epidermidis* supernatant. Results from this assay showed that most pairs (64%; 18/28) exhibited an increase in cell viability compared to *S. aureus* isolates grown in media only (Figure 7A). However, SS9 was the only isolate pair which displayed a significant increase in cell viability. This pair consisted of a strong biofilm-forming *S. aureus* strain, but a decrease in biofilm formation was seen when treated with *S. epidermidis* supernatant. Similarly, for SM17 we saw an increase in cell viability and a significant decrease in biofilm formation when *S. aureus* was exposed to *S. epidermidis* supernatant. This indicates that effector molecules in *S. epidermidis* supernatant inhibit biofilm production whilst not necessarily affecting cell viability.

SS26 and SM28 shared the same *S. aureus* strain for each pair. Decreases in biofilm formation were observed for both pairs, however, SM28 showed a larger decrease compared to SS26. Cell viability decreases were observed for both pairs, but this difference was negligible. These results further support *S. epidermidis* strains' biofilm formation inhibitory activity being site-specific, as well as their extent of biofilm formation, given that the same *S. aureus* strain was tested against *S. epidermidis* strains from either nonlesional or nasal sample sites of the same person. Likewise, SS15 and MS18 had the same strong biofilm-forming *S. epidermidis* strain isolated from nonlesional skin of a control participant. When the supernatant of this strain was incubated with a moderate biofilm-forming *S. aureus* strain from the nasal site (SS15), increases in both cell viability and biofilm formation were observed. However, when incubated with a strong biofilm-forming *S. aureus* strain from the nonlesional skin site (MS18), a decrease in cell activity and increase in biofilm formation was observed. This increase in biofilm formation in SS15 was less compared to MS18. Taken together, these results could suggest site-specific strain differences of the bacteria making up the pairs in terms of biofilm formation in participants. Both instances show that the nasal site seems to have cooperative interactions where the biofilm formation increased (MS18) or decreased to a lesser extent (SS26) compared to the other pair containing the same strain at the nonlesional site. However, this method of pairing strains was not done for all isolates at different sites and needs to be further tested to determine if this trend persists in other participants.

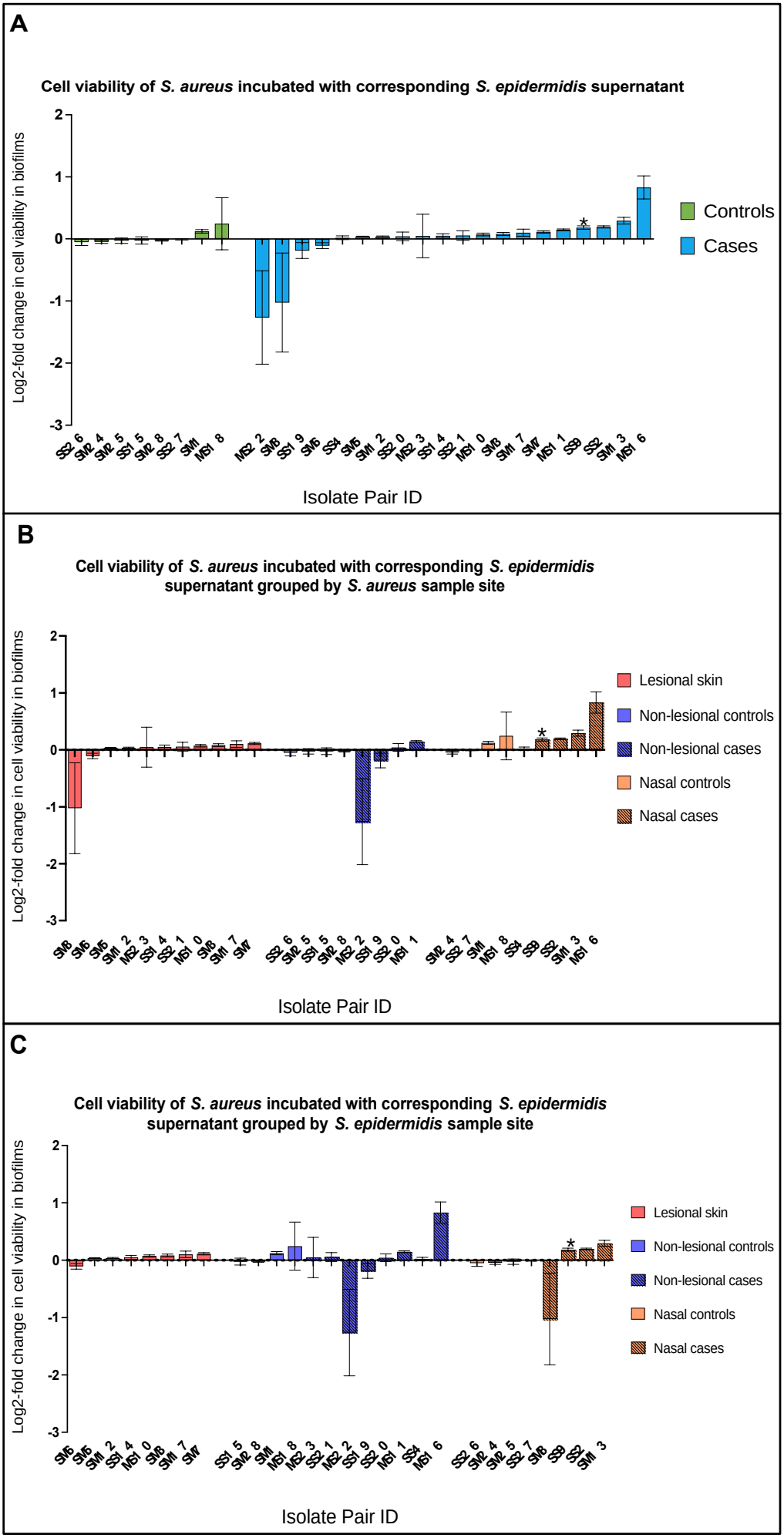


Figure 7. (A) Changes in cell viability of *S. aureus* isolates grown in biofilms incubated with corresponding *S. epidermidis* isolates compared to *S. aureus* incubated with media only. Pairs were further grouped according to the sample sites from where *S. aureus* strains (B) and *S. epidermidis* strains were isolated (C).
Isolates pairs marked with an '*' represent a $p < 0.05$.

4.4. Composition of *S. aureus* biofilms and their association with AD severity

The association of biofilm composition with AD severity has not been elucidated. When established *S. aureus* biofilms were incubated in sodium metaperiodate (Figure 8A), as this treatment disrupts the extracellular polysaccharides of biofilms, it was observed that only 18.5 % (5/27) of the selected *S. aureus* isolates showed decreases in biofilm masses compared to untreated controls. All significant results reported were fold increases from strong and moderate (S7 and M23) biofilm-forming isolates. Isolates showed a similar pattern of increases and slight decreases in biofilm formation regardless of the sample site (lesional, nonlesional or nasal sites) or AD severity.

Established biofilms treated with Proteinase K (Figure 8B) showed variable results of both increases (8/27) and decreases (19/27) of biofilm biomass, with only S24 showing a significant decrease (–1.61 log-fold change). Of the 19 isolates that demonstrated dispersal, 74% (14/19) were strong biofilm-forming *S. aureus* isolates. Isolate S8 (–3.03 log-fold change) and S7 (–2.44 log-fold change) showed the greatest dispersal when treated with PK, suggesting a more proteinaceous biofilm composition. When stratified using body site of where *S. aureus* was isolated (Figure 8C), isolates showing the largest dispersal were found at lesional skin sites, however, most isolates showed evidence of more proteinaceous biofilms. This could indicate that proteinaceous biofilm-forming isolates have a ubiquitous nature. We further stratified the data using AD severity where only 64% (9/14) of severe cases showed biofilm dispersal, compared to 80% (4/5) of moderate cases. However, these results were not significant. We also note no distinct pattern with biofilm matrix composition and AD severity. Furthermore, the increases in biofilm formation for sodium metaperiodate warrant further investigation. Overall, PK was better able to disperse the biofilm matrix than sodium metaperiodate, possibly indicating a more proteinaceous biofilm matrix.

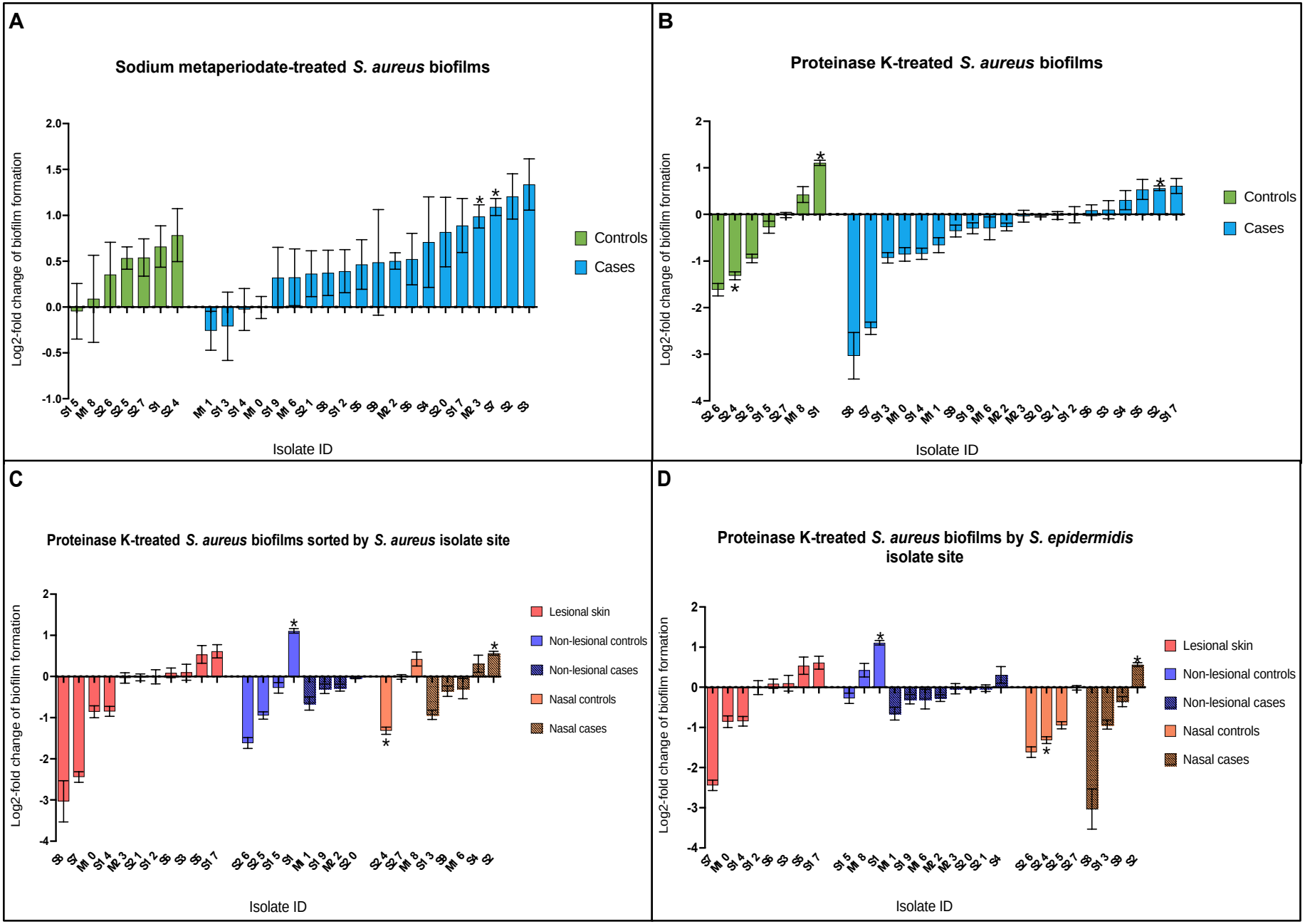


Figure 8. Change in biofilm mass of established *S. aureus* biofilms incubated with either (A) sodium metaperiodate or (B) proteinase K (PK) compared to controls incubated in TSB media only. Data was further stratified according to the isolation site of either *S. aureus* (C) or *S. epidermidis* (D). '*' represents results with an adjusted p-value of 0.05.

4.5. Protease activity of extracellular *S. epidermidis*, the presence of Esp, correlated with the effect of *S. epidermidis*-derived supernatant on corresponding *S. aureus* biofilms.

Previous results from our research group using conventional PCR to detect the presence of the *esp* gene in all *S. epidermidis* isolates from this cohort revealed a low prevalence of the gene (2.2%; 2/90). Recent studies have highlighted that carriage of the *esp* gene does not necessarily translate to its expression or *S. aureus* biofilm inhibition [68]. We sought to determine the expression of Esp in this subset of *S. epidermidis* isolates, and its effect on biofilm formation in the corresponding *S. aureus* isolates by RT-PCR for the *esp* gene.

Here, we found that of the 27 *S. epidermidis* isolates tested, 63% (17/27) were shown to express the gene. *S. epidermidis* isolated from control participants had 71% (5/7) of isolates expressing the gene, whereas 60% (12/20) *S. epidermidis* isolates from participants with AD expressed the gene. Decrease in biofilm formation upon treatment with the *S. epidermidis* supernatant was correlated with 82% (14/17) of the *S. epidermidis* isolates that express the gene. When stratified using body site, we note that lesional skin sites (88%) have a higher prevalence of isolates expressing the gene compared to non-lesional (42%) and nasal sites (63%). Furthermore, more severe AD cases (67%; 8/12) compared to moderate cases (33 %; 4/12) carried the Esp-expressing *S. epidermidis* isolates, suggesting a role for Esp in the exacerbation of AD. Furthermore, from the 19 pairs which showed a decrease in biofilm formation (Figure 6A), 14 of the corresponding *S. epidermidis* isolates, where 71% of controls (5/7) and 75% (9/12) cases, came from those pairs that had demonstrated expression of the *esp* gene.

