The effects of staphylococci on keratinocytes, with a view to understanding the pathogenesis of atopic dermatitis

Virginia-Evdokia Pappa

Submitted in accordance with the requirements for the degree of Doctor of Philosophy

The University of Leeds

Faculty of Medicine and Health

June, 2019

Declaration

The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

The right of Virginia-Evdokia Pappa to be identified as Author of this work has been asserted by her in accordance with the Copyright, Designs and Patents Act 1988.

Acknowledgements

I would like to thank my supervisors, Dr Miriam Wittmann and Dr Alex O'Neill for their valuable guidance and support throughout the project. I would also like to thank the post-doctoral researchers, Dr Adewonuola Alase and Dr Liam Sharkey for their help with the laboratory work. Special thanks to Dr James Ault and Rachel George from the mass spectrometry facility of the University of Leeds, to Dr Iain Manfield for his advice on the chromatography experiments and to Dr Justin Clarke, Ioanna Georgiou and Ciaran Neeson for their support during the final stages of the project.

I would also like to thank the MOSAR study group, who collected and pre-characterised the MOS *S. aureus* strains during the MOSAR project, the Research Group of Host-Microbe Interactions, Department of Medical Biology, Faculty of Health Sciences, UiT, Arctic University of Norway, who provided the *Lactococcus lactis* strains that were used in this project and the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA), BEI Resources, National Institute of Allergy and Infectious Diseases, NIH, for providing the NRS strains and the *S. aureus* USA300 mutant strains from the Nebraska Transposon Mutant Library (NTML).

Abstract

Background: Atopic dermatitis (AD) is a common chronic inflammatory skin disease, which significantly impairs patients' quality of life. A typical AD feature is colonisation and infection of the skin with *Staphylococcus aureus*, which leads to loss of commensal staphylococci (*Staphylococcus epidermidis*) from the skin. Although *S. epidermidis* and *S. aureus* have many similarities, *S. aureus* is a major cause of skin infections, unlike *S. epidermidis*, which is abundant in normal skin. The balance between *S. epidermidis* and *S. aureus* is disturbed in AD and there is increasing evidence that microbial dysbiosis is associated with AD pathogenesis.

Hypothesis: Skin cells respond differently to commensal staphylococci (*S. epidermidis*) and bacteria associated with skin infections, such as *S. aureus*.

Results: Cell-suspensions and filter-sterilised supernatants of most *S. aureus* strains induced human-beta-defensin (HBD)-3 production in cultured keratinocytes and skin equivalents, measured by enzyme-linked immunosorbent assay (ELISA), whereas *S. epidermidis* and a minority of *S. aureus* strains did not. The *S. aureus* factor(s) responsible for the HBD-3 induction were found to be heat-stable and likely proteinaceous. Candidate *S. aureus* proteins were identified using ion-exchange chromatography and mass spectrometry and their HBD-3-inducing capacity was tested. Phenotypic characterisation indicated compounds that may favour *S. epidermidis* over *S. aureus* growth.

Discussion: *S. epidermidis* and *S. aureus* have a different effect on HBD-3 induction in keratinocytes. This suggests that the skin has evolved an effective defence strategy against *S. aureus* (HBD-3 production) and a means of triggering this response specifically to non-commensal staphylococci by detecting a cell component unique to *S. aureus*. AD patients have increased susceptibility to *S. aureus* infection. If disruption of the skin microbial balance is proven to be able to trigger AD, or if restoration of skin flora can resolve AD, this would suggest that skin microbial dysbiosis is a key factor in AD pathogenesis.

Keywords

Atopic dermatitis, atopic eczema, *Staphylococcus aureus*, *Staphylococcus epidermidis*, skin microbiome, skin barrier, antimicrobial peptides, human-beta defensins (HBDs), HBD-3

Table of Contents

List of Tables

List of Figures

Abbreviations

The following abbreviations have been used throughout this dissertation:

AD	atopic dermatitis
Agr	accessory gene regulator
AIP	autoinducing peptide
AMP	antimicrobial peptide
ANOVA	one-way analysis of variance
AP	activator protein
BLAST	basic local alignment search tool
bp	base pair
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CFU	colony forming units
cis-UCA	cis-urocanic acid
CLA	cutaneous lymphocyte-associated antigen
CLRs	C-type lectin receptors
CLSI	Clinical and Laboratory Standards Institute
CO_2	carbon dioxide
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EEFIC	Epidemic European Fusidic acid-resistant
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FcεRI	high-affinity IgE receptor
g	gravity
g	gram
h	hour
HaCaT	human aneuploid immortal keratinocyte cell line from adult skin
HBD	human-beta defensin
IFN	interferon
Ig	immunoglobulin
IL	interleukin
JAK	janus kinase
JNK	c-Jun N-terminal kinase
kDa	kilodalton
KGM	keratinocyte growth medium
L	litre
LC-MS	liquid chromatography-mass spectrometry

M	molar
MAPK	mitogen-activated protein kinases
mAU	milli-absorbance unit
MES	2-(N-morpholino)ethanesulfonic acid
mg	milligram
MHA-II/MHB-II	Mueller-Hinton agar/broth II
MIC	minimum inhibitory concentration
min	minute
mL	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
MRSA	methicillin resistant *Staphylococcus aureus*
MSCRAMMs	microbial surface components recognising adhesive matrix molecules
NaCl	sodium chloride
NARSA	Network on Antimicrobial Resistance in *S. aureus*
NCBI	National Center for Biotechnology Information
NF-κB	nuclear factor kappa-light-chain-enhancer of activated beta cells
ng	nanogram
NLRs	NOD-like receptors
nm	nanometre
NOD	nucleotide-binding oligomerisation domain
NTML	Nebraska Transposon Mutant Library
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PenStrep	penicillin-streptomycin
pg	picogram
pH	potential of hydrogen
PIC	protease inhibitor cocktail
PM	phenotype microarray
pmol	picomolar
PrK	proteinase K
PRRs	pattern recognition receptors
PSM	phenol-soluble modulin
RANTES	regulated on activation normal T-cell expressed and secreted
RLRs	cyto-plasmic proteins retinoic acid-inducible gene-I-like receptors
RNA	ribonucleic acid
rpoB	RNA polymerase beta subunit
s	second
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*

SDS-PAGE	sodium dodecyl-suplhate polyacrylamide gel electrophoresis
SEM	standard error of mean
SPINK5	serine protease inhibitor Kazal-type 5
STAT	signal transducer and activator of transcription
TAE	tris-acetate-ethylenediaminetetraacetic acid
Th-cell/cytokine	T-helper cell/cytokine
TLR	toll-like receptor
TNF	tumour necrosis factor
TNS	trypsin-neutralising solution
TSA/TSB	tryptone soy agar/broth
TSLP	thymic stromal lymphopoietin
U	unit
UK	United Kingdom
USA	United States of America
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
γ-linolenic acid	gamma-linolenic acid
µg	microgram
µL	microlitre
µM	micromolar
µm	micrometre
°C	degrees Celsius

Thesis overview

The chapters of this thesis describe the background, the methods and the experiments performed in order to study the effects of *S. aureus* and *S. epidermidis* on keratinocytes, with a view to understanding AD. Based on the results from the experimental work, this thesis draws conclusions and makes suggestions for future work.

Chapter 1 (Introduction) describes AD and summarises the key points relevant to this project, as well as the project aims and achievements.

Chapter 2 (Materials and methods) lists the bacterial strains, skin cells and compounds used in the project, as well as the experimental approaches that were taken.

Chapter 3 (Production of AMPs in keratinocytes in response to *S. aureus* and *S. epidermidis*) reports on differences in the effects of *S. aureus* and *S. epidermidis* on the production of AMPs by keratinocytes.

Chapter 4 [Nature of the *S. aureus* HBD-3-inducing factor(s)] describes experimental approaches used in an attempt to identify *S. aureus* factor(s) that could potentially account for the induction of HBD-3 in keratinocytes.

Chapter 5 (Defining conditions to rectify staphylococcal dysbiosis: a preliminary evaluation of differing growth requirements for *S. aureus* and *S. epidermidis*) aims to identify conditions that favour the growth of *S. epidermidis* over *S. aureus*.

Chapter 6 (Conclusions) summarises the conclusions drawn from the experiments performed in this project and makes suggestions for future experimental work, in order to better understand the role of skin microbiome and *S. aureus* in the pathogenesis of AD.

Chapter 7 (References) lists the citations mentioned throughout this thesis.

Chapter 8 (Appendices and supplemental material) includes additional information on the experimental work performed in this project.

1. Introduction

1.1. The skin

Human skin is exposed to the environment and represents a barrier organ with an important defence role against external pathogens (Boer et al., 2016). It has antimicrobial, antioxidant and mechanical functions and it also controls permeability of molecules and skin hydration (Elias, 2008). It consists of three layers: the epidermis, the dermis and the hypodermis (Yousef et al., 2019).

The layers of the epidermis, starting from the outermost, are the stratum corneum, stratum lucidum (only in palms and soles), stratum granulosum, stratum spinosum and stratum basale (Figure 1-1) (Yousef et al., 2019). More than 90% of epidermal cells are keratinocytes (Eckhart et al., 2013). They are the main cells of the skin barrier and also participate in immune defence (Eyerich et al., 2018; Coates et al., 2018). The epidermis also contains melanocytes, Langerhans cells and Merkel cells (Figure 1-1) (Proksch et al., 2008; Elias, 2007; Lim et al., 2013; Gilbert, 2000; James et al., 2005; Eady and Pope, 2004; Yousef et al., 2019).

Keratinocytes of the stratum basale migrate and differentiate to form the other epidermal layers. During keratinocyte differentiation, keratinocyte stem cells in the stratum basale, which are attached to the basement membrane (extracellular matrix), produce transient amplifying suprabasal cells not attached to the membrane (Pincelli and Marconi, 2010; Dahl, 2012; Jones and Watt, 1993). These cells exit the cell cycle and start to migrate towards the upper epidermal layers (Simpson et al., 2011). Keratinocytes in the stratum spinosum are polyhedral with large nuclei. Langerhans cells, polar lipids, glycosphingolipids, free sterols, phospholipids and catabolic enzymes are also found in the stratum spinosum. Keratinocytes are attached together with desmosomes in both the stratum basale and spinosum (Mauldin and Peters-Kennedy, 2016; Yousef et al., 2019). Keratin filaments anchor the keratinocytes to the desmosomes and provide mechanical strength. Keratins 5 and 14 expressed in the stratum basale are replaced by keratins 1 and 10 in the stratum spinosum (Fuchs, 1993). Keratinocytes in the stratum granulosum have cytoplasmic granules, which contain keratin filaments,

profilaggrin and loricrin. Filaggrin induces aggregation of keratin and formation of a cornified cell envelope underneath the plasma membrane (Candi et al., 2005). Lipids accumulate inside lamellar bodies (Wikramanayake et al., 2014). Tight junction proteins (e.g. claudins and occludins) form a barrier between keratinocytes in the stratum granulosum and regulate the passage of solutes (Mauldin and Peters-Kennedy, 2016; Gonzalezmariscal, 2003; Anderson and Van Itallie, 2009; Brandner et al., 2015). During terminal differentiation, keratinocytes turn into corneocytes, when their nucleus, plasma membrane and cellular organelles disintegrate (Eckhart et al., 2013). Transglutaminase, which is calcium-activated, links involucrin, loricrin and small proline-rich proteins in an insoluble cell envelope (Candi et al., 2001), whereas the polar lipids of keratinocytes are converted into non-polar molecules, forming a lipid barrier (ceramides and fatty acids) around corneocytes (Elias, 1983; Kalinin et al., 2002). Therefore, the stratum corneum consists of anucleate corneocytes attached together with corneodesmosomes and surrounded by a cornified envelope, water-retaining keratin proteins and lipid layers. Corneocytes produce lamellar bodies, which secrete AMPs, fatty acids, cholesterol, sphingosine and ceramides and form the lipid matrix and proteases (Mauldin and Peters-Kennedy, 2016; Coates et al., 2014). Finally, during desquamation, the outermost corneocytes are shed (Wikramanayake et al., 2014). The stratum corneum is the outermost skin barrier, whereas tight junctions seal adjacent keratinocytes in the stratum granulosum, providing a second barrier (Brandner et al., 2015; Kirschner et al., 2010; Niessen, 2007).

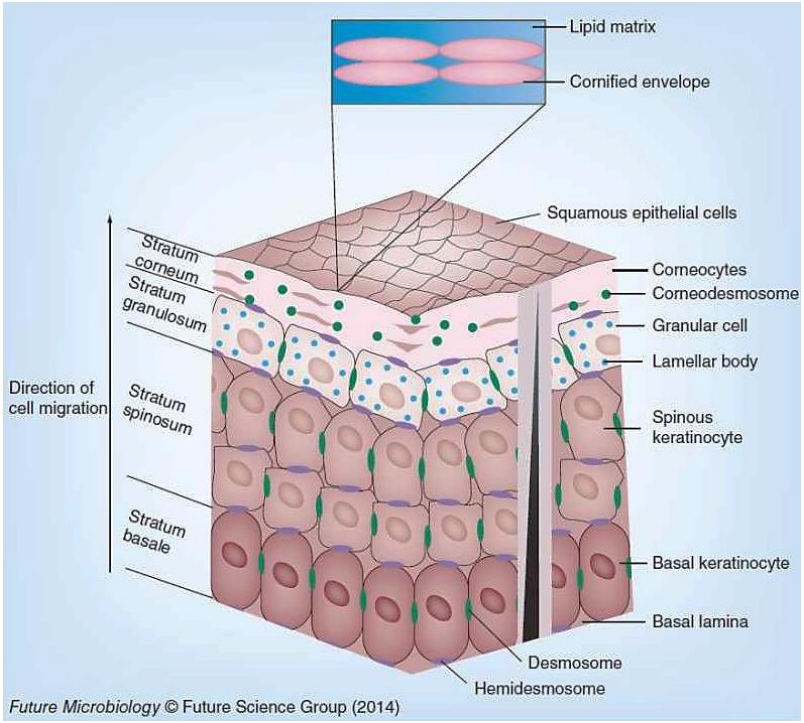


Figure 1-1: The layers of the human epidermis

The picture shows the epidermal layers, their components and the direction of cell migration [image from (Coates et al., 2014)].

The dermis, located between the epidermis and the hypodermis, consists of the papillary (outermost) and the reticular (inner) layers and it is composed of mesenchymal cells that produce the extracellular matrix (collagen, elastic fibres), as well as hair follicles, glands, vessels, macrophages, dendritic cells, mast cells and lymphocytes (Eady and Pope, 2004; Marks and Miller, 2006; James et al., 2005).

The hypodermis consists mainly of adipose cells, which provide insulation and mechanical protection to the underlying tissues and organs (Suarato et al., 2018; Gurtner et al., 2008; Ahn and Kaptchuk, 2011).

Pigmented skin shows structural differences compared to white skin. The concentration of melanin in pigmented skin melanosomes is twice as high as in white skin. Darker skin also has large melanosomes that contain more melanin and have a lower degradation rate. Melanin content offers protection against ultraviolet (UV) radiation and skin aging (Iozumi et al., 1993; Vashi et al., 2016). Although the thickness of the stratum corneum is similar in both white and pigmented skin, the stratum corneum in the latter consists of more

layers and is more compact against mechanical and chemical challenge (Rawlings, 2006). Moreover, its spontaneous desquamation is 2.5 times greater compared to white skin, due to a difference in the composition of the intercellular lipids. Pigmented skin has lower levels of ceramides, which are important for skin moisturisation and may explain the fact that water loss in pigmented skin is up to 2.7 times greater compared to white skin, leading to increased skin dryness (Berardesca and Maibach, 1988; Wan et al., 2014; Coderch et al., 2003; Sugino et al., 1993; Richards et al., 2003).