4.6. Characterisation of *S. epidermidis*-derived molecules that mediate effects on *S. aureus* growth and biofilm formation.

For the heat-treated supernatant (Figure 9A), 50% of controls and 35% of case participants' pairs showed an increase in biofilm biomass compared to control biofilm biomasses, with only SM28 and SS20 being significant increases for control and case groups, respectively. These results possibly suggest that the *S. epidermidis* effector molecules against biofilm formation are heat sensitive. Pair SS4 demonstrated an increase in biofilm formation, although not significant (Figure 6A). When treated with heat, SS4 showed a significant decrease in biofilm formation compared to the control possibly indicating that the *S. epidermidis* effector molecules of this pair are also heat-sensitive. More specifically, we could infer that these effector molecules are cooperative and heat-sensitive given that when SS4 was incubated with just the supernatant it showed an increase. No distinct grouping was observed when results were sorted according to *S. aureus*

isolate site (Figure 9B). Overall, these results suggest a variety of heat-sensitive effector molecules in *S. epidermidis* isolates that either increase or decrease the ability of *S. aureus* to form biofilms.

Incubation with *S. epidermidis* supernatant treated with sodium metaperiodate (Figure 10A) resulted in 50% of isolates from control participants showing decreases in biofilm biomass, with SS26 and SM24 being significant decreases. Similarly, 50% of case participants' pairs displayed decreases in biofilm biomass with only SM17 being significant. However, SM8 showed an increase in biomass. This data suggests no specific grouping of sodium metaperiodate-sensitive effector molecules. When stratified according to body site from where *S. epidermidis* isolates originate (Figure 10B), we note that 7/8 (88%) of pairs from the lesional skin site show increases in biofilm formation, suggesting these effector molecules could be antagonistic but sensitive to sodium metaperiodate, however, these results were not statistically significant. Further, a mixture of sodium metaperiodate effector molecules might exist, given that SM17 significantly showed a decrease in biofilm biomass compared to untreated supernatant.

Most pairs (88%; 7/8) from control participants treated with PK (Figure 11A) showed decreases in biofilm formation, with SM24 and SM18 being significant. Case participants showed 65% (13/20) of pairs with decreased biofilm biomass when treated with PK, with the only significant value being SM6 which showed in increase in biofilm biomass. When stratified by body site from where *S. aureus* was isolated (Figure 11B), we note that all lesional site isolates showed decreases, whereas, when grouped according to where *S. epidermidis* was isolated (Figure 11C) we observe only one pair with an increase in biofilm biomass either the non-lesional skin site or nasal skin site. This could indicate that these sites, for the significant pairs, have more proteinaceous effector molecules.

Interestingly, SM24 showed a significant decrease in biofilm formation for both PK- and SM-treated supernatant. Similarly, this trend was observed for heat-treated supernatant, although not significant. This could be due to a proteinaceous effector molecule being affected by heat and PK. However, decreases were observed for all treatments suggesting the possibility of multiple effector molecules with different constituents. During the initial biofilm formation analysis, we noted six pairs which gave significant results of either increases (SM5, SM6, MS23) or decreases (SS14, SM17, SS21) in biofilm biomass when incubated with the corresponding *S. aureus* supernatant.

Next, we sought to approximate the size of these potential effector molecules by incubating *S. aureus* cultures with supernatants passed through a 30 kDa filter.

Figure 12 shows a significant increase in biofilm formation of pair MS23 when incubated with supernatant that contains molecules that are <30 kDa, possibly suggesting that the molecules from *S. epidermidis* that encourage biofilm formation in *S. aureus* within this pair include those that are <30 kDa. We further note that SM17 also showed a decrease in biofilm formation for molecules <30 kDa, suggesting that these effector molecules resulting in a decrease are <30 kDa.

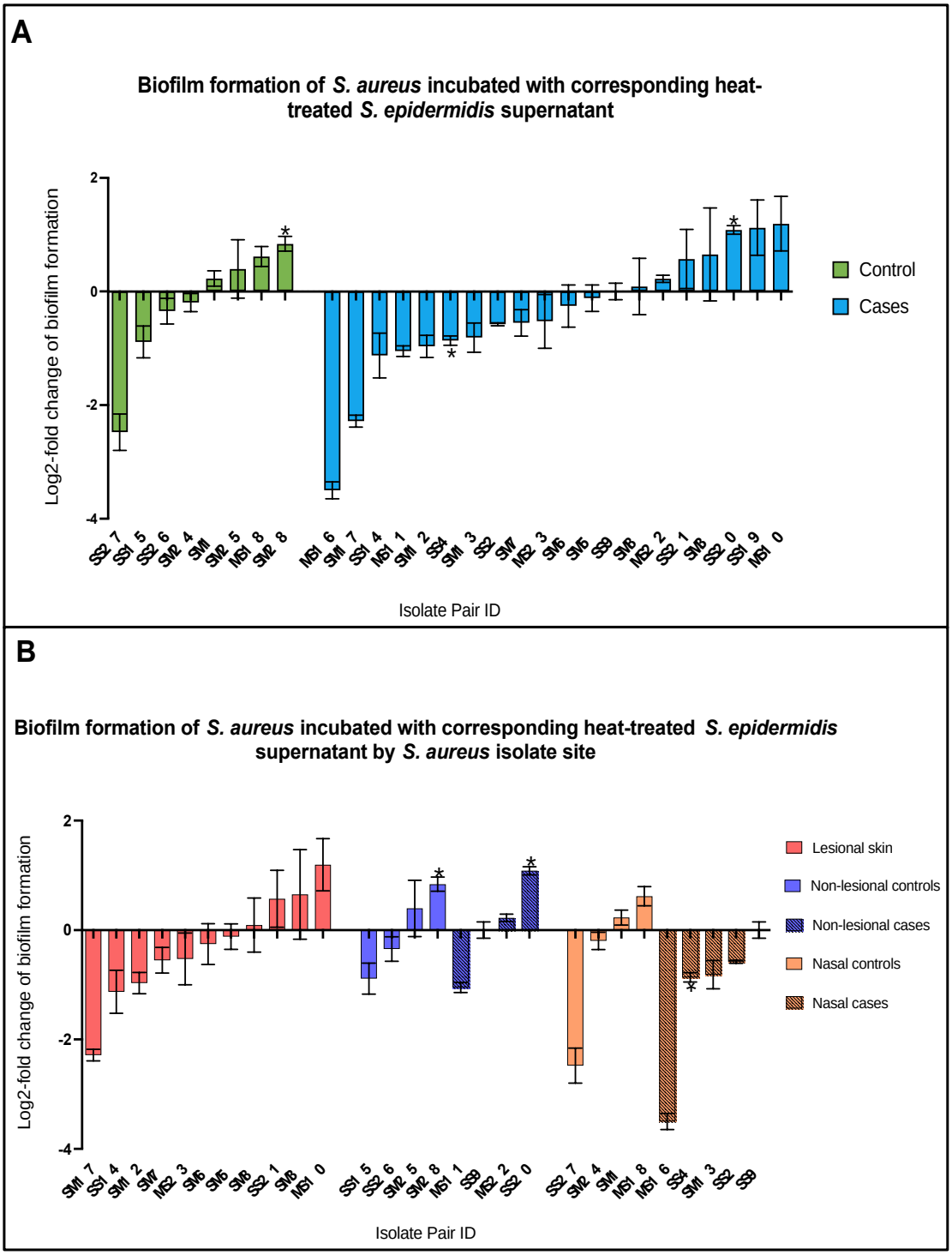


Figure 9. Changes in biofilm formation when *S. aureus* cultures were incubated with heat-treated corresponding *S. epidermidis* supernatant (A), and data further stratified by body site from where *S. aureus* was isolated (B).

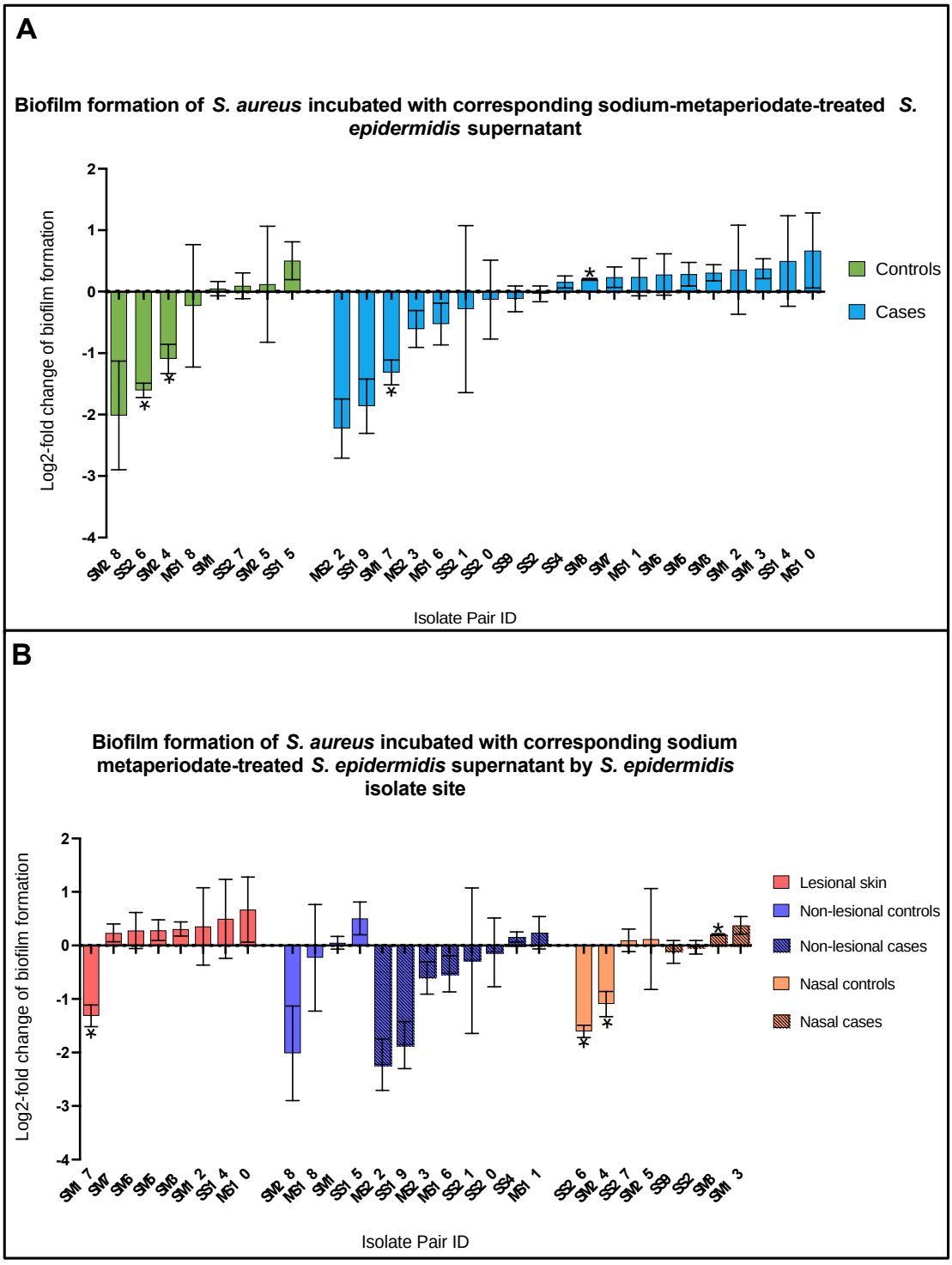


Figure 10. Changes in biofilm formation when *S. aureus* cultures were incubated with SM-treated corresponding *S. epidermidis* supernatant (A), and data further stratified using body site from where *S. epidermidis* was isolated (B).