1.2. Atopic dermatitis: general background

Atopic dermatitis (AD) is an inflammatory skin disease, characterised by immune system dysregulation and an impaired skin barrier. The prevalence of AD is rising, affecting the quality of life of more than 20% of children and around 3-10% of adults worldwide (Kolb and Ferrer-Bruker, 2019; Nutten, 2015; Leung and Bieber, 2003). Genetic predisposition and environmental factors can both affect the initiation and course of AD (Thomsen, 2014), which is characterised by periods of relapses and remissions. Acute AD usually presents with lesions characterised by erythema, oozing, excoriation and oedema due to the formation of papules and vesicles. In chronic AD, the lesions can also be pigmented and lichenified (Figure 1-2) (Leung and Bieber, 2003; Thomsen, 2014; Correale et al., 1999; Bieber, 2010). A hallmark of AD is intense pruritus, which can result in excoriations, impairment of the skin barrier integrity and infections. Pruritus can cause sleep loss and, along with skin lesions, it can lead to significant psychosocial conditions (Nutten, 2015; Absolon et al., 2003; Barnetson and Rogers, 2002; Leung and Bieber, 2003; Jeon et al., 2017).

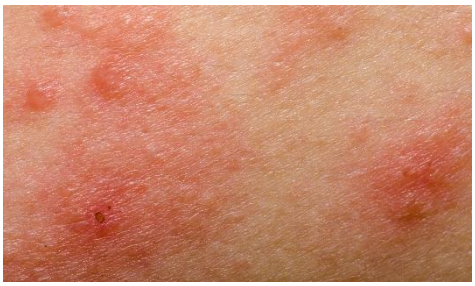
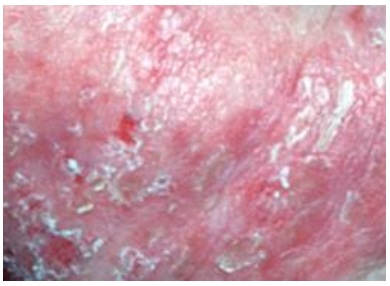
(a)  (b) 

Figure 1-2: Skin lesions in AD

Erythema, oedema, excoriation and vesicles are usually present from the acute stage of AD (a). In subacute and chronic AD, lichenification and hyperpigmentation can also appear (b) (Thomsen, 2014; Correale et al., 1999; Bieber, 2010) [images a and b adapted from (iStock.com/defun) and (Bridgett et al., 1996) respectively].

The diagnosis of AD is mainly clinical and largely based on the Hanifin-Rajka diagnostic criteria that have been used worldwide in clinical trials and in the diagnosis of AD (Hanifin and Rajka, 1980; Lee, 2016; Schram et al., 2011).

AD is a chronic condition and to date no permanent cure has been found; the available therapeutic approaches focus on the control of symptoms and infections and avoidance of potential triggering factors. The mainstay of treatment is application of moisturisers and topical glucocorticosteroids (Yarbrough et al., 2013; Leung, 2003; Van Zuuren et al., 2017). Emollients improve skin moisturisation and skin barrier integrity (Giam et al., 2016; Hon and Leung, 2013; Hon et al., 2013; Varothai et al., 2013). Topical anti-inflammatory agents such as calcineurin inhibitors (tacrolimus, pimecrolimus) and corticosteroids (hydrocortisone) inhibit mediator release from skin-resident and skin-infiltrating cells, such as T-cells (Carr, 2013; Stuetz et al., 2006; Norris, 2005; Atherton, 2003). Systemic treatments include oral glucocorticosteroids (prednisolone) and immunomodulatory drugs (calcineurin inhibitors, methotrexate) (Simon and Bieber, 2014; Megna et al., 2016). The use of biologic drugs, such as the monoclonal antibody dupilumab that binds to the IL-4 receptor-α and inhibits IL-4 and IL-13 signalling, is a promising and more disease-specific approach (Megna et al., 2016). In chronic AD, UV therapy can be beneficial (Majoie et al., 2009). Finally, antibiotics are often prescribed against infection. Although they have significant short-term benefits (Wohl et al., 2015), they are not recommended for long-term treatment, as they can disrupt the normal skin flora and lead to

the development of antibiotic-resistant strains (Błażewicz et al., 2017; Gilani et al., 2005; Friedman and Goldman, 2011; Rayner and Munckhof, 2005; Chambers and Deleo, 2009; Dethlefsen et al., 2008; Cogen et al., 2008; Kobayashi et al., 2015).

1.3. Atopic dermatitis: pathogenesis

Genetic predisposition, environmental factors, skin barrier dysfunction and alterations in the immune response have been implicated in the pathogenesis of AD (McPherson, 2016; Thomsen, 2014). The skin microbiome has also been show to play a role (Lebwohl et al., 2013; Baker, 2006; Salava and Lauerma, 2014), although the pathway of transition from the normal skin flora to a microbial imbalance and its effect on AD pathogenesis is not yet fully elucidated (Yamazaki et al., 2017; Paller et al., 2019). These factors will be analysed in more detail in the following sections.

1.3.1. Genetic factors of AD

As regards genetic background, the association of AD with various single nucleotide polymorphisms and gene mutations has been studied. Filaggrin is an important structural protein of the skin, with filaggrin mutations significantly affecting skin barrier integrity and hydration and increasing the risk of developing AD (O'Regan et al., 2008; Zaniboni et al., 2016; Leung, 2013; Palmer et al., 2006; Janssens et al., 2012; Elias et al., 2008; Milani, 2016). Up to 50% of AD patients carry filaggrin null mutations (Brown and Irwin-Mclean, 2009). The R510X and 2282del4 filaggrin null mutations, found in 9% of the European population, highly predispose to AD (Palmer et al., 2006), whereas filaggrin gene mutations in pigmented skin are 6 times less common compared to white skin (Brunner and Guttman-Yassky, 2019). The high-affinity immunoglobulin E receptor (FcεRI) is expressed mostly in dendritic and mast cells (Park et al., 2011). Polymorphisms in the β chain of *FcεRI* can result in increased surface expression of the receptor and mast cell

degranulation (Cookson and Moffatt, 2002). The serine protease inhibitor Kazal-type 5 (*SPINK5*) gene regulates skin barrier formation. Mutations in the *SPINK5* gene leading to premature termination codons are found in Netherton's disease, which is characterised by severe AD lesions and increased immunoglobulin (Ig) E serum levels (Agarwal et al., 2014; Tartaglia-Polcini et al., 2006; Bitoun et al., 2002). A functional mutation of the "regulated on activation normal T-cell expressed and secreted" (*RANTES*/*CCL5*) gene has been associated with AD, leading to increased gene expression and chemokine-induced eosinophil migration (Kato et al., 2006; Nickel et al., 2000). Furthermore, interleukin (IL)-4 and IL-13 act through their receptors IL-4R and IL-13R, which both have the IL-4Rα chain and activate the factor "signal transducer and activator of transcription 6" (STAT6), leading to production of T-helper (Th) 2 cytokines and IgE. Previous studies report an association between nucleotide polymorphisms that can alter their expression levels and AD. Two single nucleotide polymorphisms in the *IL-13* gene (rs3091307 and rs20541) and two in the *IL-13Rα1* receptor (rs2265753 and rs2254672) are associated with AD (Namkung et al., 2011; Leung et al., 2003; Shirakawa et al., 2000). Lastly, mutations of the genes encoding toll-like receptor (TLR) 2 and nucleotide-binding oligomerisation domain (NOD) 2 can increase AD severity (Ahmad-Nejad et al., 2004; Jiao et al., 2015; Macaluso et al., 2007; Wong et al., 2016).

1.3.2. Environmental factors of AD

Environmental factors have been also found to play a role in AD pathogenesis (McPherson, 2016; Thomsen, 2014). Skin dryness, infections (*S. aureus*), extreme climatic conditions (temperature, humidity, CO_2, precipitation), exposure to aero-allergens (pollen, house dust mite) and serum-specific IgE sensitisation can trigger or exacerbate AD (Leung and Bieber, 2003; Schneider et al., 2013; Langan and Irvine, 2013; Silverberg et al., 2013; Harrop et al., 2007; Sheffield et al., 2011).

IgE sensitisation is important in AD (Tanaka, 1994). Pre-school AD is associated with IgE sensitisation to food and aero-allergens (Johansson et al.,

2017). Also, patients with severe AD react to a larger panel of environmental allergens (such as house dust mite and pollen) than patients with moderate AD and have increased levels of *S. aureus*-specific IgE antibodies (Mittermann et al., 2016).

Socio-economic factors also affect AD. The prevalence of AD decreases with increasing age and shows a female-to-male preponderance (1.3 to 1) (Hatta et al., 2016; Herd et al., 1996; Fennessy et al., 2000). Moreover, AD is more frequent in urban areas, people with a family history of atopy, a higher level of education and socio-economic status, or coming from smaller families (Sybilski et al., 2015; Shaw et al., 2011; Girolomoni et al., 2003; National Collaborating Centre for Women's and Children's Health, 2007). Anxiety and psychological stress can upregulate the release of neuromediators from sensory nerves, which affect the immune response and the skin barrier and may consequently exacerbate AD (Adams et al., 2005; Suárez et al., 2012; Cacioppo et al., 1998). Also, the prevalence of AD in the population with pigmented skin has been found to be 1.2-1.7 times higher compared to the Caucasian population (Kaufman et al., 2018; Fu et al., 2014; Shaw et al., 2011).

The “hygiene hypothesis”, according to which there is less exposure to infections and allergens in early childhood due to lifestyle habits, has been postulated to play a role in the increasing AD prevalence (Baker, 2006; Williams et al., 1994).

1.3.3. The skin barrier in AD

The skin barrier is implicated in the pathogenesis of AD (McPherson, 2016). This section will analyse the lipid matrix and filaggrin protein, found in the stratum corneum, which are essential components of the skin barrier (Van Smeden and Bouwstra, 2016; McAleer and Irvine, 2013) and the pH of the skin, which plays an important role in the skin barrier function (Chan and Mauro, 2011).

1.3.3.1. Lipid matrix

The epidermis acts as a barrier against external pathogens and regulates skin hydration. Low relative humidity and high salt concentration are important for skin barrier integrity (De Goffau et al., 2009; Elias, 1983). Corneocytes in the stratum corneum are embedded in a lipid matrix arranged in “stacked bilayers” consisting of cholesterol, fatty acids and ceramides (Landmann, 1988; Hatta et al., 2006).

Omega-ω-6 and ω-3 are the main polyunsaturated fatty acids. They are structural components of cell membranes and an energy source. They also regulate cell membrane enzymes and properties and produce inflammatory regulators (leukotrienes-cytokines) (Stillwell and Wassall, 2003; Rakesh and Yung-Sheng, 2006). Delta (Δ) 5, 6 and 9 desaturases catalyse the fatty acid formation (Nakamura and Nara, 2004). Linoleic and arachidonic acids are the most common ω-6 polyunsaturated fatty acids in human skin (Trumbo et al., 2002; Nakamura and Nara, 2004; Chapkin et al., 1986; Ziboh et al., 2000). Gamma-linolenic acid (γ-linolenic acid) is produced from linoleic acid by Δ6-desaturase and it modulates matrix proteins that participate in inflammation (Rakesh and Yung-Sheng, 2006). In AD, linoleic acid increases and γ-linolenic and arachidonic acids decrease (Calder and Burdge, 2012; Calder and Miles, 2000; Manku et al., 1984). This polyunsaturated fatty acid imbalance could be caused by Δ6-desaturase dysfunction (Horrobin and Manku, 1990) or by increased use of arachidonic acid in AD inflammation (Calder, 2006).

Ceramides are composed of a sphingoid base (e.g. sphingosine) and a fatty acid and constitute up to 50% of the stratum corneum lipids. Changes in ceramide levels are correlated with skin barrier dysfunction and AD severity but not with filaggrin gene mutations (Janssens et al., 2012; Smeden et al., 2014). Sphingosine, which is potent against *S. aureus,* is reduced in AD (Parsons et al., 2012; Arikawa et al., 2002; Pruett et al., 2008; Imokawa and Ishida, 2014).

The skin barrier in AD is affected in both lesional and non-lesional skin (Proksch et al., 2006; Kelleher et al., 2015). An imbalance of the skin barrier components disrupts skin barrier integrity and increases water loss and susceptibility to pathogens (Imokawa and Ishida, 2014).

1.3.3.2. Filaggrin

Filaggrin is the major structural protein of the stratum corneum, essential for the skin barrier integrity (McAleer and Irvine, 2013). The final protein is formed after cleavage of the profilaggrin protein, during the terminal epidermal differentiation and binds to keratin filaments, leading to the formation of a keratin matrix (Palmer et al., 2006; Sandilands et al., 2009). Cis-urocanic acid (cis-UCA), a filaggrin breakdown product, which contributes to skin moisturisation and pH balance, has immunoregulatory function (Ye et al., 2014; Hart et al., 2002; Noonan and De Fabo, 1992; Sandilands et al., 2009) and can also inhibit *S. aureus* colonisation (Miajlovic et al., 2010).

Filaggrin expression is downregulated in AD skin. This is more prominent in lesional AD skin compared to non-lesional (Pellerin et al., 2013; Howell et al., 2009). Filaggrin gene expression and cis-UCA can be downregulated in AD, even without a loss-of-function mutation, as Th_2 cytokines (IL-4, IL-13), which are increased in AD, can downregulate filaggrin expression in keratinocytes (Kezic et al., 2011; Palmer et al., 2006). Also, keratinocytes exposed to IL-22 and IL-25 have reduced filaggrin expression (Niebuhr et al., 2010; Gutowska-Owsiak et al., 2011; Deleuran et al., 2012). Moreover, IL-17, IL-31 and IL-33 can downregulate filaggrin expression (Seltmann et al., 2015; Gutowska-Owsiak et al., 2012; Van Drongelen et al., 2014).

Along with filaggrin, IL-4 and IL-22 can also downregulate claudins, involucrin and loricrin proteins (Lee et al., 2014; Sehra et al., 2010; Gruber et al., 2015; Howell et al., 2009; Rabeony et al., 2014). The IL-17α cytokine was also found to downregulate not only filaggrin but also other skin barrier molecules, such as claudins (Eyerich et al., 2009; Niebuhr et al., 2011a; Toda et al., 2003; Gutowska-Owsiak et al., 2012; McAleer and Irvine, 2013). The reduced expression of filaggrin and cis-UCA in AD can increase the susceptibility to *S. aureus* (Kezic et al., 2011; Miajlovic et al., 2010; Van Drongelen et al., 2014).

1.3.3.3. Skin pH

The pH of normal skin is between 4 and 6. The acidic environment of the skin is important for an intact barrier and an effective defence. Phospholipids, fatty

acids, lamellar bodies and filaggrin breakdown products contribute to the acidic pH of the skin (Chan and Mauro, 2011; O'Regan et al., 2008). In an acidic skin pH, lipid synthesis and the activity of proteases responsible for the stratum corneum desquamation are optimal. Moreover, many AMPs are more potent, increasing the skin's defence against external pathogens. In such conditions, the growth and virulence of pathogens such as *S. aureus* are reduced (Panther and Jacob, 2015; Malik et al., 2016; Proksch, 2018). Acidic pH reduces the rate of *S. aureus* colonisation and the production of immune evasion proteins (clumping factor B, fibronectin binding protein A) (Miajlovic et al., 2010). The pH of the skin in AD increases (Eberlein-Konig et al., 2000; Seidenari and Giusti, 1995). Filaggrin gene mutations and downregulation of filaggrin and AMP production by Th_2 cytokines are responsible for the pH increase (Proksch et al., 2008; Kezic et al., 2011; Howell et al., 2009; Chieosilapatham et al., 2017). The pH of non-lesional AD skin is higher than normal skin, while the pH of lesional AD skin is even higher (Panther and Jacob, 2015; Tauber et al., 2016). The changes in the skin barrier in AD compared to healthy skin are shown in Figure 1-3.

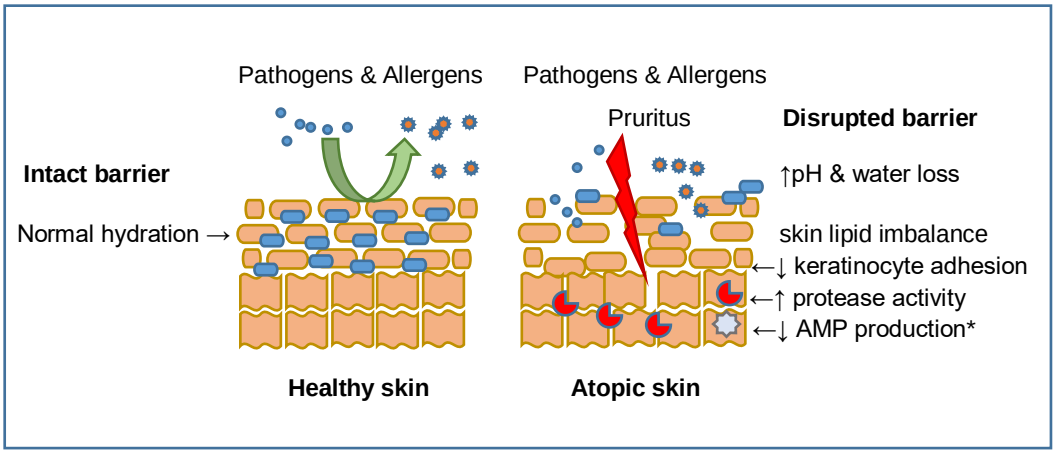


Figure 1-3: The skin barrier in normal and AD skin

The imbalance of skin lipids in AD, as well as the decrease in tight junction and cornified envelope proteins can affect the pH, hydration and integrity of the skin barrier. The increased pH alters the activity of proteases and the synthesis of lipids and AMPs. All these changes facilitate allergen penetration (Calder and Burdge, 2012; Di Nardo et al., 1998; Palmer et al., 2006; Gruber et al., 2015; Bao et al., 2016; Panther and Jacob, 2015; Clausen et al., 2018).

*AMP production in AD is lower compared to other inflammatory skin diseases.

1.3.4. The immune response in AD

The host immune response is also implicated in AD pathogenesis (Gavrilova, 2018; McPherson, 2016). The following section will describe the pattern recognition receptors (PRRs), which are important for skin defence and are expressed in keratinocytes, as well as the pathways by which keratinocytes activated by pathogens can induce expression of cytokines and AMPs (Lai and Gallo, 2008; Kuo et al., 2013).

1.3.4.1. Pattern recognition receptors (PRRs)

The immune system is divided into the innate immune system, which is mediated by PRRs and pathogen-associated molecular patterns (PAMPs) and the adaptive immune system, which is mediated by somatically generated receptors of T-cells and B-cells. The PRRs are highly conserved molecules but not antigen-specific, whereas the receptors of adaptive immunity are formed in order to recognise a specific antigen (cell-mediated via antigen-specific T-cell receptors and humoral via immunoglobulins) (Iwasaki and Medzhitov, 2004; Clark and Kupper, 2005; Miller, 2008; De Benedetto et al., 2009). Both the innate and the adaptive immune systems are implicated in AD (Biedermann et al., 2015; De Benedetto et al., 2009).

The types of PRRs of the innate immune system are TLRs, NOD-like receptors (NLRs), C-type lectin receptors (CLRs) and the cytoplasmic proteins retinoic acid-inducible gene-I-like receptors (RLRs) (Thompson et al., 2011). The most studied receptors are TLRs (De Benedetto et al., 2009), which are glycoproteins expressed in various cells, such as keratinocytes, dendritic cells, B-cells, T-cells, mast cells, macrophages and fibroblasts. Each cell type has different TLRs. The TLRs recognise PAMPs, such as lipopolysaccharides and other pathogen components. Binding of TLRs to PAMPs leads to activation of the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) pathway and of immune responses such as phagocyte activation, cytokine and AMP release and regulation of serine protease inhibitors. So far, 10 TLRs have been described, which recognise different PAMPs. Some are found on the cell membrane and recognise extracellular PAMPs, whereas

others are intracellular and recognise PAMPs that have entered the cells (Miller and Modlin, 2007; Miller, 2008; Clark and Kupper, 2005; Sugimoto et al., 2017; Takeuchi and Akira, 2010; Kawai and Akira, 2007b).

Binding of TLRs to PAMPs can activate the adaptive immune response, by activation of dendritic cells through the NF-κB pathway, leading to antigen presentation and subsequent T-cell stimulation. Depending on signals provided along with the antigen presentation, naive T-cells can be polarised to Th_1, Th_2 or T-regulatory cells (Miller, 2008; Miller and Modlin, 2007; Iwasaki and Medzhitov, 2004; Clark and Kupper, 2005; Medzhitov, 2001; Medzhitov and Janeway, 2000).

TLRs can activate the myeloid differentiation factor 88 and “IL-1-receptor associated kinases and TNF-receptor-associated” factors, leading to activation of mitogen-activated protein kinases (MAPK) and janus kinases (JAK), as well as NF-κB and activator protein (AP) 1 and subsequently induce inflammatory cytokine release (Miller, 2008; Kawai and Akira, 2007b). Also, TLRs can activate NF-κB through the “toll/IL-1 receptor domain-containing adaptor-inducing IFN-beta (β)” (TRIF) dependent pathway (Kawai and Akira, 2007a). Polymorphisms of TLRs have been found in AD patients (mainly TLR2 and TLR9) and have been associated with increased susceptibility to *S. aureus* and other infections (Miller, 2008).

The NOD1 and NOD2 receptors are cytoplasmic NLRs that recognise bacterial peptidoglycans, activate NF-κB and induce cytokine release. Since both TLRs and NODs recognise bacterial peptidoglycans, they have synergistic action (Takeuchi and Akira, 2010). Mutations of *TLR2* and *NOD2* genes can increase the AD severity through the p38 MAPK, the NF-κB and the extracellular signal-regulated kinase pathway and eosinophil and basophil activation (Ahmad-Nejad et al., 2004; Jiao et al., 2015; Macaluso et al., 2007; Wong et al., 2016).

1.3.4.2. Cytokines

Cytokines are signalling proteins, which play a role in the immune response by influencing differentiation and migration of immune cells and in epidermal

growth by regulating keratinocyte proliferation and differentiation (Cameron and Kelvin, 2013; Hanel et al., 2013; Coondoo, 2011; Nedoszytko et al., 2014). Types of cytokines include chemokines, interferons (IFN), interleukins, lymphokines and tumour necrosis factors (TNF) (Turner et al., 2014; Dinarello, 2007; Ferreira et al., 2019). In the skin they are secreted by keratinocytes, Langerhans cells, mast cells, macrophages, neutrophils, T-cells, B-cells and other cell types. They are produced upon stimulation, rather than being stored in cells and act mostly at the local level (Nedoszytko et al., 2014), although prolonged action of stimulus can lead to increased cytokine production and release in blood circulation, affecting distant body areas. Their signalling function is exerted through the corresponding receptors that are expressed on the target cells (Coondoo, 2011).

Many cytokines have been found to play an important role in AD. The hallmark of AD is an increase of T-cells polarised along the Th_2 pathway, resulting in overexpression of Th_2 cytokines (IL-4, IL-5 and IL-13). The T-cell infiltration is induced by chemoattractant factors and reaches the epidermal compartment. Keratinocytes are important cytokine producers and direct various T-cell subsets into the skin (Homey and Zlotnik, 1999; Rawlings and Harding, 2004).

Both IL-4 and IL-13 are increased in AD and act through the IL-4Rα receptor expressed on target cells. They promote CCL26 production in keratinocytes and activate T-cells, B-cells, macrophages and other cells, leading to Th_2 differentiation, IgE production by B-cells and increased infiltration of mast cells and eosinophils. They inhibit keratinocyte differentiation through STAT3 and promote cytokine production through STAT6. They also downregulate TNF-α- and IFN-γ-mediated production of the AMPs HBD-2 and HBD-3, through STAT6 activation (Albanesi et al., 2007; Amano et al., 2015). IFN-γ regulates cytokine production, increases pathogen recognition and antigen presentation and activates monocytes, macrophages, natural killer cells and apoptotic mechanisms (Schroder et al., 2004; Mühl and Pfeilschifter, 2003; Hata and Gallo, 2008). TNF-α promotes macrophage activation and nitric oxide production, thus increasing the antibacterial potency (Silva et al., 1995; Bekker et al., 2001). Downregulation of TNF-α and IFN-γ in AD can increase the susceptibility to *S. aureus* (Liu et al., 2018; Campbell and Kemp, 1997; Brauweiler et al., 2016).

Also, IL-5 promotes Th_2 differentiation and the production of IgE, eosinophils and mast cells (Brandt and Sivaprasad, 2011; Matsunaga and Yamauchi, 2016; Kagami et al., 2005; Paller et al., 2017). Pruritic symptoms in AD can be attributed to IL-31, which interacts with skin neurons expressing the IL-31 receptor A and causing itch (Cevikbas et al., 2014; Meng et al., 2018). Cytokines IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) promote the Th_2 response by activating Th_2-producing cells or through dendritic cell activation (Brandt and Sivaprasad, 2011; Wang and Liu, 2009; Saenz et al., 2010; Deleuran et al., 2012). Activated dendritic cells produce various cytokines, such as CCL-17, CCL-18 and CCL-22 and promote the Th_2 response. The CCL-17 and CCL-22 cytokines are AD markers that promote the Th_2 response through binding to the C-C chemokine receptors (CCR) type 4 and 8 (Brandt and Sivaprasad, 2011; Novak, 2012; Leung et al., 2003; Kubo et al., 2017; Nakazato et al., 2008; Kimura et al., 2014; Inngjerdingen et al., 2000; Lu et al., 2016). Also, CCL-27 acts through CCR-10 and attracts cutaneous lymphocyte-associated antigen (CLA)+ memory T-cells (Homey et al., 2000; Chen et al., 2006; Kubo et al., 2017). The T-cells are directed into the skin by means of skin-homing receptors CLA (cutaneous lymphocyte antigen) (Homey et al., 2006; Pivarcsi and Homey, 2005; Lee and Hwang, 2012). Lastly, the levels of IL-36γ cytokine are significantly increased in psoriatic lesions compared to AD, which makes this cytokine a marker of differentiation between the two inflammatory conditions (Berekmeri et al., 2018).

There are differences in the immune response in acute and chronic AD. Acute AD is characterised by a Th_2-mediated response. The increased Th_2-cytokine expression (IL-4, IL-5 and IL-13) leads to a systemic increase of eosinophils and IgE levels (Yamanaka and Mizutani, 2011; Brandt and Sivaprasad, 2011; Wittmann et al., 2014). The Th_{22} and Th_{17} responses are also upregulated in acute AD (Brunner et al., 2017). Chronic AD displays a shift from Th_2/Th_{17} towards Th_1 and T-regulatory cells (Su et al., 2017; Peng and Novak, 2015), whereas Th_{22} cells are also upregulated (Gittler et al., 2012; Eyerich and Eyerich, 2015).

Atopic skin is more frequently colonised by *S. aureus* than healthy skin (Hoeger, 2004; Higaki et al., 1999). Colonisation of AD patients with *S. aureus*

leads to more severe disease, higher T-cell expression with a greater Th_2/Th_1 ratio, production of AD cytokines such as IL-22 and TSLP and increased allergen sensitisation and skin barrier disruption (Wedi et al., 2002; Simpson et al., 2018; Nakatsuji et al., 2016). Also, the effect of *S. aureus* alpha (α) toxin on keratinocytes is increased by Th_2 cytokines, whereas *S. aureus* enterotoxin B promotes IL-31 production (Baviera et al., 2015). Pruritus triggered by IL-31 can lead to scratching, disturbance of the skin barrier and further cytokine release. As a result, skin fibronectin and laminin are more exposed to *S. aureus* cells, which bind to them. Also, IL-4 induces production of adhesin and fibronectin by fibroblasts, enhancing *S. aureus* binding and downregulates the production of AMPs that are potent against *S. aureus* (Nowicka and Grywalska, 2018; Meng et al., 2018; Simanski et al., 2013).

1.3.4.3. Antimicrobial peptides

The expression of AMPs such as HBD-2, HBD-3, cathelicidin LL-37, calprotectin (S100A8/S100A9), psoriasin (S100A7), RNAase7 and dermcidin by keratinocytes, neutrophils and other cells plays a significant role in the skin defence against pathogens (Zhang and Gallo, 2016; Schauber and Gallo, 2008; Pfalzgraff et al., 2018; Mahlapuu et al., 2016). Investigation of their role in AD is important because, compared to other inflammatory diseases, their levels do not seem to correspond to the degree of AD inflammation, resulting in increased susceptibility of AD patients to pathogen colonisation, mainly with *S. aureus* (Hata and Gallo, 2008; Ong et al., 2002; Nomura et al., 2003b; Lin et al., 2007). Upregulation of the AMPs takes place mainly in the outermost epidermis (Stoitzner et al., 1999; Percoco et al., 2013; Menzies and Kenoyer, 2005). Calprotectin, HBD-2, HBD-3 and psoriasin are produced upon stimulation. Cathelicidin LL-37 and RNase7 are both constitutively produced and induced upon inflammation, whereas dermcidin is constitutively produced and non-inducible (Heilborn et al., 2003; Zhang and Gallo, 2016; Rieg et al., 2004; Schittek, 2012; Schittek et al., 2001; Otto, 2010; Alowami et al., 2003; Ross and Herzberg, 2001). Their antimicrobial mechanisms against pathogens include disruption of the cell wall or membrane, protein inhibition or targeting of intracellular processes (Bechinger and Gorr, 2017). They also

promote keratinocyte differentiation, cytokine production and chemotaxis (Clausen et al., 2016; Harder and Schröder, 2005). Moreover, HBD-3 can increase the expression of claudins, suggesting that AMPs may also improve the skin barrier integrity in addition to their antimicrobial properties (Kiatsurayanon et al., 2014).

Production of HBDs and LL-37 by keratinocytes in AD is of great interest, due to their potency against *S. aureus* (Ong et al., 2002; Midorikawa et al., 2003; Menzies and Kenoyer, 2005; Joly et al., 2004).

Cathelicidin LL-37

The only human cathelicidin, LL-37, is expressed in immune cells, mast cells and epithelial cells. Initially, it is encoded as an inactive precursor (hCAP18) and is then cleaved in its active form in the skin by proteases kallikrein 5 and kallikrein 7 (Van Der Does et al., 2012; Sorensen et al., 2001; Yamasaki et al., 2006). LL-37 is constitutively expressed in neutrophils, whereas it is inducible in keratinocytes upon inflammation. In normal skin and non-stimulated keratinocytes the LL-37 levels are below the detection limit (Heilborn et al., 2003; Frohm et al., 1997; Love et al., 2012; Cowland et al., 1995; Dorschner et al., 2001; Kim et al., 2005; Schauber et al., 2006; Van Der Does et al., 2012; Schauber et al., 2007). LL-37 is induced after exposure to pathogens through PRRs, or to endogenous cytokines. Vitamin D can also induce LL-37 production in the skin (Love et al., 2012; Nell et al., 2004; Xhindoli et al., 2016).