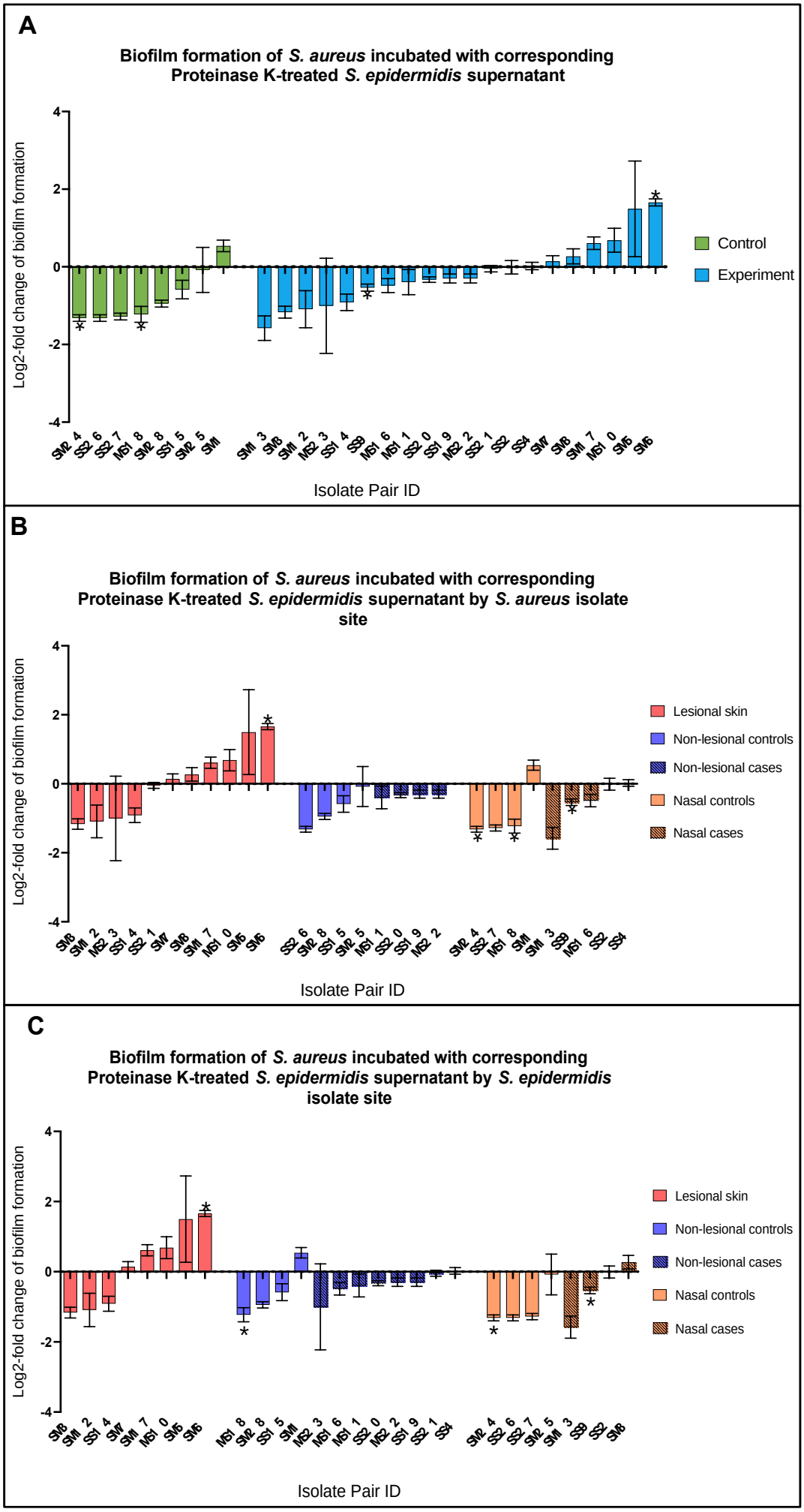


Figure 11. Changes in biofilm formation when *S. aureus* cultures were incubated with PK-treated corresponding *S. epidermidis* supernatant (A). The data was further stratified by body site from where *S. epidermidis* (B) and *S. aureus* (C) was isolated.

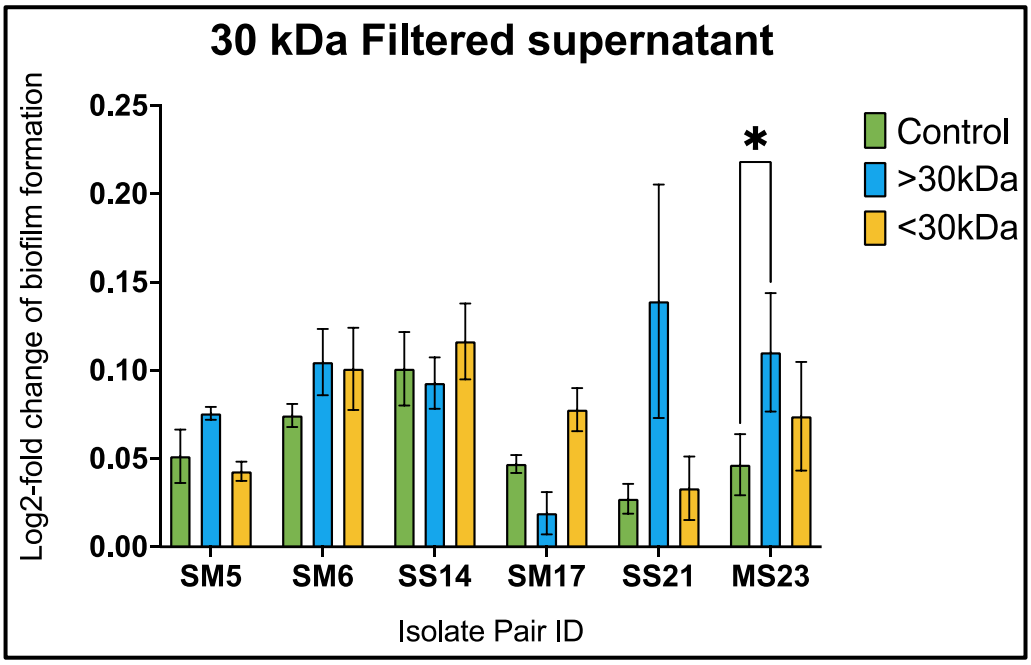


Figure 12. Biofilm formation of an initial subset of pairs. *S. epidermidis* supernatant were subjected to a 30kDa filter then incubated with the corresponding *S. aureus* cultures (Two-way ANOVA).

4.7. Determination if the observed effects of *S. epidermidis* on *S. aureus* are associated with the genetic background of *S. epidermidis* and *S. aureus*.

The genomes of both *S. epidermidis* and *S. aureus* isolates have presented varied MLST profiles. Only a subset of *S. epidermidis* isolates (M3, M5, M7, S11, S19) matched to exact STs on pubMLST, and were observed to be most closely related to these specific STs as shown in

Figure 13. These were cases with either severe or moderate AD. We note several of our isolates partially matched to different STs at ≤6 loci. Of the control *S. epidermidis* isolates, Sample 3036 (S15/S18) partially matched to 6 MLST loci with its nearest match being ST677, however, in

Figure 13 we note this isolate clusters most closely to cases, namely Sample 24 (M12), Sample 40 (S20) and Sample 16 (M8). Similarly, Sample 2 (M1) clusters more closely with cases. However, the rest of the controls appear to cluster more closely with different STs.

In

Figure 13, cases diverging from the node of subtree 9 (except Sample 34) cluster as closely related isolates from cases which have strong biofilm forming abilities. These could possibly be newly identified STs from this cohort, which will require further analysis.

CC1, CC5 and CC121 made up the clonal lineages of the control isolates, whereas the case isolates included CC15 and CC8 too. CC8 made up the majority (All isolates: 8/27, 30%; cases: 8/20, 40%) of *S. aureus* isolates, however, none of the *S. aureus* control isolates were CC8 (Table 4). The majority of the control isolates' clonal lineages were either CC5 (2/7, 29%) or CC121 (2/7, 29%). Most isolates from cases were ST8 (6/20,

30%), whereas for isolates from controls ST121 (2/7, 29%) was the majority. Moderate AD cases either CC1, CC121 and CC5, whereas severe cases were spread out across all case CCs.

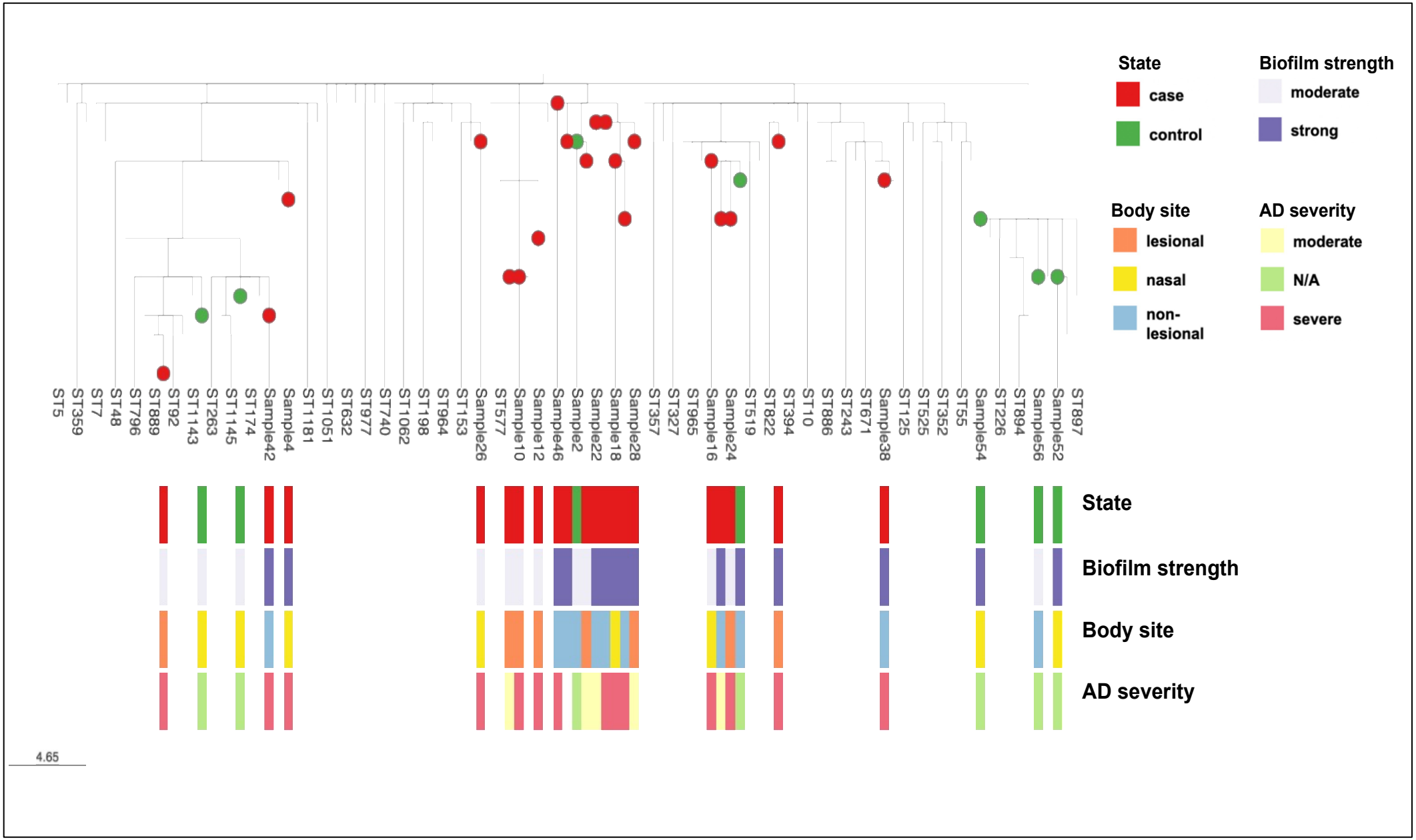


Figure 13. Dendrogram representing the ST relationship between *S. epidermidis* isolates

Table 4. Sequence types and Clonal complexes for *S. aureus* isolates in this study, obtained from PubMLST.

Isolate	Sequence type	Clonal Complex
Cases		
S2	5	CC5
S3	3	CC15
S4	7	CC8
S5	8	CC8
S6	8	CC8
S7	1	CC1
S8	612	CC8
S9	8	CC8
M10	5	CC5
M11	121	CC121
S12	8	CC8
S13	101	
S14	5	CC5
M16	8	CC8
S17	1	CC1
S19	121	CC121
S20	121	CC121
S21	8	CC8
M22	1	CC1
M23		CC15
Controls		
S1c	1	CC1
S15c	5	CC5
M18c	20	
S24c	121	CC121
S25c	121	CC121
S26c	1	CC5
S27c	88	

Note: Cells that are blank are due some results being returned with only a sequence type or clonal complex.

CC that are same have same colour font.

Chapter 5: Discussion

Staphylococcal biofilms have been noted on AD lesions [119] and , in particular, certain strains of *S. aureus* which produce strong biofilms have been associated with a pathogenic role [79, 92, 141]. Recent studies have highlighted a potential pathogenic role for *S. epidermidis* in AD development [167], and have shown its ability to augment *S. aureus* growth [92]. This indicates that some strains of *S. epidermidis* may have a synergistic relationship with *S. aureus* and promote biofilm formation. Furthermore, studies on the outcome of interactions between *S. aureus* and *S. epidermidis* have yielded mixed results [73, 76, 81]. Given the conflicting observations, we sought to investigate the effect of *S. epidermidis* supernatant on *S. aureus* biofilm formation from strains isolated from South African toddlers.

The variation displayed in our biofilm formation results supports the idea of *S. epidermidis* strain specificity contributing to the outcome of interactions [92]. When focusing on the site of origin of the *S. epidermidis* isolates, decreases in biofilm formation were observed, suggesting the inhibitory activity of *S. epidermidis* against *S. aureus* may be site-specific, and this could be explored on a larger sample scale. Pairs isolated from toddlers with either severe or moderate AD showed no distinct pattern possibly indicating that AD severity is not necessarily influenced by the outcome of biofilm formation, however, a larger sample size is needed to verify this.

Observations from isolates from nonlesional skin tended towards more strongly antagonistic interactions, possibly due to healthy participants having an abundance of commensals which prevent biofilm formation, and perhaps also colonisation by pathogenic bacteria such as *S. aureus*. Most of our isolate pairs have demonstrated an inhibitory effect on biofilm formation (75% of controls and 65% of cases, 68% overall). This is higher than the report by Iwase and colleagues [68], who described an inhibitory prevalence of co-colonising *S. epidermidis* supernatant on *S. aureus* biofilm formation at 45% (428/960). These differences are likely due to the difference in the age of participants, where in our study participants were less than 2 years old whereas they were more than 22 years old in theirs. It follows that the prevalence of *S. epidermidis* and *S. aureus* decreases with age [168]. Further, these differences can also be explained by the geo-social differences between these two settings [169]. Another hypothesis for this discrepancy could be that their isolates originated from the nasal cavities

of healthy participants [68], whereas our isolates were sourced from the nasal cavity, nonlesional and lesional skin of case and control participants.