Cathelicidin LL-37 has broad antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and viruses. Apart from its pore-forming capacity, it can interact with membrane or secreted pathogenic factors or factors released from damaged host cells and reduce their ability to induce inflammatory effects. It is also a chemoattractant for neutrophils, eosinophils, T-cells and mast cells and has immunoregulatory function. Moreover it can induce keratinocyte migration through the epidermal growth factor receptor (EGFR) and cytokine production in keratinocytes through G-protein coupled receptor and EGFR (Xhindoli et al., 2016; Turner et al., 1998; Vandamme et al., 2012; Murakami et al., 2002; Tjabringa et al., 2006; Beaumont et al., 2013; Niyonsaba et al., 2002).

Human-β Defensins

HBDs are small cationic AMPs expressed in keratinocytes and other epithelial cells (oral mucosa, urogenital, gastrointestinal and respiratory tract), as well as some leukocytes (neutrophils) (Flaherty, 2012; Mathews et al., 1999; Oppenheim et al., 2003; Harder et al., 2001; Harder et al., 1997). They are located in the epidermis and have broad-spectrum antimicrobial activity (Simanski et al., 2014; Falconer et al., 2001; Ganz, 2003).

HBD-2 and HBD-3 levels are low in normal conditions and increase upon stimulation, whereas HBD-1 is constantly produced by keratinocytes rather than being induced by cytokines or bacteria and its levels decrease in AD compared to healthy subjects (Harder and Schröder, 2005; Gambichler et al., 2006). The functions of HBD-2 and HBD-3 have been studied in more detail (Harder et al., 2001; Harder et al., 1997). Keratinocytes produce HBD-2 and HBD-3 upon exposure to pathogens via PRRs and endogenous cytokines (IL-1α, IL-17, IL-22, IL-1β, TNF-α, IL-17A/IFN-γ). Both HBD-2 and HBD-3 exert their activity through pore formation in membranes (Weinberg et al., 2012; Taylor et al., 2008; Duclohier, 1994). They can also induce further cytokine production and keratinocyte differentiation and recruit dendritic and T-cells through the CCR-6 receptor, therefore linking the innate and the adaptive immune systems (Yang et al., 1999; Oppenheim et al., 2003; Simanski et al., 2013; Dinulos et al., 2003; Mathews et al., 1999; Harder et al., 2000; Harder et al., 1997; Harder et al., 2004; Liu et al., 2002; Niyonsaba et al., 2006; Hata and Gallo, 2008; Harder et al., 2001).

Even though HBD-2 and HBD-3 belong to the same family, their induction pathways may not be identical. Various studies have been conducted using different stimulants and testing different pathways. The results are not always consistent, suggesting that different factors can trigger different pathways. The NF-κB and MAPK/AP-1 pathways mediate pathogen-induced HBD-2 expression in epithelial cells (Wehkamp et al., 2004; Hertz et al., 2003; Steubesand et al., 2009), as well as HBD-2 induction in keratinocytes upon stimulation with *Pseudomonas aeruginosa* and IL-1β (Wehkamp et al., 2006). Another study found c-Jun N-terminal kinase (JNK) and p38 MAPK pathways to be more important for HBD-2 induction than NF-κB (Krisanaprakornkit et

al., 2002). It has been reported that both commensal and pathogenic bacteria may induce HBD-2 production in keratinocytes through JNK and p38 MAPK pathways but the induction by commensal bacteria may not involve NF-κB, suggesting that different bacterial components may initially bind to the same TLRs but then trigger different pathways (Chung and Dale, 2004).

HBD-3 induction in keratinocytes (by *S. aureus* and lipoteichoic acid) and other epithelial cells, such as oral mucosa, is mediated through TLR2 receptor, p38 MAPK pathway and AP-1 transcription factor, whereas there is no indication of HBD-3 binding to NF-κB (Inthasin et al., 2018; Menzies and Kenoyer, 2006; Peng Jia et al., 2001; Steubesand et al., 2009; Sechet et al., 2018). Although most studies do not report a role for NF-κB in HBD-3 induction (Menzies and Kenoyer, 2006; Peng Jia et al., 2001; Steubesand et al., 2009; Sechet et al., 2018), one study found that HBD-3 was induced by live *S. aureus* through NF-κB, whereas HBD-3 induction by secreted *S. aureus* factors was independent of NF-κB (Wanke et al., 2011). It seems that NF-κB may not play a major role in HBD-3 induction compared to HBD-2, however, more research is needed. Clearly, there are indications of differences in the induction pathways of HBD-2 and HBD-3, which are also affected by the nature of the bacterial species.

Antimicrobial activity of AMPs

The HBD-2 defensin is more potent against Gram-negative bacteria, rather than Gram-positive, whereas the effect of HBD-3 and LL-37 on *S. aureus* and other Gram-positive bacteria is greater than the effect of HBD-2 (Schröder and Harder, 1999; Harder et al., 2000; Chung and Dale, 2004; Joly et al., 2004). The fact that HBD-3 is more potent against *S. aureus* than HBD-2 may be attributed to the increased positive charge of HBD-3 compared with HBD-2, which can counterbalance the reduced acidity of *S. aureus* surface compared to Gram-negative bacteria (Schibli et al., 2002; Dhople et al., 2006; Harder et al., 2001; Jung et al., 2011). As an increased resistance of methicillin resistant *S. aureus* (MRSA) strains to LL-37 compared to HBD-3 has been reported, HBD-3 may be more effective against *S. aureus* infections (Ouhara et al., 2008). Also, it was found that the AMP sensor system of *S. aureus* is more

limited compared to *S. epidermidis* and that both *S. epidermidis* and *S. aureus* recognise LL-37, whereas only *S. epidermidis* recognises HBD-3 (Joo and Otto, 2015). Nevertheless, HBDs and LL-37 can have a synergistic effect against *S. aureus* (Harder et al., 2001; Chen et al., 2005; Midorikawa et al., 2003; Wanke et al., 2011).

Calprotectin is mainly induced by Gram-negative bacteria but is potent against both Gram-positive and Gram-negative bacteria (Brandtzaeg et al., 1995; Abtin et al., 2010; Steinbakk et al., 1990; Van Crombruggen et al., 2016). Both RNase7 (Simanski et al., 2010; Harder and Schröder, 2002; Rademacher et al., 2016; Torrent et al., 2010) and dermcidin have broad antimicrobial activity against Gram-positive and Gram-negative bacteria (Senyürek et al., 2009; Josefson, 2001). Although psoriasin can be induced by *S. aureus*, it is mostly effective against Gram-negative bacteria (Garreis et al., 2011; Gläser et al., 2005).

Changes in AMP levels in AD

Most AMPs increase in inflammatory conditions. Research has been conducted regarding the role of AMPs in the immune response and changes in their levels in inflammatory conditions, such as AD and psoriasis (Schröder et al., 2006). Increased levels of HBD-2, HBD-3, LL-37, calprotectin, psoriasin and RNase7 have been found in AD and psoriatic lesions, however, their increase in AD is lower than in psoriasis (Figure 1-4) (Clausen et al., 2018; Harder et al., 2010; Clausen et al., 2016; Jin et al., 2014; Schonthaler et al., 2013). Dermcidin, which is constitutively expressed in normal skin, is reduced in AD (Rieg et al., 2005; Rieg et al., 2004). The relative AMP deficiency in AD favours *S. aureus* growth (Nakatsuji et al., 2017; Nowicka and Grywalska, 2018).

The levels of AMPs (HBD-2, HBD-3, LL-37, psoriasin) are increased ın lesional AD skin compared to non-lesional and healthy skin (Clausen et al., 2018; Clausen et al., 2016). In contrast to the Th_1 axis and neutrophil infiltration in psoriasis, the Th_2 axis in AD inhibits filaggrin production and AMP expression by keratinocytes through the STAT6 signalling cytokines IL-4 and IL-13 (Nomura et al., 2003a; Ong et al., 2002; Bernard et al., 2012; Di et al.,

2016; Howell et al., 2005; Howell et al., 2006b; Albanesi et al., 2007; Nomura et al., 2003b; Takeda et al., 1996).

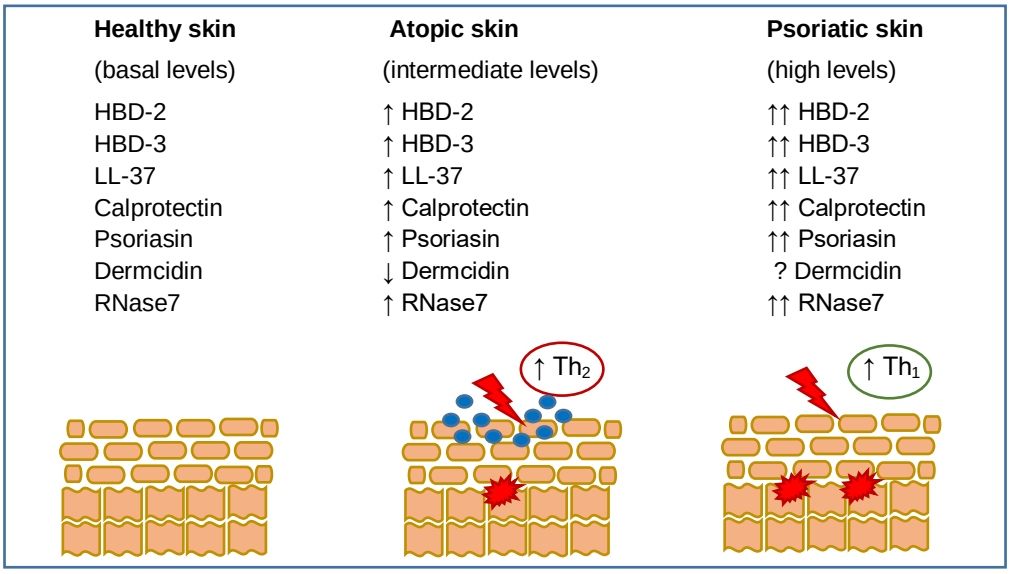


Figure 1-4: AMP expression in healthy, AD and psoriatic skin

All AMPs except dermcidin are increased in AD and psoriasis compared to healthy skin. AMP expression is higher in psoriasis than in AD. Expression of dermcidin is reduced in AD (Shiohara, 2011; Heilborn et al., 2003; Ross and Herzberg, 2001; Clausen et al., 2016).

As *S. aureus* is the most common bacterium found in the skin of the majority of AD patients and is often implicated in infection (Baldry et al., 2018; Danby, 2017; Tong et al., 2015; Bouvet et al., 2017), the potency of the main cutaneous AMPs against staphylococci, as well as changes in their levels in AD, are presented (Table 1-1).

Table 1-1: The most common AMPs which are potent against *S. aureus*

AMPs	Expressed in	Induced by	Function	Levels in AD	References
HBD-2	Activated keratinocytes	Bacteria, lipopolysaccharide, IL-1α, IL-17, IL-22, IL-1β, TNF-α, IL-17α/IFN-γ	Pore formation, cytokine production, keratinocyte differentiation, chemotaxis	Increased, but lower than in psoriasis (inhibited by IL-4/IL-13 in AD)	(Hata and Gallo, 2008; Yang et al., 2001; Niyonsaba et al., 2006; Liu et al., 2002; Harder et al., 2001; Harder et al., 2004; Harder et al., 1997; Harder et al., 2010; Ong et al., 2002; Harder et al., 2000; Mathews et al., 1999; Dinulos et al., 2003; Simanski et al., 2013; Gläser et al., 2009; Joly et al., 2004)
HBD-3	Activated keratinocytes	Bacteria, lipopolysaccharide, IL-1α, IL-17, IL-22, IL-1β, TNF-α, IL-17α/IFN-γ	Pore formation, inhibition of cell wall biosynthesis, cytokine production, keratinocyte differentiation, chemotaxis	Increased, but lower than in psoriasis (inhibited by IL-4/IL-13 in AD)	(Kisich et al., 2008; Schibli et al., 2002; Harder et al., 2001; Schröder, 2011; Simanski et al., 2013; Hata and Gallo, 2008; Yang et al., 2001; Niyonsaba et al., 2006; Liu et al., 2002; Harder et al., 2004; Harder and Schröder, 2002; Sass et al., 2010)
Cathelicidin LL-37	Keratinocytes (continually-low levels), neutrophils, activated keratinocytes, eccrine glands	Vitamin D_3, bacteria	Pore formation, Increase of membrane permeability, antimicrobial, chemoattractant & immunoregulatory function	Increased, but lower than in psoriasis (due to increased IL-4/IL-13 & reduced Vitamin D_3 in AD); similar in non-lesional AD & healthy skin	(Schauber et al., 2007; Vandamme et al., 2012; Murakami et al., 2002; Ong et al., 2002; Mallbris et al., 2010; Wang et al., 2004; Reinholz et al., 2012; Howell et al., 2006b; Jeng et al., 2009; Dorschner et al., 2001; Liu et al., 2013)

Table 1-1 (Continued)

AMPs	Expressed in	Induced by	Function	Levels in AD	References
Calprotectin (S100A8/ S100A9)	Neutrophils, activated keratinocytes	IFN-γ, TNF-α, IL-1, bacteria	Sequesters zinc, manganese & iron, keratinocyte proliferation & differentiation	Increased, but lower than in psoriasis (inhibited by IL-4/IL-13 in AD)	(Mørk et al., 2003; Ganz and Lehrer, 1997; Corbin et al., 2008; Liang et al., 2006; Hansson et al., 2014; Yui et al., 2003; Kelly et al., 1989; Aochi et al., 2011; Semprini et al., 2002; Broome et al., 2003; Nakashige et al., 2015)
Psoriasin (S100A7)	Sebaceous glands, differentiated keratinocytes	IL-17α/IFN-γ, bacteria	Pore formation, sequesters zinc	Increased, but lower than in psoriasis (inhibited by IL-4/IL-13 in AD)	(Gläser et al., 2005; Gläser et al., 2009; Broome et al., 2003; Michalek et al., 2009; Harder et al., 2010; Simanski et al., 2013; Alowami et al., 2003; Cunden et al., 2017)
Dermcidin 1L	Continually produced by eccrine glands	Not induced in epidermal cells during skin inflammation	Bacterial membrane depolarisation, keratinocyte stimulation, cytokines (TNF-α, IL8, CXCL10, CCL20)	Decreased in AD	(Niyonsaba et al., 2009; Lai et al., 2007; Rieg et al., 2005; Rieg et al., 2004; Schittek, 2012; Schittek et al., 2001; Burian and Schittek, 2015)
RNase7	Epithelia, keratinocytes (continually produced but also inducible)	TNF-α, IL-1β, IFN-γ, IL-17α/IFN-γ, bacteria	Pore formation, membrane destabilisation	Increased in AD lesional skin; similar in non-lesional AD & healthy skin	(Simanski et al., 2013; Harder and Schröder, 2005; Harder and Schröder, 2002; Harder et al., 2010; Harder et al., 2001; Becknell and Spencer, 2016)

1.3.5. The skin microbiome

The skin microbiome is associated with AD pathogenesis, although its role has not been fully elucidated yet (Paller et al., 2019; Lee et al., 2018). The skin microbiome refers to the diverse milieu of bacteria, fungi and viruses that colonise the skin. Most of them are skin commensals, which do not harm the host and protect their colonies and subsequently the skin from colonisation by pathogens. The normal skin flora consists mainly of *S. epidermidis* and other coagulase-negative staphylococci, whereas *S. aureus* prevalence is around 5% (Otto, 2010; Cogen et al., 2008; Williams et al., 1990). Different sites of the body are colonised by different microbial populations, depending on the characteristics of the area. Colonisation by pathogens affects the balance and reduces the diversity of the skin microbiome. Imbalance of the skin microbial flora has been linked to diseases (Byrd et al., 2018; Findley and Grice, 2014). Both commensals and pathogens affect the immune response and the production of AMPs (Wanke et al., 2011; Chung and Dale, 2004; Lai et al., 2010; Chung and Dale, 2008).

1.3.5.1. Skin commensals – *S. epidermidis*

Skin flora plays an important role in skin defence not only directly through colonisation resistance, whereby skin commensals inhibit colonisation of the skin by pathogens (e.g. by producing antimicrobials) but also indirectly by affecting keratinocyte functions, enhancing innate immune activity and inducing T-regulatory cells and cytokine production (Prescott et al., 2017; Zipperer et al., 2016; Nakatsuji et al., 2017; Hannigan and Grice, 2013). Although the skin flora composition depends on various factors such as gender, age, site of the body, the immune system, lifestyle and environment, staphylococci, corynebacteria and propionibacteria represent more than 60% of the skin bacteria (Scharschmidt and Fischbach, 2013; Grice and Segre, 2011; Grice et al., 2009; Yamazaki et al., 2017). Moist body sites, such as creases, are mainly colonised by staphylococci and corynebacteria, sebaceous areas by staphylococci and propionibacteria and dry areas by β-proteobacteria and flavobacteria (Costello et al., 2009; Grice et al., 2009;

Grice and Segre, 2011). Malassezia is the most common species of the fungal flora (up to 80%), found mainly in sebaceous areas (Gao et al., 2010; Paulino et al., 2008; Findley and Grice, 2014). The bacterial flora is the largest proportion of the total normal skin microbiome (Oh et al., 2014; Rodrigues-Hoffmann, 2017; Gao et al., 2010; Huffnagle and Noverr, 2013). The interactions between host and skin flora have been examined in most detail for *Staphylococcus epidermidis* (Cogen et al., 2008). Most skin commensals are opportunistic pathogens, meaning they only cause infection under specific conditions (Price et al., 2017; Cogen et al., 2010a; Lai et al., 2010).

The most abundant commensal on normal skin and mucosal surfaces is *S. epidermidis*, which comprises more than 90% of the aerobic microbial flora (Prescott et al., 2017; Otto, 2009; Otto, 2012). It has a predominantly mutualistic relationship with the host as it competes against other bacteria (e.g. *S. aureus*) by producing bacteriocins (antibacterial peptides) such as lantibiotics (epidermin, epilancin-K7, staphylococcin-1580), thus preventing pathogen colonisation (Otto, 2009; Iwase et al., 2010; Cogen et al., 2008; Peschel and Götz, 1996; Bierbaum et al., 1996). More specifically, *S. epidermidis* acts against *S. aureus* by producing phenol-soluble modulins (PSM) δ and γ, which disrupt lipid membranes and Esp-serine protease, which increases the HBD-2 activity against *S. aureus* biofilms (Vandecandelaere et al., 2014; Iwase et al., 2010; Cogen et al., 2010b).

S. epidermidis is generally characterised by low virulence. Biofilm formation is considered its most important virulence factor (Otto, 2009; Kocianova et al., 2005; Vuong and Otto, 2002). During biofilm formation, bacterial cells attach to a surface and then more groups of bacteria accumulate and form a multi-layered structure. These structures can detach from a surface and spread to neighbouring surfaces. This procedure is mediated through a variety of molecules, such as polysaccharide intercellular adhesins and other surface proteins. In addition to their role in biofilm formation, molecules such as poly-γ-glutamic acid also offer protection from host defence mechanisms (e.g. AMPs and neutrophils) and external pathogens (Sabaté-Brescó et al., 2017; Li et al., 2005; McKenney et al., 2000; Cogen et al., 2008; Otto, 2009; Kocianova et al., 2005). Adhesion of *S. epidermidis* to host molecules such as fibrinogen, fibronectin, collagen and elastin is succeeded through cell

wall-anchored proteins (adhesins) (Sabaté-Brescó et al., 2017; Arrecubieta et al., 2007; Brennan et al., 2009; Arciola et al., 2003).

The accessory gene regulator (Agr) quorum sensing system of *S. epidermidis* detects the expression of an autoinducing peptide (AIP) produced during cell growth. In increased colony densities it promotes the expression of PSMβs, which detach *S. epidermidis* biofilms from a surface and promotes colonisation to other areas. The *S. epidermidis* Agr system also produces pheromones, which inhibit the Agr system of other bacteria (Boles and Horswill, 2008; Olson et al., 2014; Otto, 2001; Otto et al., 2001; Otto et al., 1999).

Despite the harmonic interaction with the host, *S. epidermidis* can become pathogenic under specific conditions, such as in presence of medical devices, in immunocompromised patients and in neonates. Biofilm formation is the main cause of its pathogenicity and production of virulence factors such as PSMs is kept low, in accordance with the commensal nature of *S. epidermidis* (Otto, 2012; Namvar et al., 2014; Dong and Speer, 2014; Sabaté-Brescó et al., 2017; Büttner et al., 2015).

In conclusion, *S. epidermidis* as a skin commensal can protect human host from external pathogens. Reduction of *S. epidermidis* skin colonies (e.g. with antibiotics) can disrupt the skin microbial balance (Cogen et al., 2008).

1.3.5.2. Skin pathogens – *S. aureus*

Pathogens are microorganisms that live at the expense of a host and cause disease (Walter et al., 2002). While *S. aureus* is rarely found on healthy skin, it is one of the most common causes of systemic, skin, soft-tissue and respiratory tract infections (Lowy, 1998; Moreillon et al., 1995; Cogen et al., 2008). However, persistent nasal carriage of *S. aureus* in healthy people can reach up to 30% (Zanger et al., 2011; Sakr et al., 2018).

In order to protect itself from the host, *S. aureus* has developed a variety of mechanisms, including epithelial barrier disruption, inhibition of AMPs and opsonisation, neutrophil-chemotaxis interference, and neutrophil-cytolysis

(Iwatsuki et al., 2006). Growth and toxin production of *S. aureus* are optimal at 37°C and pH 7.0 (Wirtanen and Salo, 2016). The virulence factors produced by *S. aureus* are either secreted or surface-associated and include superantigens (enterotoxins A-C, toxic shock syndrome toxin-1), cytotoxins (hemolysins α-δ), enzymes (proteases, lipases, nucleases) and surface proteins (fibronectin-binding proteins). The main mechanisms that *S. aureus* has developed against the host have been identified (Table 1-2) (Iwatsuki et al., 2006; Foster, 2005; Lyon and Novick, 2004). *S. aureus* superantigens are produced by more than 70% of *S. aureus* strains (Bröker et al., 2016; Becker et al., 2003). They are exotoxins that bridge the major histocompatibility complex and the variable region of the T-cell receptor β chain, leading to polyclonal T-cell activation. This results in massive production of inflammatory mediators. As there are only around 25 types of variable region of T-cell receptor β chain and the *S. aureus* superantigens bind to more than one type of β chain, they can activate up to 25% of T-cells, whereas specific binding to the major histocompatibility complex of other antigens activates less than 0.01% of T-cells (Fraser and John, 2016; Llewelyn and Cohen, 2002; Leung et al., 1995).

Table 1-2: *S. aureus* protective mechanisms against host

Factor	Function
Exfoliative toxins	Disrupt cell-to-cell cohesion; allow staphylococci to spread on the skin (Iwatsuki et al., 2006; Cogen et al., 2008)
Enterotoxins A-C & toxic shock syndrome toxin-1	Cause T-cell activation; inhibit eosinophil apoptosis (Iwatsuki et al., 2006; Cogen et al., 2008; Wedi et al., 2002)
Agr system	Regulates toxin & biofilm production (Traber et al., 2008)
Chemotaxis inhibitory protein of staphylococci	Affects neutrophil chemotaxis (De Haas et al., 2004)
Extracellular adherence protein	Inhibits leucocyte recruitment & extravasation (Chavakis et al., 2002)
α-toxin	Damages cell membranes & causes apoptosis of B-cells, T-cells & monocytes; affects signalling between innate & adaptive system, leading to T-cell activation (Otto, 2014; Nygaard et al., 2012; Niebuhr et al., 2011b)

The Agr quorum sensing system of *S. aureus* consists of both secreted and surface proteins and regulates the *S. aureus* virulence (Traber et al., 2008; Wang and Tom, 2016). The AIPs produced by *S. aureus* cells provide positive feedback to the Agr system. During late-log and stationary phases, when cell densities are increased, the Agr system is activated leading to inhibition of biofilm formation and secretion of virulence factors (Pollitt et al., 2014; Thoendel et al., 2011; Novick, 2003; Carnes et al., 2010; Qazi et al., 2001). In contrast, in low cell densities (with consequent low AIP production) or in a high-glucose environment the Agr system is repressed, leading to increased biofilm formation and reduced production of virulence factors (Boles and Horswill, 2008). Mutations of the Agr system result in increased biofilm production (Vuong et al., 2000; Pollitt et al., 2014; Kielian et al., 2001).

1.3.5.3. Phenol-soluble modulins (PSMs)

PSMs are amphipathic, α-helical peptides produced by staphylococci and play an important role in their virulence (Cheung et al., 2014). Based on their length, PSMs are divided in α-type (20-25 amino-acids) and β-type (43-45 amino-acids). Most α-type PSMs have a neutral or positive charge, whereas β-type PSMs have a negative charge (Cheung et al., 2014). It has been found that *S. aureus* produces PSMα1-PSMα4, PSMβ1, PSMβ2 and δ-toxin (PSMγ), (Wang et al., 2007; Cheung et al., 2014), whereas *S. epidermidis* produces PSMα, PSMβ1, PSMβ2, PSMδ, PSMε, and δ-toxin (Cheung et al., 2014; Mehlin et al., 1999; Vuong et al., 2004b; Yao et al., 2005). The PSMβs and δ-toxin are similar between *S. aureus* and *S. epidermidis* (Cheung et al., 2014).

At micromolar concentrations, PSMs disrupt the membranes of host cells, leading to cell-lysis, whereas at nanomolar concentrations they induce neutrophil recruitment by binding to the formyl peptide receptor 2 of the immune cells, leading to chemokine release (Berube et al., 2014; Kretschmer et al., 2010; Cheung et al., 2014; Cogen et al., 2010a; Syed et al., 2015; Giese et al., 2011). PSMs also contribute to biofilm detachment and to bacterial expansion and colonisation (Periasamy et al., 2012; Dastgheyb et al., 2015). The fact that the Agr system regulates PSMs plays a role in the differences in

PSM production by commensal and pathogenic bacteria (Le and Otto, 2015; Cheung et al., 2014; Queck et al., 2008; Vuong et al., 2004a). Although *S. epidermidis* can produce cytolytic PSMs (PSMδ), it usually produces less aggressive forms (PSMβs). In contrast, *S. aureus* produces more cytolytic PSMαs and less PSMβs (Cheung et al., 2010; Otto, 2009; Wang et al., 2011; Wang et al., 2007). The capacity of *S. epidermidis* to lyse neutrophils is reduced compared to *S. aureus*. In contrast to *S. aureus* strains that produce highly cytolytic PSMαs, *S. epidermidis* evades neutrophil killing with passive methods such as extracellular polymers that inhibit the uptake of *S. epidermidis* by neutrophils, biofilm formation, the SepA protease and the Aps AMP sensor. This indicates that, when it comes to host colonisation, *S. epidermidis* has a more passive profile compared to *S. aureus* (Cheung et al., 2010; Otto, 2009; Wang et al., 2011; Wang et al., 2007).

Although δ-toxin is similar between *S. epidermidis* and *S. aureus*, *S. epidermidis* δ-toxin can bind to AMPs (HBD-2, HBD-3, LL-37) and increase their antimicrobial activity, unlike *S. aureus* δ-toxin (Cogen et al., 2010a). *S. epidermidis* PSMδ and δ-toxin have shown similarities in the structure and mode of action to LL-37. In addition, they can help maintain the normal skin flora, as they are potent mainly against pathogens such as *S. aureus* (Cogen et al., 2010b).

1.3.5.4. The skin microbiome in AD

The skin microbiome plays an important role in AD pathogenesis (Paller et al., 2019; Lee et al., 2018; Salava and Lauerma, 2014). In AD the susceptibility to external pathogens is increased (Hata and Gallo, 2008) and the microbial diversity is decreased (Nakatsuji et al., 2017; Salava and Lauerma, 2014; Kong et al., 2012). Pathogen colonisation is facilitated by changes that take place during AD, such as the defective skin barrier and the shift in skin immune response (Th_2 axis, AMP deficiency) (Nowicka and Grywalska, 2018; Tomczak et al., 2019). The predominant pathogen in AD is *S. aureus* (Figure 1-5), followed by β-hemolytic Streptococci (Chiu et al., 2009; David and Cambridge, 1986), whereas *S. epidermidis* populations are reduced (Higaki et al., 1999). Malassezia species are part of the normal skin flora but may also

be implicated in the pathogenesis of AD, where specific IgE sensitisation of Malassezia allergen is commonly found (Glatz et al., 2017; Gaitanis et al., 2013; Rodrigues-Hoffmann, 2017; Harada et al., 2015; Kekki et al., 2013). Also, AD increases the predisposition to herpes simplex virus-1 and vaccinia virus (Kim et al., 2013).

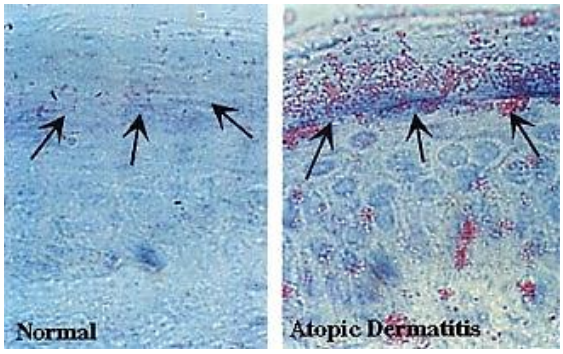


Figure 1-5: *S. aureus* adherence in normal and AD skin

Adherence of *S. aureus* (arrows) is observed mainly in the stratum corneum and is increased in AD compared to normal skin [image from (Cho et al., 2001)].

In lesional AD skin, *S. aureus* prevalence is around 91%, whereas coagulase-negative staphylococci are the second most prevalent (9%) (Baldry et al., 2018; Danby, 2017; Abeck and Mempel, 1998; Aly et al., 1977). In non-lesional AD skin, the prevalence of coagulase-negative staphylococci is around 60% and of *S. aureus* about 30% (Aly et al., 1977). Moreover, *S. aureus* is more abundant in acute than in chronic AD lesions (Leyden et al., 1974; Kong et al., 2012). The *S. aureus* densities in lesional AD skin may exceed 10^7 organisms/cm^2 and in non-lesional AD skin are around 10^3-10^4 organisms/cm^2 (Aly et al., 1977; Leyden et al., 1974; Zollner et al., 2000; Tomi et al., 2005; Williams et al., 1990; Kong et al., 2012; Di Domenico et al., 2018). The dysfunctional skin barrier in AD and the increased Th_2 cytokines, which downregulate AMPs, along with *S. aureus* virulence factors promote *S. aureus* colonisation (Kisich et al., 2008; Miajlovic et al., 2010). Prevalence of *S. aureus* in anterior nares of AD patients is significantly higher than in healthy people and nasal colonisation can contribute to further colonisation of AD skin (Chiu et al., 2010; Nishijima et al., 1997; Williams et al., 1998).

Around 30% to 60% of *S. aureus* strains on AD skin secrete superantigens (Breuer et al., 2002; Breuer et al., 2000; Bunikowski et al., 2000). Colonisation with *S. aureus* and mainly toxin-producing strains is positively correlated with AD severity (Nowicka and Grywalska, 2018; Tomczak et al., 2019). Enterotoxins produced by *S. aureus* can increase the skin reaction to common allergens in AD patients (Langer et al., 2007). *S. aureus* α-toxin promotes Th_{17} and Th_1 cell activation and IFN-γ production, leading to chronification of AD (Wichmann et al., 2009; Breuer et al., 2004; Breuer et al., 2005; Niebuhr et al., 2011a; Tauber et al., 2016).