Analyses of our cell-free supernatant has noted anti-*S. aureus* activity being strain-specific [170]. This corroborates the hypothesis by Vandecandelaere et al [73] that the variation seen in reduced biofilm biomasses after treatment with protease-producing *S. epidermidis* is due to strain-specific differences. Likewise, our data shows variation in *S. aureus* biofilm biomass, regardless of the biofilm-producing strength, thereby, supporting this hypothesis. Taken together, these results reinforce the idea that the outcome of interactions between *S. aureus* and *S. epidermidis* isolates are *S. epidermidis* strain-specific.

Although the presence of the *esp* was reported to be ubiquitous, its expression does not necessarily correlate with inhibitory effects on *S. aureus* biofilm formation [76]. Even though our previous work yielded a low prevalence for the presence of the *esp* gene, our subset reports a high expression of the gene via RT-qPCR. Notably, the previous study's protocol was a conventional PCR [159] which had required optimization, therefore, the low percentage of isolates exhibiting presence of the gene may not be the true representation of the overall prevalence of the gene. Given these discrepancies, an optimised PCR protocol is necessary for the clinical assessment of isolates. Esp secreted from inhibitory-type *S. epidermidis* has been shown to inhibit biofilm formation and nasal colonisation by *S. aureus* [68]. Similarly, in the present study, when *S. epidermidis* isolates from corresponding pairs were stratified using body-site, particularly, at nasal site, decreases in biofilm biomass were noted. Furthermore, about 63% (5/8) of *S. epidermidis* isolates from those pairs expressed the *esp* gene. However, analysis of biofilm formation of a subset of pairs incubated with the 30 kDa filtered supernatant (Figure 7) did not exhibit distinct differences in biofilm formation between the different sizes. Given that Esp is the most well-characterized antagonist of biofilm formation for *S. epidermidis* and that its size is 27 kDa, further characterisation is warranted to determine what other molecules could be mediating these effects using a larger sample size.

A study that investigated the competitive bacterial colonisation of *S. aureus* and *S. epidermidis* in newborns by conducting an analysis on Esp and general protease activity concluded that the protection against *S. aureus* was more likely to be from one or more proteases other than Esp [162]. This is supported by our data characterising the molecules

from *S. epidermidis* supernatant which displayed an effect on biofilm formation. For example, in pair SM1, the corresponding *S. epidermidis* isolate showed expression of the *esp* gene and increases in biofilm formation when the supernatant was treated with heat and PK, which could explain the decrease in biofilm formation without treated supernatant. However, we also observed an increase in biofilm formation when the supernatant was treated with sodium metaperiodate which could indicate a different molecule also influencing biofilm formation. A similar trend was observed with SM8, which also had a decreased cell viability of *S. aureus* which led us to believe that apart from these factors only influencing biofilm formation, they could affect cell viability too.

However, a study that investigated the effect of boiling supernatants on biofilm formation suggested that the increase of growth could be due to a slight increase in pH [171]. Further, they suggested that specific microbe-produced heat-sensitive metabolites could have evaporated or degraded resulting in increased growth [171]. As a result, it would be useful to analyse the contribution of other proteases and molecules as our data shows potential to implicate multiple mechanisms contributing to the outcome of interactions. Given that Esp further undergoes post-translational modifications [172] which may hinder its early activity, future experiments should also consider a more proteomic-based approach to analyse the effect of Esp on biofilm formation inhibition.

Given the differences observed with the effect of *S. epidermidis* on *S. aureus,* we sought to determine if there was a genetic basis for these observations. Our results mirror reports of *S. aureus* strain exhibiting monoclonality whereas *S. epidermidis* strains present diverse strain compositions [81, 173]. We further report the possibility of new strains in our cohort as many of our isolates did not completely match established MLST profiles. Furthermore, the heterogeneity between the *S. epidermidis* strains provides a basis for the variation observed in the outcome of interactions between tested pairs.

We noted that two *S. epidermidis* strains isolated from urban, female cases at lesional skin sites were both ST789, however, these pairs, SM5 and SM7, presented with severe and moderate AD, respectively. The *S. aureus* strains that make up SM5 and SM7 were ST8 (CC8) and ST1 (CC1), respectively. Biofilm formation results showed that SM7 had a decrease biomass, however, SM5 showed an increase. Taken together, these observations could

support the idea from Byrd and colleagues [81] that variation in clonal lineages of *S. aureus* colonising participants with AD could play a role in the differential outcome of severity in AD. However, these results are speculative and require more rigorous testing.

Although most studies report an inhibitory effect of cell-free supernatant of *S. epidermidis* on *S. aureus* biofilms [68, 73, 74], we reported increases in biofilm formation from some pairs as well. A possible reason for this could be due to the QS systems facilitating interspecies crosstalk. According to Williams *et al.*, commensal CoNS species' AIPs exhibit strong inhibitory effects on *S. aureus agr* activity, leading to the reduced ability of *S. aureus* to induce protease activity, thereby preventing biofilm dispersal [62, 122]. Conversely, repression of the *agr* system demonstrated an increase in biofilm formation [174], which could be why we observe these increases in biofilm formation. *S. epidermidis agr* type I can only inhibit *S. aureus agr* type I to III [175]. CC8 was the most abundant *S. aureus* clonal lineage overall, and was found exclusively in severe cases, whereas CC1 and CC121 were more often found in moderate cases. Certain *S. aureus* lineages may correlate with different *agr* types, such *agr* group 1 with CC8, group II with CC5, CC12 and CC15, and group IV with CC121 [176]. The pairs which showed increased biofilm production were either CC8, CC5, CC15 or CC121, highlighting the possibility that the corresponding *S. epidermidis'* AIPs play a role in biofilm formation via the QS system. The QS system would require high ratio of AIPs from *S. epidermidis agr* type I to effectively inhibit *S. aureus* virulence. However, *S. epidermidis agr* type I was found to be less abundant than to *S. aureus* in AD skin [62], which potentially explains the exacerbation of AD disease severity. Conversely, in our study, interactions were examined with the same starting concentrations of both species, however *S. aureus* was diluted whereas *S. epidermidis* supernatant was not, hence the effect of AIP abundance could be taken into account to explain these increases in biofilm production.

As previous biofilm studies highlighted the importance of either PIA-dependent or protein-dependent biofilms in disease pathogenesis in hospital acquired infections or medical devices [107, 117, 118], we sought to determine if the composition of biofilms have an association with AD severity. Our results have shown that when these *S. aureus* biofilms are incubated with sodium metaperiodate, which disrupts PIA-dependent biofilms, most *S. aureus* biofilms did not display dispersal, indicating potentially *ica*-independent biofilms. PK dispersed most

biofilms at varying degrees indicating that isolates form proteinaceous biofilm matrices. Similar to results from Glatthard *et al.* and Yadav *et al.*, we note that some *S. aureus* biofilms (M11 and S13) showed dispersal (decrease in biofilm biomass compared to control) after treatment with sodium metaperiodate or PK (Figure 8A and Figure 8B) [74, 177], indicating these could be biofilms matrices that are a result of both protein- and PIA- associated molecules, or mixed biofilms. We also highlight some isolates which did not show dispersal by either PK or sodium metaperiodate such as S1 or S2. A study suggested that absorbances observed after treatment with PK or sodium metaperiodate may be attributed to extracellular DNA, which is also a significantly contributing factor to biofilm formation [178]. Therefore, future studies should include DNAse treatment to determine the contribution of eDNA to biofilm matrices. Taken together, these results highlight proteinaceous biofilms in our isolates. It has been shown that biofilm formation in MRSA is *ica*- independent and requires protein adhesins, whereas in MSSA strains PIA/PNAG has a more important role [179, 180]. Skin colonisation by MRSA is a challenge for AD treatment, and can result in skin and soft tissue infections [181]. Therefore, information regarding the biofilm composition of our isolates has important implications for preventing recurring over-colonisation and spreading of *S. aureus* to new environments, as well as informing AD treatment strategies and antibiotic selection in our populations.

Our study does present some limitations. Firstly, as we are conducting a cross-sectional study, temporal assessments of the interactions between *S. aureus* and *S. epidermidis* will not be permitted; and potential changes in the sequence types of the co-colonising *S. epidermidis* populations could not be assessed. Secondly, biofilm production demonstrated in *in vitro* experiments may not accurately represent *in vivo* biofilm production, owing to differences in adherence and maturation mechanisms, and the overall conditions encountered during skin and nasal colonisation [182]. In our experiments, the polystyrene surface of the microtiter plates and TSB broth in which biofilms were grown, represent a markedly different environment than the skin and anterior nares, which would promote biofilm production via adherence to host matrix molecules, as well as exhibit a stimulated host immune response. As a result, these differences could affect the translatability of our study into clinical settings. However, these experiments were designed to accumulate knowledge and so their immediate translatability should only be taken into consideration once sufficient data

generation has occurred to warrant more clinical studies. We used controls diluted in fresh TSB, whereas experiments were incubated in supernatant from overnight cultures of *S. epidermidis*. Therefore, when we conducted experiments, it was difficult to determine the full extent of the effect of *S. epidermidis* supernatant on *S. aureus* cultures. Future experiments could explore the use of fluorescent *S. aureus* strains or recombinant reporter strains for various key growth and virulence genes could be used to observe the full effect of *S. epidermidis* on *S. aureus* strains [183]. Microorganisms rarely exist in isolation, so despite our focus being on co-colonising S. *epidermidis'* interactions with *S. aureus*, the role of other members of the skin microbiota are not considered. Hence, future studies should investigate these complex interactions which will allow for the development of more microbiome-balanced interventions.

Chapter 6: Conclusions

Given the important role of bacterial interactions in AD, this study sought to assess the effect of skin and nasal *S. epidermidis* on the growth dynamics, particularly biofilm formation, of co-colonising *S. aureus* isolated from South African AD toddlers. We report variation in the *S. aureus* biofilm formation results, independent of the biofilm-producing strength, potentially suggesting the outcome of interactions between these two species are *S. epidermidis* strain-specific.

Although the expression of the *esp* gene was observed in a number of *S. epidermidis* isolates, results from supernatants treated with either heat, PK or SM suggest the possibility of multiple effector molecules with different constituents contributing to the outcomes of interaction. As a result, studies should employ, however not limit themselves to, a more proteomic-based approach to determine these effector molecules. This study also contributes novel genotypic data on *S. epidermidis* strains colonising South African toddlers. *S. aureus* strains exhibit monoclonality whereas *S. epidermidis* strains present diverse strain compositions, which could be the basis for the differential outcomes observed. Future research should apply a holistic approach to microbiome studies to understand the different factors influencing the outcome of interactions leading to biofilm production, and how this translates to AD disease severity. By enhancing the current understanding of these interactions, new drug targets can be identified, and improved and sustainable practices can be developed to inform more targeted approaches for AD therapeutics design

Chapter 7: References

1. Nutten, S., *Atopic Dermatitis: Global Epidemiology and Risk Factors.* Annals of Nutrition & Metabolism, 2015. **66**.
2. Eichenfield, L.F., et al., *Guidelines of care for the management of atopic dermatitis: section 2. Management and treatment of atopic dermatitis with topical therapies.* Journal of the American Academy of Dermatology, 2014. **71**(1): p. 116-132.
3. Volke, A., et al., *12-month prevalence of atopic dermatitis in resource-rich countries: a systematic review and meta-analysis.* Scientific reports 2022. **12**(1): p. 15125.
4. (IHME), I.f.H.M.a.E. *GBD 2019 Cause and Risk Summary: Atopic dermatitis - Level 4 cause*. 2020 [cited 2020 9 February 2023]; Available from: https://www.healthdata.org/results/gbd_summaries/2019/atopic-dermatitis-level-4-cause.
5. Mallol, J., et al., *The International Study of Asthma and Allergies in Childhood (ISAAC) Phase Three: a global synthesis.* Allergologia et immunopathologia, 2013. **41**(2): p. 73-85.
6. Williams, H., et al., *Is eczema really on the increase worldwide?* Journal of Allergy and Clinical Immunology, 2008. **121**(4): p. 947-954. e15.
7. Odhiambo, J.A., et al., *Global variations in prevalence of eczema symptoms in children from ISAAC Phase Three.* The Journal of allergy and clinical immunology, 2009. **124**(6): p. 1251-8.e23.
8. Laughter, M.R., et al., *The global burden of atopic dermatitis: lessons from the Global Burden of Disease Study 1990-2017.* The British journal of dermatology, 2021. **184**(2): p. 304-309.
9. Deckers, I.A., et al., *Investigating international time trends in the incidence and prevalence of atopic eczema 1990–2010: a systematic review of epidemiological studies.* PloS one, 2012. **7**(7).
10. Annesi-Maesano, I., et al., *Time trends in prevalence and severity of childhood asthma and allergies from 1995 to 2002 in France.* Allergy, 2009. **64**(5): p. 798-800.
11. Falade, A., et al., *Prevalence and severity of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema in 6-to 7-year-old Nigerian primary school children: the international study of asthma and allergies in childhood.* Medical Principles and Practice, 2004. **13**(1): p. 20-25.
12. Zar, H.J., et al., *The changing prevalence of asthma, allergic rhinitis and atopic eczema in African adolescents from 1995 to 2002.* Pediatric allergy and immunology, 2007. **18**(7): p. 560-565.
13. Esamai, F., S. Ayaya, and W. Nyandiko, *Prevalence of asthma, allergic rhinitis and dermatitis in primary school children in Uasin Gishu district, Kenya.* East African medical journal, 2002. **79**(10): p. 514-518.
14. Gittler, J.K., et al., *Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis.* The Journal of allergy and clinical immunology, 2012. **130**(6): p. 1344-54.
15. Suárez-Fariñas, M., et al., *Nonlesional atopic dermatitis skin is characterized by broad terminal differentiation defects and variable immune abnormalities.* The Journal of allergy and clinical immunology, 2011. **127**(4): p. 954-64.e1-4.