Patients with AD can develop specific sensitisation and IgE responses to *S. aureus* superantigens (McAleer and Irvine, 2013; Llewelyn and Cohen, 2002; Tomi et al., 2005; Zollner et al., 2000; Bunikowski et al., 2000). Treatment against *S. aureus* in AD patients even without clinical signs of superinfection can improve the disease. Nevertheless, eradication of *S. aureus* is not possible and recolonisation can be seen after 1-2 months, indicating the need for a more effective and long-term treatment (Breuer et al., 2002; Huang et al., 2009; Gong et al., 2006; Machura et al., 2008; Ryan et al., 2013).

There is a significant overuse of topical and systemic antibiotics in particular in primary care, which has increased the prevalence of *S. aureus* resistant strains (Błażewicz et al., 2017; Llor and Bjerrum, 2014; Ventola, 2015; Shallcross and Davies, 2014). Increased resistance of *S. aureus* strains in AD patients mainly to fusidic acid but also to methicillin and other antibiotics compared to healthy people has been reported (Harkins et al., 2018; Jung et al., 2015; Park et al., 2016). Moreover, antibiotics also act against *S. epidermidis* and other bacteria of the normal skin flora, leading to reduced microbial diversity and increased *S. aureus* colonisation after the antibiotic treatment, which suggests that antibiotics fail to restore the microbial balance and the proper recolonisation of the skin (Kobayashi et al., 2015; Friedman and Goldman, 2011; Gilani et al., 2005; SanMiguel et al., 2017). A systematic review emphasised the importance of *S. aureus* on AD course and the need for further research regarding the association of *S. aureus* with AD pathogenesis (Totte et al., 2016).

1.4. The project

1.4.1. Project background

Pathogenesis of AD

Genetic predisposition, environmental factors, skin barrier, immune response and skin microbiome are all implicated in the pathogenesis of AD (Table 1-3). Nevertheless, it has not yet been elucidated which factor is the driving cause of AD and which factors are secondary (McPherson, 2016; Salava and Lauerma, 2014; Thomsen, 2014). Deeper understanding of the contribution of these factors to AD will support the development of more targeted and effective treatments for patients (McPherson, 2016).

Two main hypotheses regarding AD pathogenesis have been proposed (Figure 1-6). The “inside-outside” theory describes the immune system dysregulation, including a shift towards a predominance of Th_2 responses, a weakening of the skin barrier under the influence of IL-4, IL-13, IL-33 and a switch of the antibody production of the IgE isotype (class switch) with the development of specific IgE against innocuous antigens (Brüssow, 2016; Elias et al., 2008; Boguniewicz and Leung, 2006; Ong et al., 2002; Cork et al., 2009). The “outside-inside” theory claims that a defective skin barrier allows pathogens to penetrate the skin and trigger a deregulated immune response (Brüssow, 2016; Elias et al., 2008). The “outside-inside” pathway can cause immune system dysregulation (“inside-outside” theory), leading to a vicious circle (Figure 1-6) (Elias, 2008; Elias et al., 2008).

Table 1-3: Summary of the factors implicated in AD pathogenesis

Pathogenesis of AD	
Genetic factors	
- Filaggrin gene mutations	→ affect skin barrier integrity & hydration
- *FcεRI* gene polymorphisms	→ mast cell degranulation
- *SPINK5* gene mutations	→ ↑ IgE
- *CCL5* gene mutations	→ ↑ eosinophil migration
- *IL-13* & *IL-13Rα1* single nucleotide polymorphisms	→ ↑ IgE
- *TLR2* & *NOD2* gene mutations	→ ↑ cytokines & eosinophils
Environmental factors	
socio-economic factors, skin dryness, infection, extreme climatic conditions, aero-allergens, specific IgE sensitisation (*S. aureus* superantigens)	→ affect skin barrier integrity & pathogen penetration
Skin barrier	
- ↑ cholesterol & linoleic acid ↓ tight junction & cornified cell envelope proteins, ceramides, γ-linolenic & arachidonic acids	→ affect skin barrier integrity
- ↓ cis-UCA & sphingosine	→ ↓ skin antimicrobial activity
- ↑ pH	→ affects activity of proteases, AMPs, lipid synthesis & pathogen colonisation
Immune response	
- shift towards Th_2 axis	→ ↑ Th_2 cytokines, Th_2 cells, mast cells ↑ eosinophils, IgE ↓ keratinocyte differentiation, filaggrin, cis-UCA, loricrin, involucrin changes in lipid composition & ↑ pH ↑ adhesin, fibronectin, *S. aureus* binding
- downregulation of AMPs (compared to inflammation degree)	→ ↑ pathogen colonisation
Skin microbiome	
- ↓ skin commensals (*S. epidermidis*) & microbial diversity	→ ↑ pathogen colonisation
- ↑ *S. aureus* colonisation	→ ↑ T-cell expression with greater Th_2/Th_1 ratio, cytokine production (IL-22, TSLP, IL-31) & allergen sensitisation, pruritus & skin barrier disruption
- *S. aureus* superantigens	→ T-cell activation & ↑ cytokine production

References: Genetic factors (Palmer et al., 2006; Cookson and Moffatt, 2002; Tartaglia-Polcini et al., 2006; Bitoun et al., 2002; Kato et al., 2006; Namkung et al., 2011; Ahmad-Nejad et al., 2004; Wong et al., 2016); Environmental factors (Leung and Bieber, 2003; Schneider et al., 2013; Langan and Irvine, 2013; Silverberg et al.,

2013; Harrop et al., 2007; Sheffield et al., 2011; Sybilski et al., 2015; Shaw et al., 2011; Zollner et al., 2000; Bunikowski et al., 2000); Skin barrier (Calder and Burdge, 2012; Di Nardo et al., 1998; Imokawa et al., 1991; Arikawa et al., 2002; Kezic et al., 2011; Palmer et al., 2006; Gruber et al., 2015; Bao et al., 2016; Panther and Jacob, 2015; Miajlovic et al., 2010); Immune response (Ahmad-Nejad et al., 2004; Wong et al., 2016; Albanesi et al., 2007; Amano et al., 2015; Nomura et al., 2003b); Skin microbiome (Kong et al., 2012; Chiu et al., 2009; Higaki et al., 1999; Wedi et al., 2002; Simpson et al., 2018; Nakatsuji et al., 2016; Baviera et al., 2015; Wichmann et al., 2009)

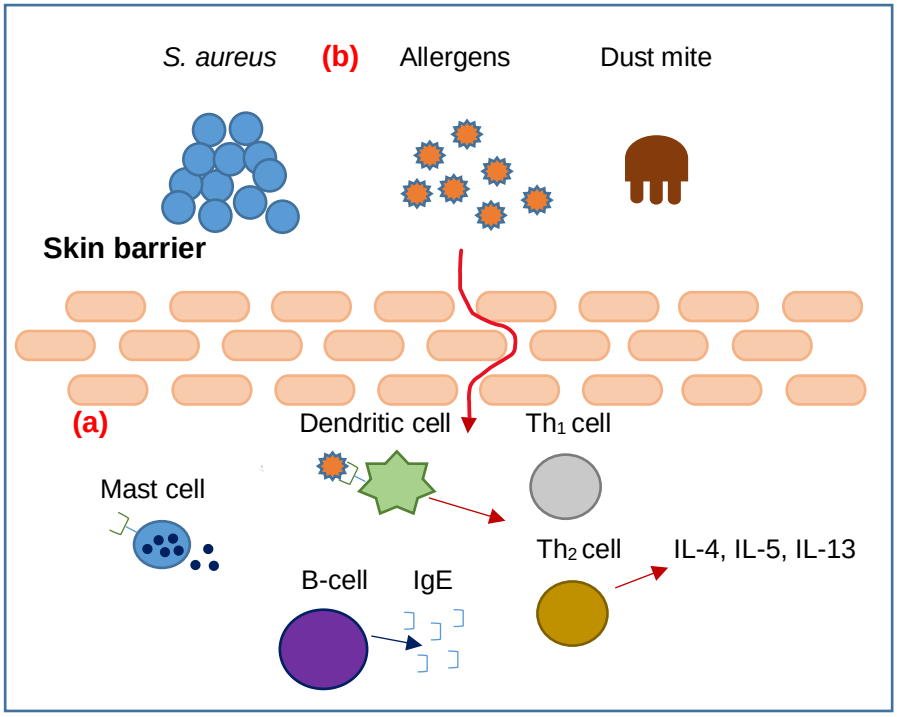


Figure 1-6: Inside-outside and outside-inside theory

a. "Inside-outside" theory: When allergens come in contact with dendritic cells, they induce their migration to the dermis and promote cytokine production. A predisposition to elevated Th_2 cells can lead to increased Th_2 cytokine production (IL-4, IL-5, IL-13). Allergen presentation by dendritic cells in the context of Th_2 cytokines also induces the production of allergen-specific IgE from B-cells, which activates mast cells and induces inflammatory molecules. The inflammation compromises the skin barrier. **b.** "Outside-inside" theory: Skin barrier defects allow allergens to penetrate the skin and trigger inflammatory mediators (Th_2 cells and Th_2 cytokines) (Cork et al., 2009; Brüssow, 2016; Elias et al., 2008).

Microbial dysbiosis and medical conditions

There is a growing body of literature, which suggests that microbial dysbiosis is implicated in a broad range of medical conditions (Carding et al., 2015; Wang et al., 2017; Belizário et al., 2018). There is increasing evidence that

dysbiosis of the gut microbiome may play a role in the pathogenesis of intestinal diseases (Olivares et al., 2015; De Palma et al., 2010; Krogius-Kurikka et al., 2009; Miquel et al., 2013), metabolic disorders (Larsen et al., 2010; Ley et al., 2006; Vrieze et al., 2012), disorders of the central nervous system (Hsiao et al., 2013; Desbonnet et al., 2010; Neufeld et al., 2011) and autoimmune conditions (Mu et al., 2017). Oral microbial dysbiosis has also been found to play a role in oral infections and systemic conditions (Sudhakara et al., 2018; Van Der Meulen et al., 2016). Although more research is needed regarding the exact role of the microbiome in these disorders, several studies have noticed that there is an increased prevalence of microbial dysbiosis in people affected with such conditions and that restoration of the microbial flora may improve the symptoms (Walker et al., 2011; Carding et al., 2015; Belizário et al., 2018).

Microbial dysbiosis and AD

The defective skin barrier and immune system dysregulation in AD are associated with skin microbial imbalance and *S. aureus* colonisation, which is the most common pathogen in AD, although their correlation is not clear yet (Chiu et al., 2009; Harder et al., 2010; Rieg et al., 2005; Paller et al., 2019; Lee et al., 2018).

Whether or not there is a causative relationship between *S. aureus* and AD, needs to be investigated. Research results are conflicting. Studies have reported that imbalance of skin microbial flora may precede the development of AD (Kennedy et al., 2017) and that restoration of the skin microbial balance in AD exacerbations precedes the improvement of AD lesions (Kong et al., 2012). Skin microbial dysbiosis characterised by increased *S. aureus* colonisation, has been also shown to trigger atopic-like inflammation in mice (Kobayashi et al., 2015). However, other researchers failed to show that treatment of *S. aureus* can improve AD (Bath-Hextall et al., 2010).

Furthermore, even though *S. aureus* and *S. epidermidis* belong to the same genus and have many similarities (Méric et al., 2015), they have different effects on the skin. This is also supported by the fact that normal skin flora consists mainly of *S. epidermidis* and *S. aureus* prevalence is around 5%

(Otto, 2010; Cogen et al., 2008; Williams et al., 1990), whereas in AD *S. aureus* prevalence is more than 90% (Baldry et al., 2018; Chiu et al., 2009; David and Cambridge, 1986). Therefore, this fact raises the question of whether skin has developed specific mechanisms that maintain *S. aureus* colonisation low in normal skin and whether defects in these mechanisms lead to microbial dysbiosis and increased *S. aureus* colonisation, which may potentially trigger AD. Most scientific research investigates the role of *S. aureus* in AD by performing studies involving AD patients (Totte et al., 2016). This project involved both *S. epidermidis* and *S. aureus*, focused on the role of *S. aureus* at the cellular level and investigated differences in the effects of *S. aureus* and *S. epidermidis* on keratinocytes, with a view to understanding the role of *S. aureus* in the pathogenesis of AD.

1.4.2. Hypothesis, aims and achievements of the project

Hypothesis: *S. epidermidis*, which is a skin commensal and *S. aureus*, which is associated with skin infections, trigger different responses in keratinocytes and differential induction of AMPs. An atopic environment can alter the response of keratinocytes to *S. aureus*.

Aims: The main aim of the project was to investigate the interaction of staphylococci with skin cells, focusing on the AMP production by keratinocytes, when exposed to *S. aureus* and *S. epidermidis*. A second aim was to identify compounds that favour the growth of *S. epidermidis* over *S. aureus*.

Achievements:

a. *S. aureus* but not *S. epidermidis* induces HBD-3 production in keratinocytes

b. Induction of HBD-2 and LL-37 production in keratinocytes by *S. aureus* and *S. epidermidis* is similar

c. The factor(s) produced by *S. aureus* responsible for the HBD-3 production in keratinocytes are proteinaceous, most likely heat-stable and surface-associated/secreted

2. Materials and methods

2.1. Materials

Manufacturers/suppliers of materials used in this study are indicated within this chapter and their addresses are given in Appendices and supplemental material, 8.1.

2.2. Staphylococci used in this study

The *S. aureus* and *S. epidermidis* strains used in this project are shown in Table 2-1.

Two presumptive *S. epidermidis* strains were self-isolated from the forearm by members of the laboratory of Dr Alex O'Neill, by plating swabs onto mannitol-salt agar (Sigma-Aldrich). The individuals from which the strains were isolated had no skin disease or other chronic condition. The identity of these strains was confirmed as *S. epidermidis* by amplification of the RNA polymerase β subunit (*rpoB*) by polymerase chain reaction (PCR) and DNA sequencing (analysed in chapter 2.2.2.3) (Drancourt and Raoult, 2002) and named SE3 and SE9, respectively.

The NRS strains and the *S. aureus* USA300 mutant strains from the Nebraska Transposon Mutant Library (NTML) were obtained from the Network on Antimicrobial Resistance in *S. aureus* (NARSA) repository (BEI Resources, c2019). The MOS strains are a mix of hospital-associated, community-associated and livestock-associated *S. aureus* strains, collected and pre-characterised during the MOSAR project by the MOSAR trial investigators group (Lee et al., 2013; Kime et al., 2019). The *S. aureus* WC003 and WC006 strains were purchased from Woundchek.

All other staphylococci used in this project are part of a collection held in the laboratory of Dr Alex O'Neill, University of Leeds.

Table 2-1: Staphylococcal strains used in this project

(a) Strain	***S. epidermidis*** Comments	Source/Reference
NCTC11047	Isolated from human nose	(Public Health England), (Banner et al., 2007)
NCTC6513	Isolated from human nose	(Public Health England), (Sanyal and Greenwood, 1993),
7765746	Methicillin resistant strain	(Laboratory of Dr Alex O'Neill)
SE3	Isolated from healthy skin	(Current project)
SE9	Isolated from healthy skin	(Current project)
(b) Strain	***S. aureus*** **Comments**	**Source/Reference**
USA300	Highly virulent clinical isolate, methicillin resistant, implicated in recurrent skin infections	(Azarian et al., 2016; Fey et al., 2013; Boyle-Vavra and Daum, 2007)
SH1000	Common laboratory strain with well characterised virulent repertoire, used in cutaneous infections studies	(O'Neill, 2010), (Horsburgh et al., 2002; O'Neill, 2010; Clarke et al., 2007)
UAMS-1	Biofilm forming clinical isolate	(Savage et al., 2013a; Sassi et al., 2015)
WC003	V8 protease positive clinical isolate	(Woundchek)
WC006	V8 protease positive clinical isolate	(Woundchek)
WV1	SH1000 variant with loss-of-function *sigB* gene mutation (deletion of 650-770 *sigB* nucleotides), increased *agr* function, highly proteolytic, unable to form biofilms	(Savage et al., 2013b)
LPV1	SH1000 variant loss of *agr* function due to *agrA* missense mutation, biofilm forming, deficient in colony spreading	(Savage et al., 2013b)
CS6-EEFIC*	AD clone, implicated in impetigo, *fusB* gene, resistant to fucidic acid	(O'Neill et al., 2007a; Pereira, 2014)
805	AD clones, *fusC* gene, resistant to fucidic acid	(O'Neill et al., 2007b)
1128		
602		
1076		
1083		
860		
851		
979		
NRS720	Methicillin resistant clinical isolate	(BEI Resources, c2019)
NRS786	Clinical isolate	(BEI Resources, c2019)
NRS65	Clinical isolate, glycopeptide-intermediate **	(BEI Resources, c2019)

Table 2-1 (Continued)

(b) Strain	***S. aureus*** Comments	Source/Reference
MOS283	Methicillin resistant clinical isolates	(Lee et al., 2013; Kime et al., 2019)
MOS148		
MOS287		
MOS457		
MOS259		
MOS229		
MOS135		
MOS53		
MOS153		
MOS227		
MOS165		
MOS412		
MOS208		
MOS128		

(c) Strain Name	***S. aureus* USA300 mutants** *** Gene description	Accession number
NE24	secretory antigen precursor SsaA (*ssaA*)	SAUSA300_2503
NE33	LPXTG-motif cell wall surface anchor family protein	SAUSA300_2589
NE67	immunodominant antigen B (*isaB*)	SAUSA300_2573
NE460	autolysin (*atl*)	SAUSA300_0955
NE710	thermonuclease precursor	SAUSA300_0776
NE778	glycerophosphoryl diester phosphodiesterase (*glpQ*)	SAUSA300_0862
NE925	superantigen-like protein 7	SAUSA300_0401
NE987	secretory antigen precursor SsaA (*ssaA*)	SAUSA300_2249
NE1035	secretory antigen precursor SsaA (*ssaA*)	SAUSA300_2253
NE1109	RNA polymerase sigma factor SigB (*rpoF*)	SAUSA300_2022
NE1241	thermonuclease (*nuc*)	SAUSA300_1222
NE1289	SdrD protein (*sdrD*)	SAUSA300_0547
NE1408	phiSLT ORF 87-like protein, putative DNA-binding protein	SAUSA300_1430
NE1532	accessory gene regulator protein A (*agrA*)	SAUSA300_1992
NE1544	glycerophosphoryl diester phosphodiesterase family protein	SAUSA300_1020

* Epidemic European Fusidic acid-resistant Impetigo Clone

** Intermediate-level minimum inhibitory concentration (MIC) to the glycopeptide drugs vancomycin and teicoplanin

*** Source: (BEI Resources, c2019); Reference: (Bose et al., 2013)

2.2.1. Culturing staphylococcal strains

All staphylococcal strains were stored in cryovials (Thermo-Fisher Scientific) at -80°C as glycerol stocks, with 20% (w/v) glycerol (Sigma-Aldrich). According to the needs of each experiment, the staphylococcal strains were propagated using either Mueller-Hinton agar/broth II (MHA-II/MHB-II, Oxoid) or tryptone soy agar/broth (TSA/TSB, Oxoid).

In order to culture *S. aureus* strains, TSA or MHA-II agar plates were prepared (Sarstedt). For the NTML strains, agar was supplemented with 10 µg/mL erythromycin (Sigma-Aldrich). Strains were streaked for individual colonies from glycerol stocks using a 1 µL-inoculating loop (Sarstedt). The plates were incubated overnight (18 h) in a static incubator at 37^{o}C and then stored at 4^{o}C. Aliquots of 9 mL TSB or MHB-II were inoculated with individual colonies and incubated at 37^{o}C for 18 h with vigorous aeration.

2.2.2. Verifying the identity of staphylococcal species

2.2.2.1. Gram staining and pastorex test

In order to verify the identity of staphylococci, Gram staining and coagulase tests were performed for the previously identified staphylococcal strains that were used in this project (Table 2-1). Crystal violet, Lugol's iodine, carbol fuchsin (Pro Lab Diagnostics) and acetone (Sigma-Aldrich) were used during Gram staining (Matheson, 1999). In order to identify whether a strain is coagulase-positive or coagulase-negative, the pastorex test (Bio-rad) was used according to the manufacturer’s protocol (Appendices and supplemental material, 8.2).

2.2.2.2. Susceptibility testing – minimum inhibitory concentration (MIC) determination

In order to evaluate the susceptibility of staphylococcal strains to antimicrobial molecules, the MIC protocol was followed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Cockerill et al., 2012) (Appendices and supplemental material, 8.2). The panel of molecules chosen

for MICs are shown in Table 2-2. All compounds were purchased from Sigma-Aldrich, except arachidonic acid (Biovision).

Table 2-2: Peptides and fatty acids used in this project

Compound	Solvent/Solubility	Compound	Solvent/Solubility
Arachidonic acid	100 mg/mL 100% (v/v) ethanol	**D-alanine**	50 mg/mL sterile water
Cis-UCA	10 mg/mL sterile water	**L-alanine**	50 mg/mL sterile water
Erythromycin	50 mg/mL 50% (v/v) ethanol	**L-citrulline**	50 mg/mL sterile water
Linoleic acid	50 mg/mL sterile water	**D-aspartic acid**	50 mg/mL 1M NaOH
γ-linolenic acid	100 mg/mL DMSO*	**L-homoserine**	50 mg/mL sterile water
Phe-Ala	50 mg/mL sterile water	**Leu-Ala**	50 mg/mL sterile water
Ala-Phe	50 mg/mL 80% acetic acid		

* DMSO: dimethyl sulfoxide

2.2.2.3. PCR amplification and DNA sequencing[1]

Genomic DNA isolation of the staphylococcal strains was performed with the PurElute Bacterial Genomic Kit (EdgeBio) in general accordance with the manufacturer's instructions. However, following resuspension of the cell pellet in spheroblast buffer, lysostaphin was added at a final concentration of ~ 0.1 mg/mL and held at 37°C for 30 min to facilitate cell lysis. Post-isolation, DNA concentration was measured by UV spectrophotometry at 260 nm using a P300 nanophotometer (Implen). The purity of the DNA (absence of protein and salt/solvent contaminants) was determined from the absorbance ratios at 260 nm / 280 nm and 260 nm / 230 nm, respectively (Sambrook and Russell, 2001).

[1]PCR amplification-DNA sequencing was performed by Dr Liam Sharkey, post-doctoral researcher in the University of Leeds.

For PCR, oligonucleotide primers were synthesised by Eurofins (Appendices and supplemental material, 8.3), resuspended in 10 mM Tris-Cl (pH 8.5) to a final concentration of 100 pmol/µL and stored at -20°C. PCRs were performed in a Techne-TC-3000 thermal cycler (Bibby Scientific) with Q5 High-Fidelity DNA Polymerase (New England Biolabs). Each reaction was composed of 1 X Q5 reaction buffer, 200 µM dNTPs (Promega), 0.5 µM forward primer, 0.5 µM reverse primer, 10 ng genomic DNA, and 0.02 U/µL Q5 polymerase. Reactions were run for 30 cycles of 98°C for 10 s, 61°C for 20 s and 72°C for 20 s. Primer annealing temperatures were calculated using the NEB-Tm calculator (New England Biolabs, c2019).

PCR products were analysed by electrophoresis through a 1.2% (w/v) agarose gel in tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer (Thermo-Fisher Scientific) and 4 µL of SYBR Safe DNA stain (10,000X concentrate in DMSO, Thermo-Fisher Scientific) at 90 volts for 30 min. The DNA from the PCR reactions was purified using a QIAquick PCR Purification Kit (Qiagen), according to manufacturer's instructions and DNA sequencing was performed on the purified PCR product (Genewiz-Sanger sequencing, Beckman-Coulter Genomics). The sequences were subsequently searched against the National Center for Biotechnology Information (NCBI) genomic database using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990; Altschul et al., 1997).

2.2.3. Phenotype microarrays

After confirming their identity, *S. aureus* USA300, *S. aureus* CS6-EEFIC, *S. epidermidis* NCTC11047 and *S. epidermidis* NCTC6513 were sent to Biolog for phenotypic characterisation. The strains were tested using 10 phenotype microarray (PM) panels designed to interrogate metabolic pathways along with ionic, osmotic and pH effects (PM-1 to PM-10), and 10 panels to assess the sensitivity to various antimicrobials with different mechanisms of action (PM-12 to PM-20). A patented redox technology with cell respiration (nicotinamide adenine dinucleotide production) as a universal reporter was used (Biolog Inc, c2019; Bochner, 2009; Bochner, 2001).

2.3. Skin cells

2.3.1. Growth media

Primary normal human epidermal keratinocytes (isolated from single adult donors) and dermal fibroblasts (isolated from adult skin) were purchased from PromoCell. All cells used were between passages 3 and 5. The HaCaT cells (a human aneuploid immortal keratinocyte cell line from adult skin) were obtained from the laboratory of Dr Wittmann, University of Leeds.

Both HaCaT cells and fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM) with 1 g/L glucose and sodium pyruvate, supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS, Gibco, Thermo-Fisher Scientific) and 1% (v/v) penicillin-streptomycin (PenStrep 10,000 U/mL, Gibco, Thermo-Fisher Scientific). The primary keratinocytes were cultured in keratinocyte growth medium (KGM, PromoCell) supplemented with 0.004% (v/v) bovine pituitary extract, 0.125 ng/mL recombinant human epidermal growth factor, 5 µg/mL recombinant human insulin, 0.33 µg/mL hydrocortisone, 0.39 µg/mL epinephrine, 10 µg/mL recombinant human transferrin and 0.06 mM $CaCl_2$ (all products were from PromoCell).

2.3.2. Storage and thawing of skin cells

In order to freeze cells, a suspension with around 1 million cells was harvested by centrifugation at 400 x *g* for 3 min to remove the growth medium. The cell pellet was resuspended in 1 mL freezing medium (PromoCell) and stored overnight at -80^{o}C in a freezing container (Thermo-Fisher Scientific). The cells were then transferred to the liquid nitrogen tank for long-term storage.

In order to thaw cells, the cryovial obtained from the liquid nitrogen tank was thawed in a 37^{o}C waterbath and the suspension was transferred into a T75 or a T150 flask (Corning, Appleton Woods) containing the appropriate medium, as described above. For the T75 flasks, 15 mL of medium was used, whereas for the T150 flasks, 20 mL of medium was used. Cells were incubated at 37^{o}C with 5% CO_2 and medium was changed every 2-3 days. When the flasks were about 80% confluent, cells were passaged.

2.3.3. Skin cell passage

Primary keratinocytes

The growth medium from the flask was aspirated and the cells were washed twice with 10 mL of phosphate buffered saline (PBS, Gibco, Thermo-Fisher Scientific). Then, 5 mL of 0.02% versene-ethylenediaminetetraacetic acid (EDTA, Thermo-Fisher Scientific) was added and the flask was incubated at 37^{o}C with 5% CO_2, until the majority of cells appeared to detach from each other (~ 3 min). The versene-EDTA was aspirated and 3 mL of 0.05% trypsin-EDTA (Thermo-Fisher Scientific) was added to the flask. Cells were then incubated again at 37^{o}C with 5% CO_2 until the majority of cells appeared to detach from the flask (~ 3 min) and trypsin was then inactivated by addition of 3 mL of trypsin-neutralising solution (TNS, Lonza).

HaCaT cells and fibroblasts

The growth medium from the flask was aspirated and the cells were washed twice with 10 mL of PBS. Then, 5 mL of 0.05% trypsin-EDTA was used for the T150 flask and 3 mL for the T75 flask. The flask was incubated at 37^{o}C with 5% CO_2, until the majority of cells appeared to detach from each other (round up) and from the flask (~ 6-10 min). An equal volume of DMEM containing 10% (v/v) FBS and 1% (v/v) PenStrep was used to inactivate the trypsin (5 mL for the T150 flask and 3 mL for the T75 flask).

Final steps for all cell types

For all cell types, the suspension was transferred into a 50 mL-falcon tube (Corning, Appleton Woods). Cells were harvested by centrifugation at 400 x *g* for 3 min. The supernatant was then removed and the cell pellet resuspended in 10 mL of growth medium. In order to define the number of cells in the suspension, 10 µL of this suspension was mixed with an equal volume of trypan blue dye (Sigma-Aldrich) and introduced into a Hawksley hemocytometer (Camlab) with a cover slip (Thermo-Fisher Scientific). After defining the number of cells per mL of cell-suspension, approximately 25,000

cells were seeded onto 24-well plates (25,000 cells in 0.5 mL of medium in each well, Appleton Woods) and were incubated at 37ºC with 5% CO_2. When the confluence reached 70-80% (170,000 cells per well approximately), the plates were stimulated (usually within 1-3 days).

2.3.4. Stimulation of skin monolayers

About 30 min before stimulation, the medium in the wells of the HaCaT cultures was replaced with serum-free DMEM (DMEM with 1% PenStrep) and the medium in the wells of the primary keratinocyte cultures with KGM not containing epidermal growth factor and hydrocortisone. The 24-well plates were then stimulated with whole cultures (~ 10^7 CFU) or filter-sterilised supernatants (at a final dilution ratio of 1:50 in DMEM or KGM, unless stated otherwise) and incubated at 37ºC with 5% CO_2 (for 20 h when staphylococcal cells were added and for 24 h when only supernatant was added). As *S. aureus* densities in atopic skin were found to be around 10^7 CFU/cm^2 (Leyden et al., 1974), this number of bacteria was chosen in order to approximate the staphylococcal densities of inflamed skin. As controls, one well was left non-stimulated (DMEM or KGM), whereas TSB staphylococcal growth medium was added to another well. The suspensions were collected in 1.5 mL collection tubes (Eppendorf) and cleared by centrifugation at 500 x *g* for 5 min, to remove cell debris. These supernatants were then stored at -20ºC.

In order to create atopic-like conditions, HaCaT monolayers were incubated with 10, 20 and 50 ng/mL of recombinant IL-4 (Peprotech) for 1 h, prior to staphylococcal stimulation.

Lastly, a protease inhibitor cocktail (PIC) from Sigma-Aldrich was used to block proteolytic activity of *S. aureus* supernatant.

2.3.5. 3D skin equivalent models

The 3D skin equivalent models were produced by Dr Adewonuola Alase, post-doctoral researcher in the Wellcome Trust Brenner Building (St. James's

University Hospital, Leeds, UK), according to the “Vienna Model” (Seltmann et al., 2015; Mildner et al., 2006; Gschwandtner et al., 2013). Approximately 20,000 fibroblasts and 200,000 keratinocytes were seeded per cell culture insert (3 µm pore size, Corning, Appleton Woods). During stimulation, the surface of the skin equivalent was pierced with a 21 g needle (Terumo). Skin equivalents were stimulated with ~ 10^6 CFU of *S. aureus* USA300 or *S. epidermidis* NCTC6513, resuspended in PBS and incubated for 16 h at 37°C with 5% CO_2. PBS without bacterial cells was added to skin equivalents as a negative control. The culture medium was collected, cleared by centrifugation at 500 x *g* for 5 min and stored at -20°C.