16. Schmitt, J., et al., *Assessment of clinical signs of atopic dermatitis: a systematic review and recommendation.* The Journal of allergy and clinical immunology, 2013. **132**(6): p. 1337-47.
17. Hanifin, J.M., et al., *The Eczema Area and Severity Index-A Practical Guide.* Dermatitis, 2022. **33**(3): p. 187-192.
18. Wickett, R.R. and M.O. Visscher, *Structure and function of the epidermal barrier.* American journal of infection control, 2006. **34**(10): p. S98-S110.
19. Pouillot, A., et al., *The stratum corneum: a double paradox.* Journal of cosmetic dermatology, 2008. **7**(2): p. 143-148.
20. Fartasch, M., *Epidermal barrier in disorders of the skin. .* Microscopy Research and Technique, 1997.
21. van Smeden, J. and J.A. Bouwstra, *Stratum Corneum Lipids: Their Role for the Skin Barrier Function in Healthy Subjects and Atopic Dermatitis Patients.* Current problems in dermatology, 2016. **49**: p. 8-26.
22. van Smeden, J., et al., *The importance of free fatty acid chain length for the skin barrier function in atopic eczema patients.* Experimental dermatology, 2014. **23**(1): p. 45-52.
23. Danso, M., et al., *Altered expression of epidermal lipid bio-synthesis enzymes in atopic dermatitis skin is accompanied by changes in stratum corneum lipid composition.* Journal of dermatological science, 2017. **88**(1): p. 57-66.
24. Candi, E., R. Schmidt, and G. Melino, *The cornified envelope: a model of cell death in the skin.* Nature reviews. Molecular cell biology, 2005. **6**(4): p. 328-40.
25. Yang, G., et al., *Skin Barrier Abnormalities and Immune Dysfunction in Atopic Dermatitis.* International Journal of Molecular Sciences, 2020. **21**(8): p. 2867.
26. Sasaki, T., et al., *Filaggrin loss-of-function mutations are not a predisposing factor for atopic dermatitis in an Ishigaki Island under subtropical climate.* Journal of dermatological science, 2014. **76**(1): p. 10-5.
27. Løset, M., et al., *Genetics of Atopic Dermatitis: From DNA Sequence to Clinical Relevance.* Dermatology, 2019. **235**(5): p. 355-364.
28. Margolis, D.J., et al., *Uncommon Filaggrin Variants Are Associated with Persistent Atopic Dermatitis in African Americans.* The Journal of investigative dermatology, 2018. **138**(7): p. 1501-1506.
29. Winge, M.C., et al., *Novel filaggrin mutation but no other loss-of-function variants found in Ethiopian patients with atopic dermatitis.* The British journal of dermatology, 2011. **165**(5): p. 1074-80.
30. Thawer-Esmail, F., et al., *South African amaXhosa patients with atopic dermatitis have decreased levels of filaggrin breakdown products but no loss-of-function mutations in filaggrin.* The Journal of allergy and clinical immunology, 2014. **133**(1): p. 280-2.e1-2.
31. Rippke, F., E. Berardesca, and T.M. Weber, *pH and Microbial Infections.* Current problems in dermatology, 2018. **54**: p. 87-94.
32. Lambers, H., et al., *Natural skin surface pH is on average below 5, which is beneficial for its resident flora.* International Journal of Cosmetic Science, 2006. **28**(5): p. 359-370.
33. Costa, F.G. and A.R. Horswill, *Overcoming pH defenses on the skin to establish infections.* PLoS pathogens, 2022. **18**(5): p. e1010512.

34. Mempel, M., et al., *Role of Staphylococcus aureus surface-associated proteins in the attachment to cultured HaCaT keratinocytes in a new adhesion assay.* The Journal of investigative dermatology, 1998. **111**(3): p. 452-6.
35. Clausen, M.-L., et al., *Staphylococcus aureus colonization in atopic eczema and its association with filaggrin gene mutations.* British Journal of Dermatology, 2017. **177**(5): p. 1394–1400.
36. Van Drongelen, V., et al., *Reduced filaggrin expression is accompanied by increasedStaphylococcus aureuscolonization of epidermal skin models.* Clinical & Experimental Allergy, 2014. **44**(12): p. 1515–1524.
37. Hülpüsch, C., et al., *Skin pH–dependent Staphylococcus aureus abundance as predictor for increasing atopic dermatitis severity.* Allergy, 2020. **75**(11): p. 2888-2898.
38. Badloe, F.M.S., et al., *IgE autoantibodies and autoreactive T cells and their role in children and adults with atopic dermatitis.* Clinical and Translational Allergy, 2020. **10**(1): p. 34.
39. Biedermann, T., et al., *Regulation of T Cell Immunity in Atopic Dermatitis by Microbes: The Yin and Yang of Cutaneous Inflammation.* Frontiers in immunology, 2015. **6**: p. 353.
40. Werfel, T., et al., *Cellular and molecular immunologic mechanisms in patients with atopic dermatitis.* Journal of allergy and clinical immunology, 2016. **138**(2): p. 336-349.
41. Furue, M., et al., *Atopic dermatitis: immune deviation, barrier dysfunction, IgE autoreactivity and new therapies. .* Allergology international., 2017. **66**(3): p. 398-403.
42. Brunner, P.M., E. Guttman-Yassky, and D.Y. Leung, *The immunology of atopic dermatitis and its reversibility with broad-spectrum and targeted therapies. .* Journal of Allergy and Clinical Immunology, 2017. **139**(4): p. S65-S76.
43. Tshikantwa, T.S., et al., *Current Trends and Potential Applications of Microbial Interactions for Human Welfare.* Frontiers in microbiology, 2018. **9**: p. 1156.
44. Baishya, J., et al., *The impact of intraspecies and interspecies bacterial interactions on disease outcome.* Pathogens, 2021. **10**(2): p. 96.
45. Dréno, B., et al., *Microbiome in healthy skin, update for dermatologists.* Journal of the European Academy of Dermatology and Venereology : JEADV, 2016. **30**(12): p. 2038-2047.
46. Parlet, C.P., M.M. Brown, and A.R. Horswill, *Commensal Staphylococci Influence Staphylococcus aureus Skin Colonization and Disease.* Trends in Microbiology, 2019.
47. Ong, P.Y., et al., *Endogenous antimicrobial peptides and skin infections in atopic dermatitis.* The New England journal of medicine, 2002. **347**(15): p. 1151-60.
48. Lai, Y., et al., *Activation of TLR2 by a small molecule produced by Staphylococcus epidermidis increases antimicrobial defense against bacterial skin infections.* Journal of Investigative Dermatology, 2010. **130**(9): p. 2211-2221.
49. Loomis, K.H., et al., *A mixed community of skin microbiome representatives influences cutaneous processes more than individual members.* Microbiome, 2021. **9**(1): p. 22.
50. Naik, S., et al., *Compartmentalized control of skin immunity by resident commensals.* Science, 2012. **337**(6098): p. 1115-9.

51. Chen, Y.E., M.A. Fischbach, and Y. Belkaid, *Skin microbiota-host interactions.* Nature 2018. **553**(7689): p. 427–436.
52. Grice, E.A., et al., *Topographical and temporal diversity of the human skin microbiome.* Science, 2009. **324**(5931): p. 1190-2.
53. Egert, M., & Simmering, R. , *The Microbiota of the Human Skin.* Advances in Experimental Medicine and Biology, 2016: p. 61-81.
54. Thomas, C.L. and P. & Fernández-Peñas, *The microbiome and atopic eczema:More than skin deep.* Australasian Journal of Dermatology, 2016. **58**(1): p. 18-24.
55. Grice, E.A. and J.A. Segre, *The skin microbiome.* Nature reviews. Microbiology, 2011. **9**(4): p. 244-53.
56. Totté, J.E.E., Pardo, L.M., Fieten, K.B., Vos, M.C., van den Broek, T.J., Schuren, F.H.J. and Pasmans, S.G.M.A., , *Nasal and skin microbiomes are associated with disease severity in paediatric atopic dermatitis.* British Journal of Dermatology, 181(4), pp.796-804., 2019. **181**(4): p. 796-804.
57. Zhou, Y., et al., *Exploration of bacterial community classes in major human habitats.* Genome biology, 2014. **15**(5): p. R66.
58. Laux, C., A. Peschel, and B. Krismer, *Staphylococcus aureus Colonization of the Human Nose and Interaction with Other Microbiome Members.* Microbiology spectrum, 2019. **7**(2).
59. Nakatsuji, T., et al., *Antimicrobials from human skin commensal bacteria protect against Staphylococcus aureus and are deficient in atopic dermatitis.* Science translational medicine, 2017. **9**(378): p. eaah4680.
60. Sugimoto, S., et al., *Staphylococcus epidermidis Esp degrades specific proteins associated with Staphylococcus aureus biofilm formation and host-pathogen interaction.* Journal of bacteriology, 2013. **195**(8): p. 1645–1655.
61. Gallo, R.L. and L.V. Hooper, *Epithelial antimicrobial defence of the skin and intestine.* Nature Reviews Immunology, 2012. **12**(7): p. 503-516.
62. Williams, M.R., et al., *Quorum sensing between bacterial species on the skin protects against epidermal injury in atopic dermatitis.* Science translational medicine, 2019. **11**(490): p. eaat8329.
63. Ong, P.Y. and D.Y. Leung, *Bacterial and viral infections in atopic dermatitis: a comprehensive review.* Clinical reviews in allergy & immunology, 2016. **51**(3): p. 329-337.
64. Bitschar, K., et al., *Lugdunin amplifies innate immune responses in the skin in synergy with host- and microbiota-derived factors.* Nature communications, 2019. **10**(1): p. 2730.
65. Zipperer, A., et al., *Human commensals producing a novel antibiotic impair pathogen colonization.* Nature, 2016. **535**(7613): p. 511-6.
66. Schaumburg, F., et al., *New epidemiology of Staphylococcus aureus infection in Africa. Clinical microbiology and infection* Clinical microbiology and infection, 2014. **20**(7): p. 589–596.
67. Kong, H.H., et al., *Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. .* Genome research, 2012. **22**(5): p. 850-859.
68. Iwase, T., et al., *Staphylococcus epidermidis Esp inhibits Staphylococcus aureus biofilm formation and nasal colonization.* Nature, 2010. **465**(7296): p. 346-349.

69. Cogen, A.L., et al., *Selective antimicrobial action is provided by phenol-soluble modulins derived from Staphylococcus epidermidis, a normal resident of the skin.* The Journal of investigative dermatology, 2010. **130**(1): p. 192-200.
70. Newstead, L.L., et al., *Staphylococcal-Produced Bacteriocins and Antimicrobial Peptides: Their Potential as Alternative Treatments for Staphylococcus aureus Infections.* Antibiotics (Basel, Switzerland), 2020. **9**(2).
71. Schittek, B., *The antimicrobial skin barrier in patients with atopic dermatitis.* Current problems in dermatology, 2011. **41**: p. 54-67.
72. Brogden, K.A., *Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?* Nature reviews microbiology, 2005. **3**(3): p. 238-250.
73. Vandecandelaere, I., et al., *Protease production by Staphylococcus epidermidis and its effect on Staphylococcus aureus biofilms.* Pathogens and disease, 2014. **70**(3): p. 321-31.
74. Glatthardt, T., et al., *Small Molecules Produced by Commensal Staphylococcus epidermidis Disrupt Formation of Biofilms by Staphylococcus aureus.* Applied and environmental microbiology, 2020. **86**(5).
75. Chen, C., et al., *Secreted proteases control autolysin-mediated biofilm growth of Staphylococcus aureus.* The Journal of biological chemistry, 2013. **288**(41): p. 29440-52.
76. Fredheim, E., Flægstad, T., Askarian, F., & Klingenberg, C. , *Colonisation and interaction between S. epidermidis and S. aureus in the nose and throat of healthy adolescents.* European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology,, 2015. **34**(1).
77. Ogonowska, P., et al., *Colonization With Staphylococcus aureus in Atopic Dermatitis Patients: Attempts to Reveal the Unknown.* Frontiers in microbiology, 2020. **11**: p. 567090.
78. Sonesson, A., et al., *Identification of bacterial biofilm and the Staphylococcus aureus derived protease, staphopain, on the skin surface of patients with atopic dermatitis.* Scientific reports, 2017. **7**(1).
79. Di Domenico, E.G., et al., *Inflammatory cytokines and biofilm production sustain Staphylococcus aureus outgrowth and persistence: a pivotal interplay in the pathogenesis of Atopic Dermatitis.* Scientific reports, 2018. **8**(1): p. 9573.
80. Shi, B., et al., *The skin microbiome is different in pediatric versus adult atopic dermatitis.* Journal of Allergy and Clinical Immunology, 2016. **138**(4): p. 1233-1236.
81. Byrd, A.L., et al., *Staphylococcus aureus and Staphylococcus epidermidis strain diversity underlying pediatric atopic dermatitis.* Science translational medicine, 2017. **9**(397): p. eaal4651.
82. Lebon, A., Labout, J.A., Verbrugh, H.A., Jaddoe, V.W., Hofman, A., van Wamel, W.J., van Belkum, A. and Moll, H.A., *Role of Staphylococcus aureus nasal colonization in atopic dermatitis in infants: the Generation R Study.* Archives of pediatrics & adolescent medicine, 2009. **163**(8): p. 745-749.
83. Francuzik, W., et al., *Propionibacterium acnes Abundance Correlates Inversely with Staphylococcus aureus: Data from Atopic Dermatitis Skin Microbiome.* Acta dermato-venereologica, 2018. **98**(5).
84. Kwon, S., et al., *Changes in Lesional and Non-lesional Skin Microbiome During Treatment of Atopic Dermatitis.* Acta dermato-venereologica, 2019. **99**(3): p. 284-290.

85. Ndhlovu, G.O.N., et al., *Molecular epidemiology of Staphylococcus aureus in African children from rural and urban communities with atopic dermatitis.* BMC infectious diseases,, 2021. **21**(1): p. 348.
86. Ndhlovu, G.O.N., et al., *Skin and nasal colonization of coagulase-negative staphylococci are associated with atopic dermatitis among South African toddlers.* PLoS One, 2022. **17**(3): p. e0265326.
87. Nakatsuji, T., Chen, T. H., Two, A. M., Chun, K. A., Narala, S., Geha, R. S., Hata, T. R., & Gallo, R. L. , *Staphylococcus aureus Exploits Epidermal Barrier Defects in Atopic Dermatitis to Trigger Cytokine Expression.* The Journal of investigative dermatology, 2016. **136**(11): p. 2192–2200.
88. Williams, M.R., et al., *Staphylococcus aureus Induces Increased Serine Protease Activity in Keratinocytes.* Journal of Investigative Dermatology, 2017. **137**(2): p. 377-384.
89. Cardona, I.D., S.H. Cho, and D.Y. Leung, *Role of bacterial superantigens in atopic dermatitis : implications for future therapeutic strategies.* American journal of clinical dermatology, 2006. **7**(5): p. 273-9.
90. Howell, M.D., Boguniewicz, M., Pastore, S., Novak, N., Bieber, T., Girolomoni, G., & Leung, D. Y. , *Mechanism of HBD-3 deficiency in atopic dermatitis.* Clinical immunology (Orlando, Fla.), 2006. **121**(3): p. 332-338.
91. Sieprawska-Lupa, M., Mydel, P., Krawczyk, K., Wójcik, K., Puklo, M., Lupa, B., Suder, P., Silberring, J., Reed, M., Pohl, J., Shafer, W., McAleese, F., Foster, T., Travis, J., & Potempa, J. , *Degradation of human antimicrobial peptide LL-37 by Staphylococcus aureus-derived proteinases.* Antimicrobial agents and chemotherapy, 2004. **48**(12): p. 4673-4679.
92. Gonzalez, T., et al., *Biofilm propensity of Staphylococcus aureus skin isolates is associated with increased atopic dermatitis severity and barrier dysfunction in the MPAACH pediatric cohort.* Allergy, 2021. **76**(1): p. 302-313.
93. Donlan, R.M. and J.W. Costerton, *Biofilms: survival mechanisms of clinically relevant microorganisms.* Clinical microbiology reviews, 2002. **15**(2): p. 167-93.
94. Flemming, H.C. and J. Wingender, *The biofilm matrix.* Nature reviews microbiology, 2010. **8**(9): p. 623-33.
95. Yin, W., et al., *Biofilms: The Microbial "Protective Clothing" in Extreme Environments.* International journal of molecular sciences, 2019. **20**(14).
96. Archer, N.K., et al., *Staphylococcus aureus biofilms: properties, regulation, and roles in human disease.* Virulence, 2011. **2**(5): p. 445-59.
97. Zheng, Y., et al., *Colonization of medical devices by staphylococci.* Environmental microbiology, 2018. **20**(9): p. 3141-3153.
98. Del Pozo, J.L., *Biofilm-related disease.* Expert review of anti-infective therapy, 2018. **16**(1): p. 51-65.
99. Shigeta, M., et al., *Permeation of antimicrobial agents through Pseudomonas aeruginosa biofilms: a simple method.* Chemotherapy, 1997. **43**(5): p. 340-5.
100. Bagge, N., et al., *Dynamics and spatial distribution of beta-lactamase expression in Pseudomonas aeruginosa biofilms.* Antimicrobial agents and chemotherapy, 2004. **48**(4): p. 1168-74.
101. Schilcher, K. and A.R. Horswill, *Staphylococcal Biofilm Development: Structure, Regulation, and Treatment Strategies.* Microbiology and molecular biology reviews : MMBR, 2020. **84**(3).

102. Moormeier, D.E. and K.W. Bayles, *Staphylococcus aureus biofilm: a complex developmental organism.* Molecular microbiology, 2017. **104**(3): p. 365-376.
103. Otto, M., *Staphylococcal Biofilms.* Microbiology Spectrum, 2018. **6**(4).
104. Weidenmaier, C., et al., *Role of teichoic acids in Staphylococcus aureus nasal colonization, a major risk factor in nosocomial infections.* Nature medicine, 2004. **10**(3): p. 243-5.
105. Winstel, V., et al., *Wall Teichoic Acid Glycosylation Governs Staphylococcus aureus Nasal Colonization.* mBio, 2015. **6**(4): p. e00632.
106. Baur, S., et al., *A nasal epithelial receptor for Staphylococcus aureus WTA governs adhesion to epithelial cells and modulates nasal colonization.* PLoS pathogens, 2014. **10**(5): p. e1004089.
107. Fluckiger, U., et al., *Biofilm formation, icaADBC transcription, and polysaccharide intercellular adhesin synthesis by staphylococci in a device-related infection model.* Infection and immunity, 2005. **73**(3): p. 1811-9.
108. Mack, D., et al., *Essential functional role of the polysaccharide intercellular adhesin of Staphylococcus epidermidis in hemagglutination.* Infection and immunity, 1999. **67**(2): p. 1004-8.
109. O'Gara, J.P., *ica and beyond: biofilm mechanisms and regulation in Staphylococcus epidermidis and Staphylococcus aureus.* FEMS microbiology letters, 2007. **270**(2): p. 179-88.
110. Toledo-Arana, A., et al., *Staphylococcus aureus develops an alternative, ica-independent biofilm in the absence of the arlRS two-component system.* Journal of Bacteriology, 2005. **187**(15): p. 5318-29.
111. Fitzpatrick, F., H. Humphreys, and J.P. O'Gara, *Evidence for icaADBC-independent biofilm development mechanism in methicillin-resistant Staphylococcus aureus clinical isolates.* Journal of Clinical Microbiology, 2005. **43**(4): p. 1973-6.
112. Hennig, S., S. Nyunt Wai, and W. Ziebuhr, *Spontaneous switch to PIA-independent biofilm formation in an ica-positive Staphylococcus epidermidis isolate.* International journal of medical microbiology : IJMM, 2007. **297**(2): p. 117-22.
113. Rohde, H., et al., *Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of Staphylococcus epidermidis and Staphylococcus aureus isolated from prosthetic hip and knee joint infections.* Biomaterials, 2007. **28**(9): p. 1711-20.
114. Formosa-Dague, C., et al., *Zinc-dependent mechanical properties of Staphylococcus aureus biofilm-forming surface protein SasG.* Proceedings of the National Academy of Sciences of the United States of America, 2016. **113**(2): p. 410-5.
115. Banner, M.A., et al., *Localized tufts of fibrils on Staphylococcus epidermidis NCTC 11047 are comprised of the accumulation-associated protein.* Journal of bacteriology, 2007. **189**(7): p. 2793-804.
116. Geoghegan, J.A., et al., *Role of surface protein SasG in biofilm formation by Staphylococcus aureus.* Journal of bacteriology, 2010. **192**(21): p. 5663-73.
117. Nguyen, H.T.T., T.H. Nguyen, and M. Otto, *The staphylococcal exopolysaccharide PIA - Biosynthesis and role in biofilm formation, colonization, and infection.* Computational and structural biotechnology journa, 2020. **18**: p. 3324-3334.
118. Yarawsky, A.E., et al., *The biofilm adhesion protein Aap from Staphylococcus epidermidis forms zinc-dependent amyloid fibers.* The Journal of biological chemistry, 2020. **295**(14): p. 4411-4427.

119. Allen, H.B., et al., *The presence and impact of biofilm-producing staphylococci in atopic dermatitis.* JAMA Dermatology 2014. **150**(3): p. 260-5.
120. Periasamy, S., et al., *How Staphylococcus aureus biofilms develop their characteristic structure.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(4): p. 1281-6.
121. Whitchurch, C.B., et al., *Extracellular DNA required for bacterial biofilm formation.* Science, 2002. **295**(5559): p. 1487.
122. Boles, B.R. and A.R. Horswill, *Agr-mediated dispersal of Staphylococcus aureus biofilms.* PLoS pathogens, 2008. **4**(4): p. e1000052.
123. Wilson, C., et al., *Quantitative and Qualitative Assessment Methods for Biofilm Growth: A Mini-review.* Research & reviews. Journal of engineering and technology, 2017. **6**(4).
124. Dalecki, A.G., C.L. Crawford, and F. Wolschendorf, *Targeting Biofilm Associated Staphylococcus aureus Using Resazurin Based Drug-susceptibility Assay.* Journal of visualized experiments : JoVE, 2016(111).
125. Van den Driessche, F., et al., *Optimization of resazurin-based viability staining for quantification of microbial biofilms.* Journal of microbiological methods, 2014. **98**: p. 31-4.
126. Reichhardt, C. and M.R. Parsek, *Confocal Laser Scanning Microscopy for Analysis of Pseudomonas aeruginosa Biofilm Architecture and Matrix Localization.* Frontiers in microbiology, 2019. **10**: p. 677.
127. Schlafer, S. and R.L. Meyer, *Confocal microscopy imaging of the biofilm matrix.* Journal of microbiological methods, 2017. **138**: p. 50-59.
128. Haney, E.F., M.J. Trimble, and R.E.W. Hancock, *Microtiter plate assays to assess antibiofilm activity against bacteria.* Nature protocols, 2021. **16**(5): p. 2615-2632.
129. Xu, Z., et al., *Crystal Violet and XTT Assays on Staphylococcus aureus Biofilm Quantification.* Current microbiology, 2016. **73**(4): p. 474-82.
130. Alonso, B., et al., *Comparison of the XTT and resazurin assays for quantification of the metabolic activity of Staphylococcus aureus biofilm.* Journal of microbiological methods, 2017. **139**: p. 135-137.
131. Brown, H.L., et al., *Tetrazolium reduction allows assessment of biofilm formation by Campylobacter jejuni in a food matrix model.* Journal of applied microbiology, 2013. **115**(5): p. 1212-21.
132. da Silva, W.J., et al., *Improvement of XTT assay performance for studies involving Candida albicans biofilms.* Brazilian dental journal, 2008. **19**(4): p. 364-9.
133. Chandra, J., et al., *Interaction of Candida albicans with adherent human peripheral blood mononuclear cells increases C. albicans biofilm formation and results in differential expression of pro-and anti-inflammatory cytokines.* Infection and immunity, 2007. **75**(5): p. 2612-2620.
134. Jesaitis, A.J., et al., *Compromised host defense on Pseudomonas aeruginosa biofilms: characterization of neutrophil and biofilm interactions.* Journal of immunology, 2003. **171**(8): p. 4329-39.
135. Kristian, S.A., et al., *Biofilm formation induces C3a release and protects Staphylococcus epidermidis from IgG and complement deposition and from neutrophil-dependent killing.* The Journal of infectious diseases, 2008. **197**(7): p. 1028-35.

136. Thurlow, L.R., et al., *Staphylococcus aureus biofilms prevent macrophage phagocytosis and attenuate inflammation in vivo.* Journal of Immunology, 2011. **186**(11): p. 6585-96.
137. Hanke, M.L., et al., *Targeting macrophage activation for the prevention and treatment of Staphylococcus aureus biofilm infections.* Journal of immunology 2013. **190**(5): p. 2159-68.
138. Scherr, T.D., et al., *Staphylococcus aureus Biofilms Induce Macrophage Dysfunction Through Leukocidin AB and Alpha-Toxin.* mBio, 2015. **6**(4).
139. Carvalhais, V., et al., *Dormancy within Staphylococcus epidermidis biofilms: a transcriptomic analysis by RNA-seq.* Applied microbiology and biotechnology, 2014. **98**(6): p. 2585-96.
140. Schommer, N.N., et al., *Staphylococcus epidermidis uses distinct mechanisms of biofilm formation to interfere with phagocytosis and activation of mouse macrophage-like cells 774A.1.* Infection and immunity, 2011. **79**(6): p. 2267-76.
141. Blicharz, L., Michalak, M., Szymanek-Majchrzak, K., Młynarczyk, G., Skowroński, K., Rudnicka, L., & Samochocki, Z. , *The Propensity to Form Biofilm in vitro by Staphylococcus aureus Strains Isolated from the Anterior Nares of Patients with Atopic Dermatitis: Clinical Associations.* Dermatology, 2020: p. 1-7.
142. Manandhar, S., et al., *Phenotypic and genotypic characterization of biofilm producing clinical coagulase negative staphylococci from Nepal and their antibiotic susceptibility pattern.* Annals of clinical microbiology and antimicrobials,, 2021. **20**(1): p. 41.
143. Allen, H.B. and J.L. Mueller, *A novel finding in atopic dermatitis: film-producing Staphylococcus epidermidis as an etiology.* International journal of dermatology, 2011. **50**(8): p. 992-3.
144. Allen, H., et al., *Eccrine sweat duct occlusion by Staphylococcal-derived biofilms: an unexpected signature finding of eczema in dermatologic diseases.* Journal of Clinical and Experimental Dermatology Research, 2015. **6**(310): p. 2.
145. Akiyama, H., et al., *Confocal laser scanning microscopic observation of glycocalyx production by Staphylococcus aureus in skin lesions of bullous impetigo, atopic dermatitis and pemphigus foliaceus.* British Journal of Dermatology, 2003. **148**(3): p. 526-32.
146. Cavalcante, F.S., et al., *Methicillin-resistant Staphylococcus aureus from infected skin lesions present several virulence genes and are associated with the CC30 in Brazilian children with atopic dermatitis.* Virulence, 2021. **12**(1): p. 260-269.
147. Vuong, C., et al., *Increased colonization of indwelling medical devices by quorum-sensing mutants of Staphylococcus epidermidis in vivo.* The Journal of infectious diseases, 2004. **190**(8): p. 1498-505.
148. Gupta, V.K., Paul, S., & Dutta, C., *Geography, Ethnicity or Subsistence-Specific Variations in Human Microbiome Composition and Diversity.* Frontiers in microbiology, 2017. **8**(1162).
149. de Lusignan, S., et al., *The epidemiology of eczema in children and adults in England: A population-based study using primary care data.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2021. **51**(3): p. 471-482.

150. von Hertzen, L.C., & Haahtela, T. (2004). , *Immunization and atopy: possible implications of ethnicity.* The Journal of allergy and clinical immunology, 2004. **113**(401-406).
151. Meylan, P., et al., *Skin Colonization by Staphylococcus aureus Precedes the Clinical Diagnosis of Atopic Dermatitis in Infancy.* The Journal of investigative dermatology, 2017. **137**(12): p. 2497-2504.
152. Kennedy, E.A., et al., *Skin microbiome before development of atopic dermatitis: Early colonization with commensal staphylococci at 2 months is associated with a lower risk of atopic dermatitis at 1 year.* The Journal of allergy and clinical immunology, 2017. **139**(1): p. 166-172.
153. Smith, F.J., et al., *Loss-of-function mutations in the gene encoding filaggrin cause ichthyosis vulgaris.* Nature genetics, 2006. **38**(3): p. 337-42.
154. Levin, M.E., et al., *Environmental factors associated with allergy in urban and rural children from the South African Food Allergy (SAFFA) cohort.* Journal of Allergy and Clinical Immunology, 2020. **145**(1): p. 415-426.
155. Ndhlovu, G.O.N., *Bacteriome interactions in pediatric atopic dermatitis in a rural and urban South African cohort*, in *Department of Molecular and Cell biology*. 2023, University of Cape Town.
156. Williams, H.C., et al., *The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. III. Independent hospital validation.* The British journal of dermatology, 1994. **131**(3): p. 406-16.
157. Kunz, B., et al., *Clinical validation and guidelines for the SCORAD index: consensus report of the European Task Force on Atopic Dermatitis.* Dermatology, 1997. **195**(1): p. 10-9.
158. Dube, F.S., et al., *Detection of Streptococcus pneumoniae from Different Types of Nasopharyngeal Swabs in Children.* PLoS One, 2013. **8**(6): p. e68097.
159. Moonsamy, R.T., *Colonisation dynamics of Staphylococcus epidermidis in paediatric atopic dermatitis in rural and urban South African settings*, in *Department of Molecular and Cell Biology*. 2020, University of Cape Town: Unpublished
160. Tecan, *Automated solution for monitoring growth of Staphylococcus aureus. Optimizing bacterial growth studies on the Infinite® 200 PRO multimode reader platform*
using permanent shaking and heating. 2012. **1**.
161. Uršič, V., V. Tomič, and M. Košnik, *Effect of different incubation atmospheres on the production of biofilm in methicillin-resistant Staphylococcus aureus (MRSA) grown in nutrient-limited medium.* Current microbiology, 2008. **57**(4): p. 386-390.
162. Lee, D.C., et al., *Bacterial Colonization of the Hospitalized Newborn: Competition Between Staphylococcus aureus and Staphylococcus epidermidis.* The Pediatric infectious disease journal, 2019. **38**(7): p. 682-686.
163. Izano, E.A., et al., *Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in Staphylococcus aureus and Staphylococcus epidermidis biofilms.* Applied and environmental microbiology, 2008. **74**(2): p. 470-6.
164. Sager, M., et al., *Characterization of Biofilm Formation in [Pasteurella] pneumotropica and [Actinobacillus] muris Isolates of Mouse Origin.* PLoS One, 2015. **10**(10): p. e0138778.

165. Ikeda, Y., et al., *PCR-based identification of Staphylococcus epidermidis targeting gseA encoding the glutamic-acid-specific protease.* Canadian journal of microbiology, 2004. **50**(7): p. 493-8.
166. Thomas, J.C., et al., *Improved multilocus sequence typing scheme for Staphylococcus epidermidis.* Journal of clinical microbiology, 2007. **45**(2): p. 616-9.
167. Cau, L., et al., *Staphylococcus epidermidis protease EcpA can be a deleterious component of the skin microbiome in atopic dermatitis.* Journal of Allergy and Clinical Immunology, 2021. **147**(3): p. 955-966.e16.
168. Liu, Q., et al., *Staphylococcus epidermidis Contributes to Healthy Maturation of the Nasal Microbiome by Stimulating Antimicrobial Peptide Production.* Cell host & microbe, 2020. **27**(1): p. 68-78.e5.
169. den Heijer, C.D., et al., *Prevalence and resistance of commensal Staphylococcus aureus, including meticillin-resistant S aureus, in nine European countries: a cross-sectional study.* The Lancet. Infectious diseases, 2013. **13**(5): p. 409-15.
170. Hardy, B.L., et al., *Antimicrobial Activity of Clinically Isolated Bacterial Species Against Staphylococcus aureus.* Frontiers in microbiology, 2019. **10**: p. 2977.
171. Babrud, R.B., et al., *The effect of Lactobacillus reuteri cell free supernatant on growth and biofilm formation of Paenibacillus larvae.* Iranian Journal of Veterinary Research, 2019. **20**(3): p. 192.
172. Martínez-García, S., et al., *Extracellular proteases of Staphylococcus epidermidis: roles as virulence factors and their participation in biofilm.* APMIS : acta pathologica, microbiologica, et immunologica Scandinavica, 2018. **126**(3): p. 177-185.
173. Zhou, Y., et al., *Heterogeneous regulation of Staphylococcus aureus by different Staphylococcus epidermidis agr types in atopic dermatitis.* Journal of Investigative Dermatology, 2023.
174. Vuong, C., et al., *Impact of the agr quorum-sensing system on adherence to polystyrene in Staphylococcus aureus.* The Journal of infectious diseases, 2000. **182**(6): p. 1688-93.
175. Paharik, A.E., et al., *Coagulase-Negative Staphylococcal Strain Prevents Staphylococcus aureus Colonization and Skin Infection by Blocking Quorum Sensing.* Cell host & microbe, 2017. **22**(6): p. 746-756.e5.
176. Holtfreter, S., et al., *Clonal distribution of superantigen genes in clinical Staphylococcus aureus isolates.* Journal of clinical microbiology, 2007. **45**(8): p. 2669-80.
177. Yadav, M.K., et al., *In vitro Multi-Species Biofilms of Methicillin-Resistant Staphylococcus aureus and Pseudomonas aeruginosa and Their Host Interaction during In vivo Colonization of an Otitis Media Rat Model.* Frontiers in cellular and infection microbiology, 2017. **7**: p. 125.
178. Brady, A.J., et al., *Antibiotic susceptibility of planktonic- and biofilm-grown staphylococci isolated from implant-associated infections: should MBEC and nature of biofilm formation replace MIC?* Journal of medical microbiology, 2017. **66**(4): p. 461-469.
179. O'Neill, E., et al., *Association between methicillin susceptibility and biofilm regulation in Staphylococcus aureus isolates from device-related infections.* Journal of Clinical Microbiology, 2007. **45**(5): p. 1379-88.
180. McCarthy, H., et al., *Methicillin resistance and the biofilm phenotype in Staphylococcus aureus.* Frontiers in cellular and infection microbiology, 2015. **5**: p. 1.

181. Kim, J., et al., *Interactions Between Atopic Dermatitis and Staphylococcus aureus Infection: Clinical Implications.* Allergy, asthma & immunology research, 2019. **11**(5): p. 593-603.
182. Otto, M., *Staphylococcal infections: mechanisms of biofilm maturation and detachment as critical determinants of pathogenicity.* Annual review of medicine, 2013. **64**: p. 175-88.
183. Toledo-Silva, B., et al., *Metabolites of bovine-associated non-aureus staphylococci influence expression of Staphylococcus aureus agr-related genes in vitro.* Veterinary Research, 2021. **52**(1): p. 62.

Chapter 8: Supplementary data

Supplementary Table 1: Untransformed data for biofilm formation of S. aureus isolates incubated with corresponding S. epidermidis supernatant compared to S. aureus incubated with media.

Pair	Control			Experiment		
	1	2	3	1	2	3
SM1	0,0908	0,1122	0,1221	0,1018	0,0792	0,1193
SS2	0,0886	0,0863	0,0909	0,0792	0,0627	0,0764
SM3	0,091	0,1018	0,1027	0,0953	0,0871	0,0959
SS4	0,053	0,065	0,0703	0,068	0,0885	0,0794
SM5	0,0493	0,0517	0,0597	0,0886	0,0916	0,1187
SM6	0,0653	0,0624	0,0783	0,1079	0,0988	0,1356
SM7	1,0812	0,0599	0,0644	0,0577	0,0762	0,0915
SM8	0,9893	0,3299	0,9538	0,0566	0,0839	0,0699
SS9	0,1036	0,1623	0,1317	0,0939	0,0925	0,1295
MS10	0,1309	0,1781	0,1213	0,1238	0,1422	0,2143
MS11	0,0869	0,1051	0,1201	0,1276	0,1252	0,1167
SM12	0,0977	0,0992	0,1133	0,0874	0,108	0,0981
SM13	0,1412	0,1236	0,1751	0,089	0,0879	0,0901
SS14	0,1963	0,1458	0,1888	0,0801	0,0908	0,1069
SS15	0,0837	0,072	0,091	0,0867	0,1101	0,075
MS16	0,114	0,1739	0,1186	0,1101	0,0808	0,0863
SM17	0,2331	0,2748	0,2608	0,1214	0,1164	0,0935
MS18	0,1648	0,127	0,1213	0,2675	0,1285	0,1646
SS19	0,6025	0,3634	0,1929	0,3233	0,3078	0,2927
SS20	0,1482	0,0957	0,0963	0,1685	0,1507	0,1449
SS21	0,1891	0,2051	0,1561	0,0823	0,1109	0,1073
MS22	0,4735	0,4166	0,2413	0,3484	0,3898	0,3586
MS23	0,1845	0,1179	0,1511	0,2625	0,1733	0,2429
SM24	0,1898	0,1729	0,1665	0,2207	0,2008	0,1053
SM25	0,1416	0,0807	0,0864	0,0608	0,057	0,0542
SS26	0,1993	0,2475	0,243	0,0801	0,3034	0,0624
SS27	0,2065	0,1256	0,1085	0,064	0,0747	0,0891
SM28	0,2184	0,2032	0,9494	0,2941	0,1179	0,1285

Supplementary Table 2: Untransformed data for cell viability of S. aureus isolates grown in biofilms incubated with corresponding S. epidermidis isolates compared to S. aureus incubated with media only.

Pair	Control			Experiment		
	1	2	3	1	2	3
SM1	22011,83	23937,83	24310,83	24925,83	25610,83	25904,83
SS2	21398,8333	23284,8333	24415,8333	26159,8333	26160,8333	26669,8333
SM3	24296,83	24884,83	24322,83	26318,83	25422,83	25987,83
SS4	24345,8333	26234,8333	26746,8333	25936,8333	25645,8333	26735,8333
SM5	24544,8333	25618,8333	26974,8333	26358,8333	26191,8333	26516,8333
SM6	24235,8333	26331,8333	26553,8333	23423,8333	23356,8333	24794,8333
SM7	24102,8333	24842,8333	25156,8333	26698,8333	26357,8333	27069,8333
SM8	24865,8333	27053,8333	26469,8333	6770,83333	17482,8333	17851,8333
SS9	22616,83	23413,83	22675,83	25555,83	25872,83	26506,83
MS10	23850,8333	25880,8333	25891,8333	26137,8333	26374,8333	26922,8333
MS11	23681,83	26496,83	25687,83	25989,83	26094,83	25724,83
SM12	19769,8333	21096,8333	23109,8333	26034,8333	25231,8333	27169,8333
SM13	22671,8333	25524,8333	24984,8333	24688,8333	25921,8333	25119,8333
SS14	23637,8333	25649,8333	23797,8333	-2660,1667	-1690,1667	1565,83333
SS15	24697,83	25238,83	26900,83	24967,83	24263,83	26312,83
MS16	4953,83	15850,83	12192,83	17671,83	22615,83	18689,83
SM17	25469,8333	26961,8333	26882,8333	27214,8333	28368,8333	29496,8333
MS18	1058,83333	5518,83333	1377,83333	4212,83333	2354,83333	3124,83333
SS19	14982,8333	10065,8333	7484,83333	8775,83333	9401,83333	10482,8333
SS20	25474,8333	28683,8333	29009,8333	27302,8333	30006,8333	28352,8333
SS21	26061,8333	27458,8333	28342,8333	26720,8333	29658,8333	28754,8333
MS22	16198,8333	16267,8333	23970,8333	12990,8333	8087,83333	4576,83333
MS23	22594,83	17618,83	21498,83	16301,83	26380,83	22312,83
SM24	26555,83	26859,83	28300,83	25835,83	26880,83	26488,83
SM25	26859,8333	27936,8333	27979,8333	26598,8333	28081,8333	26542,8333
SS26	25344,8333	26454,8333	26473,8333	25090,8333	26165,8333	24264,8333
SS27	25730,8333	27233,8333	22630,8333	25011,8333	25259,8333	24949,8333
SM28	26035,8333	27988,8333	26510,8333	26331,8333	26602,8333	26231,8333

Supplementary Table 3: Untransformed data for biofilm mass of established S. aureus biofilms incubated with proteinase K (PK) compared to controls incubated in TSB media only.

Pair	Control			Experiment		
	1	2	3	1	2	3
SM1	0,1876	0,1976	0,2799	0,2862	0,3457	0,3388
SS2	0,0662	0,0677	0,0628	0,0605	0,0749	0,0612
SM3	0,289	0,5909	0,5032	0,1837	0,2093	0,2263
SS4	0,2881	0,2916	0,2835	0,3026	0,2712	0,3052
SM5	0,1105	0,1055	0,1106	0,8187	0,1722	0,2055
SM6	0,1053	0,1011	0,098	0,3273	0,3374	0,2991
SM7	0,109	0,1848	0,1587	0,15	0,1835	0,1677
SM8	0,0901	0,1618	0,1904	0,1628	0,1666	0,208
SS9	0,0901	0,09	0,0888	0,0666	0,0606	0,0588
MS10	0,1035	0,1667	0,1354	0,183	0,2764	0,2035
MS11	0,2602	0,3739	0,2259	0,2767	0,2136	0,1764
SM12	0,4027	0,3587	0,1821	0,2119	0,1108	0,1377
SM13	0,2624	0,1631	0,1884	0,0858	0,0552	0,0679
SS14	0,2807	0,2678	0,223	0,1599	0,1193	0,1335
SS15	0,1206	0,0752	0,0825	0,0552	0,0748	0,0572
MS16	0,0839	0,099	0,0763	0,0714	0,0571	0,0579
SM17	0,056	0,0585	0,0577	0,0908	0,096	0,0773
MS18	0,2331	0,2748	0,2608	0,1214	0,1164	0,0935
SS19	0,0826	0,067	0,0723	0,0658	0,0588	0,0562
SS20	0,6025	0,3634	0,1929	0,3233	0,3078	0,2927
SS21	0,4735	0,4166	0,2413	0,3484	0,3898	0,3586
MS22	0,0826	0,067	0,0723	0,0658	0,0588	0,0562
MS23	0,1993	0,2475	0,243	0,0801	0,3034	0,0624
SM24	0,1474	0,1459	0,1261	0,0547	0,0538	0,06
SM25	0,1898	0,1729	0,1665	0,2207	0,2008	0,1053
SS26	0,1474	0,1459	0,1261	0,0547	0,0538	0,06
SS27	0,0496	0,1978	0,1166	0,0467	0,0508	0,0526
SM28	0,1293	0,1145	0,0973	0,0605	0,055	0,0618

Supplementary Table 4: Untransformed data for biofilm mass of established S. aureus biofilms incubated with sodium metaperiodate (SM) compared to controls incubated in TSB media only.

Pair	Control			Experiment		
	1	2	3	1	2	3
S1c	0,1691	0,1213	0,1938	0,2863	0,2714	0,2131
S2	0,1802	0,1716	0,2337	0,3698	0,4907	0,5033
S3	0,1485	0,2319	0,2602	0,5111	0,4584	0,6683
S4	0,1004	0,0892	0,1247	0,1569	0,128	0,2495
S5	0,0991	0,1362	0,1362	0,1375	0,1901	0,1906
S6	0,1197	0,1688	0,171	0,2181	0,2687	0,1819
S7	0,133	0,1309	0,125	0,2858	0,2878	0,2562
S8	0,0937	0,1639	0,1385	0,1895	0,1402	0,1877
S9	0,1067	0,118	0,0886	0,2321	0,1175	0,1151
M10	0,1222	0,1165	0,1025	0,1146	0,1223	0,1038
M11	0,1396	0,1397	0,0985	0,1154	0,1137	0,0889
S12	0,1419	0,1248	0,1114	0,1537	0,1991	0,1473
S13	0,2194	0,1991	0,2071	0,1962	0,2213	0,1349
S14	0,2525	0,1932	0,205	0,177	0,2321	0,2345
S15c	0,1381	0,1224	0,113	0,1509	0,1165	0,0996
M16	0,0772	0,0637	0,0635	0,1082	0,0804	0,0713
S17	0,1175	0,1243	0,1201	0,24	0,2617	0,1774
M18c	0,0837	0,0697	0,0595	0,0645	0,1104	0,0606
S19	0,1954	0,182	0,1249	0,2688	0,1985	0,1715
S20	0,0963	0,117	0,0833	0,1427	0,158	0,2346
S21	0,1785	0,1143	0,106	0,1639	0,207	0,1474
M22	0,1422	0,146	0,1002	0,192	0,1878	0,1709
M23	0,1154	0,1025	0,1091	0,2001	0,2377	0,2125
S24c	0,1299	0,1134	0,1023	0,1581	0,2141	0,2305
S25c	0,111	0,1051	0,0961	0,1533	0,1623	0,1374
S26c	0,3348	0,2425	0,3685	0,3072	0,4335	0,492
S27c	0,365	0,3838	0,1595	0,3852	0,5101	0,4343

Supplementary Table 5: Supplementary table 5: Untransformed data of biofilm formation when S. aureus cultures were incubated with heat-treated corresponding S. epidermidis supernatant.

Pair	Control			Experiment		
SM1	0,0731	0,0827	0,1102	0,0953	0,1023	0,1151
SS2	0,1474	0,1373	0,1246	0,0932	0,0914	0,0896
SM3	0,0809	0,073	0,0977	0,1347	0,0739	0,2297
SS4	0,1496	0,1252	0,1441	0,0808	0,0775	0,0719
SM5	0,0972	0,194	0,1641	0,1302	0,1249	0,1682
SM6	0,13	0,1575	0,1222	0,0906	0,151	0,1094
SM7	0,0993	0,1314	0,247	0,0903	0,1153	0,123
SM8	0,0979	0,1233	0,0905	0,0753	0,1464	0,1226
SS9	0,1898	0,1766	0,2093	0,2158	0,1783	0,1836
MS10	0,0624	0,0619	0,0898	0,1131	0,2158	0,1783
MS11	0,1364	0,1174	0,1067	0,0622	0,0574	0,0547
SM12	0,1725	0,1554	0,1389	0,0915	0,0788	0,0699
SM13	0,1228	0,1349	0,1262	0,0867	0,0735	0,0606
SS14	0,1522	0,2409	0,375	0,1364	0,0855	0,1378
SS15	0,1783	0,1275	0,1319	0,0809	0,0641	0,0946
MS16	0,4206	0,2256	0,2676	0,0283	0,0289	0,0239
SM17	0,5722	0,3642	0,5755	0,1102	0,1051	0,0955
MS18	0,0118	0,0221	0,0242	0,0342	0,0274	0,028
SS19	0,0189	0,025	0,0346	0,046	0,0842	0,0477
SS20	0,0468	0,0391	0,0557	0,1001	0,1054	0,0949
SS21	0,0256	0,0194	0,0277	0,0265	0,033	0,0536
MS22	0,0318	0,0337	0,0369	0,0417	0,0399	0,0381
MS23	0,0383	0,0567	0,0554	0,0376	0,0243	0,0462
SM24	0,0243	0,0505	0,0311	0,03	0,0348	0,028
SM25	0,0382	0,0302	0,0247	0,0271	0,0487	0,0517
SS26	0,0498	0,062	0,0439	0,0455	0,0341	0,0439
SS27	0,3377	0,1823	0,1719	0,0458	0,0321	0,0484
SM28	0,06	0,0667	0,0712	0,1302	0,1153	0,1094

Supplementary Table 6: Supplementary table 6: Untransformed data of biofilm formation when S. aureus cultures were incubated with SM-treated corresponding S. epidermidis supernatant.

Pair	Control			Experiment		
S1c	0,1691	0,1213	0,1938	0,2863	0,2714	0,2131
S2	0,1802	0,1716	0,2337	0,3698	0,4907	0,5033
S3	0,1485	0,2319	0,2602	0,5111	0,4584	0,6683
S4	0,1004	0,0892	0,1247	0,1569	0,1280	0,2495
S5	0,0991	0,1362	0,1362	0,1375	0,1901	0,1906
S6	0,1197	0,1688	0,1710	0,2181	0,2687	0,1819
S7	0,1330	0,1309	0,1250	0,2858	0,2878	0,2562
S8	0,0937	0,1639	0,1385	0,1895	0,1402	0,1877
S9	0,1067	0,1180	0,0886	0,2321	0,1175	0,1151
M10	0,1222	0,1165	0,1025	0,1146	0,1223	0,1038
M11	0,1396	0,1397	0,0985	0,1154	0,1137	0,0889
S12	0,1419	0,1248	0,1114	0,1537	0,1991	0,1473
S13	0,2194	0,1991	0,2071	0,1962	0,2213	0,1349
S14	0,2525	0,1932	0,2050	0,1770	0,2321	0,2345
S15c	0,1381	0,1224	0,1130	0,1509	0,1165	0,0996
M16	0,0772	0,0637	0,0635	0,1082	0,0804	0,0713
S17	0,1175	0,1243	0,1201	0,2400	0,2617	0,1774
M18c	0,0837	0,0697	0,0595	0,0645	0,1104	0,0606
S19	0,1954	0,1820	0,1249	0,2688	0,1985	0,1715
S20	0,0963	0,1170	0,0833	0,1427	0,1580	0,2346
S21	0,1785	0,1143	0,1060	0,1639	0,2070	0,1474
M22	0,1422	0,1460	0,1002	0,1920	0,1878	0,1709
M23	0,1154	0,1025	0,1091	0,2001	0,2377	0,2125
S24c	0,1299	0,1134	0,1023	0,1581	0,2141	0,2305
S25c	0,1110	0,1051	0,0961	0,1533	0,1623	0,1374
S26c	0,3348	0,2425	0,3685	0,3072	0,4335	0,4920
S27c	0,3650	0,3838	0,1595	0,3852	0,5101	0,4343

Supplementary Table 7: Untransformed data of biofilm formation when S. aureus cultures were incubated with PK-treated corresponding S. epidermidis supernatant.

Pair	Control			` Experiment		
S1c	0,0908	0,1122	0,1221	0,2299	0,2443	0,2270
S2	0,0886	0,0863	0,0909	0,1288	0,1360	0,1278
S3	0,0910	0,1018	0,1027	0,1171	0,0906	0,1109
S4	0,0530	0,0650	0,0703	0,0662	0,0812	0,0873
S5	0,0493	0,0517	0,0597	0,0663	0,0792	0,0892
S6	0,0633	0,0619	0,0588	0,0618	0,0715	0,0627
S7	1,0812	0,0599	0,0644	0,0666	0,0770	0,0787
S8	0,9893	0,3299	0,9538	0,0623	0,1070	0,1189
S9	0,1036	0,1623	0,1317	0,1029	0,1134	0,0951
M10	0,1309	0,1781	0,1213	0,0884	0,0725	0,0774
M11	0,0869	0,1051	0,1201	0,0665	0,0587	0,0733
S12	0,0662	0,0677	0,0628	0,0605	0,0749	0,0612
S13	0,1412	0,1236	0,1751	0,0715	0,0762	0,0836
S14	0,1963	0,1458	0,1888	0,1014	0,1050	0,0895
S15c	0,0837	0,0720	0,0910	0,0629	0,0669	0,0750
M16	0,1140	0,1739	0,1186	0,1091	0,0937	0,1315
S17	0,056	0,0585	0,0577	0,0908	0,096	0,0773
M18c	0,0421	0,0541	0,0551	0,0658	0,0770	0,0612
S19	0,0826	0,067	0,0723	0,0658	0,0588	0,0562
S20	0,0580	0,0639	0,0596	0,0599	0,0592	0,0582
S21	0,0660	0,0574	0,0561	0,0624	0,0587	0,0556
M22	0,0697	0,0744	0,0680	0,0618	0,0551	0,0591
M23	0,07	0,0665	0,063	0,0611	0,0622	0,0717
S24c	0,1474	0,1459	0,1261	0,0547	0,0538	0,06
S25c	0,1293	0,1145	0,0973	0,0605	0,055	0,0618
S26c	0,3422	0,3174	0,2450	0,1035	0,0885	0,1043
S27c	0,0620	0,0883	0,0587	0,0660	0,0707	0,0704