2.4. Enzyme-linked immunosorbent assays (ELISA)

In order to measure the levels of the AMPs produced by skin cells when stimulated with staphylococci, enzyme-linked immunoassays (ELISA) were used. Compared to Western Blot, the ELISA test has shown better sensitivity and reproducibility and lower variability and has been widely used in similar experiments (Pempkowiak, 2001; Oh et al., 2017; Ommori et al., 2013). Sandwich ELISA assays were chosen. In these assays, two antibodies are used, which bind to different sites of the antigen and provide higher sensitivity and specificity compared to direct ELISA (Jordan, 2000). Initially, the plate was coated with the capture antibody. The sample was added, followed by the detection antibody. An enzyme-linked secondary antibody then bound to the detection antibody. Lastly, the substrate was added, and was converted by the enzyme to a detectable form (Cox et al., 2004).

The HBD-2 and HBD-3 ELISA kits were from Peprotech, whereas the human IL-8 ELISA kit was from Biolegend. The LL-37 ELISA kit was from Hycult Biotech. The manufacturer’s protocol was followed in each case. The detection limit for the HBD-2 ELISA is 31.25 pg/mL, for the HBD-3 ELISA is 62.5 pg/mL, for the IL-8 kit is 15.6 pg/mL and for the LL-37 kit is 100 pg/mL.

2.5. Toxi-light assay

The ToxiLight™ Non-destructive Cytotoxicity BioAssay Kit (Lonza) was used in order to define the toxicity of staphylococcal supernatants towards skin cells. The kit measures the activity of adenylate kinase, a protein released into the supernatant when cells die (Olsson et al., 1983; Crouch et al., 1993).

After collection of the supernatants, remaining cells were lysed using Triton X-100 (Sigma-Alrich), in order to determine the adenylate kinase which was contained in living cells (not released into the supernatant). Briefly, plates were washed with 200 µL of PBS followed by addition of 100 µL of 10% (v/v) Triton X-100 in PBS in each of the 24 wells. The plate was incubated at room temperature with shaking for 20 min. Lysates were centrifuged at 16,000 x *g* for 1 min. All supernatants and lysates were stored in 1.5 mL Eppendorf tubes at -20^{o}C. The Toxilight protocol was followed according to the manufacturer's instructions. Duplicate determinations were performed on each sample. For each plate, 10 mL assay buffer was added to the vial containing the lyophilised adenylate kinase detection reagent which was equilibrated at room temperature for at least 15 min. The samples were loaded into a 96-well plate (Appleton Woods) in duplicates (20 µL of the supernatants or 10 µL of the lysates – 1:2 dilution) and then 100 µL of the assay buffer was added to each well. The luminescence was determined for each well with 1 s intervals, using the Mithras Microplate Reader (Berthold Technologies) and the Microwin programme (Berthold Technologies). Toxicity was measured by dividing the luminescence value for the supernatant by the sum of the luminescence values for the supernatant and the lysate.

2.6. Protein inactivation

2.6.1. Heat inactivation

Supernatant from *S. aureus* overnight TSB culture was incubated at 100^{o}C for 10 min in a thermocycler and then filter-sterilised using a 0.22 µm Millex-GP Syringe Filter Unit (Merck).

2.6.2. Proteinase K treatment

Proteinase K (PrK) was purchased from Sigma-Aldrich. Supernatants from *S. aureus* SH1000 (SA) were incubated in a thermocycler for 1 h at 37ºC with 33.3 µg/mL PrK non-heated (SA-PrK) or heated at 100ºC for 10 min (SA + PrK-heat). As controls, an aliquot of SA-PrK was heated at 100ºC for 10 min after incubation [(SA+PrK)-heat] to deactivate PrK and also SA was incubated at 37ºC for 1 h (SA37). HaCaT monolayers were stimulated with these samples (maximum PrK concentration in HaCaT monolayers: 0.999 µg/mL).

2.7. The SdrD and IsaB proteins

In an attempt to produce the SdrD protein, *Lactococcus lactis* expressing *sdrD* was obtained from the Research Group of Host-Microbe Interactions, Department of Medical Biology, Faculty of Health Sciences, UiT, The Arctic University of Norway (Askarian et al., 2017). Lactococcal wild-type strains, strains with an empty vector (pMG36e) and with a vector containing the *sdrD* insert were cultured in M17 medium (Sigma-Aldrich) supplemented with 0.5% (w/v) glucose and 0.5% (w/v) sucrose (Sigma-Aldrich), according to the recommendation of the research group that produced the mutant strains. For the strains that carried the plasmid, media was also supplemented with 10 µg/mL erythromycin (Sigma-Aldrich) to ensure plasmid maintenance. Both agar plates and broth cultures were incubated at 30ºC for 18 h (with vigorous aeration for broth cultures).

In order to investigate the effect of IsaB protein on skin cells, recombinant IsaB protein was obtained from MyBiosource (IsaB derived from *S. aureus* strain MRSA252).

2.8. Fractionation and characterisation of staphylococcal supernatant

2.8.1. Ion-exchange chromatography

S. aureus SH1000 was propagated in TSB overnight at 37ºC with vigorous aeration. The following day, 4 mL of the culture was added to 800 mL TSB, which was then incubated for 18 h at 37ºC with vigorous aeration. The cells were then harvested via centrifugation at 5000 x *g* for 20 min. The supernatant was collected and 1 M filter-sterilised 2-(N-morpholino)ethanesulfonic acid (MES) buffer (Sigma-Aldrich) was added in order to obtain a final concentration of 50 mM. The pH was adjusted to 5.5 and the supernatant was filter-sterilised. Then, 600 mL of this *S. aureus* SH1000 supernatant was loaded onto two ion-exchange columns, interconnected in series: a cation-exchange column (5 mL-HiTrap SP HP, GE Healthcare) and an anion-exchange column (5 mL-HiTrap Q HP, GE Healthcare). These columns were attached to an Äkta Purifier system (GE Healthcare). The columns were then uncoupled and eluted separately using a 0-100% gradient (5 column volumes) of lysis buffer B (20 mM MES and 1 M NaCl, pH 5.5) and buffer A (20 mM MES and 50 mM NaCl, pH 5.5) as a diluent. All solutions were filter-sterilised (0.22 µm Millipore Stericup filter units, Merck) and de-gassed for 30 min prior to fractionation. The fractions were collected, filter-sterilised and stored in 1.5 mL collection tubes at -80ºC. Solutions and fractions were filtered using 0.22 µm Millex-GP Syringe Filter Unit.

2.8.2. SDS-PAGE

Samples of fractions of SH1000 supernatant, containing 1 X RunBlue LDS sample buffer (Expedeon) and 20 mM dithiothreitol (DTT, Expedeon) were denatured by heating to 100°C for 5 min prior to separation by SDS-PAGE (Sambrook and Russell, 2001). The protein ladder that was used was blue prestained protein standard, broad range (11-190 kDa, New England Biolabs). The gel was run at 160 volts for 65 min and was then stained with InstantBlue (Expedeon).

2.8.3. Size-exclusion chromatography

The Äkta Purifier system was also used to perform size-exclusion chromatography. A single fraction produced from the ion-exchange chromatography was loaded onto a Superdex 75 (16/60) column (GE Healthcare) pre-equilibrated with buffer A (20 mM MES and 50 mM NaCl, pH 5.5), with a flow rate of 0.6 mL/min. Fractions were collected at 3 mL intervals.

2.8.4. Mass spectrometry

The protein concentration of fractions produced by ion-exchange chromatography was determined by the Bradford method (Bradford, 1976). Briefly, 1 mL of the Bradford dye (Bio-rad) and 20 µL of each fraction were mixed. The absorbance at 595 nm was measured using P300 nanophotometer (Implen). Bradford dye was used as a blank. Then, aliquots of these fractions were sent for mass spectrometry.

Liquid chromatography-mass spectrometry (LC-MS) was performed by Leeds mass spectrometry core facility and the proteins that were detected in each fraction were identified. The protocols are described in Appendices and supplemental material, 8.4.

2.9. Graphs and statistical analysis

Unless stated otherwise, all graphs were created using GraphPad Prism 7 (GraphPad Prism version 7.05 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). Values plotted are means unless otherwise indicated and the error bars represent the standard error of mean (SEM).

Statistical analysis was also performed with GraphPad Prism 7 using one-way analysis of variance (ANOVA), with the significant difference between groups determined by Tukey post-hoc test. The “n” value represents the number of independent experiments. Data are expressed as mean ± SEM. A probability

value of * $p<0.05$ was considered statistically significant; * $p<0.05$, ** $p<0.01$, *** $p<0.001$. In order to perform statistics, values that were not measurable by the assays (below the detection limit) were set to the lowest detection limit value of the assay.

3. Production of AMPs in keratinocytes in response to *S. aureus* and *S. epidermidis*

3.1. Introduction

The predominant pathogen in AD is *S. aureus*, found in more than 90% of AD patients (Baldry et al., 2018; Chiu et al., 2009; David and Cambridge, 1986). In contrast, microbial diversity and especially the presence of *S. epidermidis*, which is ordinarily the most abundant skin commensal, are reduced in AD (Nakatsuji et al., 2017; Salava and Lauerma, 2014; Kong et al., 2012; Higaki et al., 1999; Otto, 2012; Geoghegan et al., 2018). Although a causative relationship between microbial dysbiosis and AD has yet to be proven (Paller et al., 2019; Lee et al., 2018), an association between *S. aureus* colonisation and immune system dysregulation in AD has been reported (Di Domenico et al., 2018; Tauber et al., 2016; Salava and Lauerma, 2014; Harder et al., 2010; Rieg et al., 2005; Harkins et al., 2019).

Keratinocytes are the main skin cells that produce cytokines and AMPs, mainly in response to stimuli, such as bacteria (Zhang and Gallo, 2016; Schauber and Gallo, 2008; Schröder, 2010; Suter et al., 2009; Pfalzgraff et al., 2018; Yousef et al., 2019). Cytokines are important signalling molecules for the skin immune response (Nedoszytko et al., 2014; Coondoo, 2011). The IL-8 cytokine is a neutrophil attractant in the epidermis and its levels increase in AD (Vlahopoulos et al., 1999; Barker et al., 1991; Jiang et al., 2012; Amarbayasgalan et al., 2013). In non-stimulated keratinocytes, IL-8 is usually non-detectable. It is not stored in cells but produced and released upon stimulation e.g. with staphylococci (Takematsu and Tagami, 1993; Mohamadzadeh et al., 1994; Sasaki et al., 2003). Keratinocyte production of HBD-2, HBD-3 and LL-37 in AD has been widely studied, due to the potency of these AMPs against *S. aureus*, the most common pathogen in AD and the fact that their levels are downregulated in AD. It has been shown that HBD-3 is more potent against *S. aureus* compared to HBD-2 and LL-37 (Baldry et al., 2018; Ong et al., 2002; Midorikawa et al., 2003; Menzies and Kenoyer, 2005; Joly et al., 2004; Nomura et al., 2003b; Howell et al., 2005).

Although the exact mechanisms of AMP induction by bacteria have not been elucidated, it appears that the induction pathways differ, depending on the AMP and even the bacterial species (Steubesand et al., 2009; Chung and Dale, 2004; Chung and Dale, 2008). *S. epidermidis* has been reported to induce HBD-2 and HBD-3 expression through TLR2, EGFR and NF-κB signaling pathways, whereas *S. aureus* triggers mainly phosphatidylinositol 3-kinase and MAPK signaling pathways instead of NF-κB and induces HBD-2 via IL-1 and HBD-3 via EGFR activation (Sorensen et al., 2005; Lai et al., 2010; Wanke et al., 2011; Ommori et al., 2013).

The differentiation stage of keratinocytes may affect their reaction to stimuli. Most studies found that HBD-2 is more readily induced in differentiated keratinocytes compared to non-differentiated keratinocytes, in contrast to HBD-3 (Krisanaprakornkit et al., 2003; Liu et al., 2002; Menzies and Kenoyer, 2005; Dinulos et al., 2003). However, other studies have reported upregulation of both HBD-2 and HBD-3 in differentiated keratinocytes (Harder et al., 2004), increased HBD-3 but not HBD-2 expression (Abiko et al., 2003), or no effect of the differentiation stage at all (Frye et al., 2001). Other factors may account for the different findings in projects, such as different experimental conditions (determination of mRNA expression or AMP protein product) or stimulants (cytokines, staphylococcal cells or supernatant) (Liu et al., 2002). Although differentiated keratinocytes in outermost epidermal layers are the first line defence, *S. aureus* may inhibit terminal differentiation of keratinocytes (Son et al., 2014) and skin barrier disruption in AD can increase skin penetration of *S. aureus* and other pathogens towards deeper epidermal layers with nucleated keratinocytes (Agrawal and Woodfolk, 2014; Eckert et al., 2002). Therefore, it is important to investigate various keratinocyte differentiation stages and the interaction of pathogens with host cells from deeper layers as well (Popov et al., 2014).

Keratinocyte monolayers and 3D skin equivalents are both widely used for the investigation of skin pathophysiology but have differences in phenotype, cell signalling and metabolic functions (Zanoni et al., 2014; Mazzoleni et al., 2009; Smalley et al., 2006). A 3D skin equivalent model has a differentiated stratified epidermis and dermal fibroblasts and is therefore more similar to human skin compared to a 2D keratinocyte monolayer (Rademacher et al., 2018). In

contrast, 2D keratinocyte monolayers grow in a more controlled environment and are more reproducible and easier to manipulate. Growth media can also be distributed evenly, allowing a more symmetric cell distribution (Edmondson et al., 2014). A reliable model to study keratinocyte response is the HaCaT immortalised human keratinocyte cell line, which exhibits lower variability compared to primary keratinocytes isolated from human epidermis (Colombo et al., 2017).

A question raised in this project was whether increased *S. aureus* colonisation and microbial dysbiosis, which result in inflammatory responses, can trigger AD. In order to investigate differences in the effect of *S. aureus* and *S. epidermidis* on AMP keratinocyte production and to better understand the role of *S. aureus* in AD pathogenesis, 3D skin equivalent models and keratinocyte monolayers were stimulated with staphylococcal cells or supernatants. Most previous studies investigated only *S. aureus* (Midorikawa et al., 2003; Menzies and Kenoyer, 2005), or just one *S. aureus* and one *S. epidermidis* strain (Lai et al., 2010; Holland et al., 2009). However, as bacterial strains even from the same species exhibit genotypic and phenotypic differences and may trigger different immune responses (Gill et al., 2005; Fux et al., 2005; Sela et al., 2018), the experiments in this project used a variety of clinical and laboratory *S. aureus* and *S. epidermidis* strains, including both methicillin susceptible and resistant strains. Moreover, most previous studies focused on AMP gene expression (Haisma et al., 2013; Wanke et al., 2011). Nevertheless, as increased mRNA expression does not necessarily depict increased protein production, due to several regulation stages between gene transcription and translation of protein (Vogel and Marcotte, 2012; Greenbaum et al., 2003), this project evaluated the AMP protein production by ELISA.

Although AMP levels on lesional AD skin are increased compared to non-lesional and health skin, they are reduced compared to those seen in other inflammatory diseases, such as psoriasis (Méndez-Samperio, 2013). The Th_2 milieu in AD, as opposed to the Th_1 axis in psoriasis, may downregulate AMP expression (Clausen et al., 2018; Clausen et al., 2016; Hijnen et al., 2013; Nomura et al., 2003a; Nomura et al., 2003b). *S. aureus* can promote Th_2 responses (Mandron et al., 2006) and IL-4 can downregulate gene expression of HBD-2 and HBD-3 in keratinocytes, induced by other cytokines (Albanesi

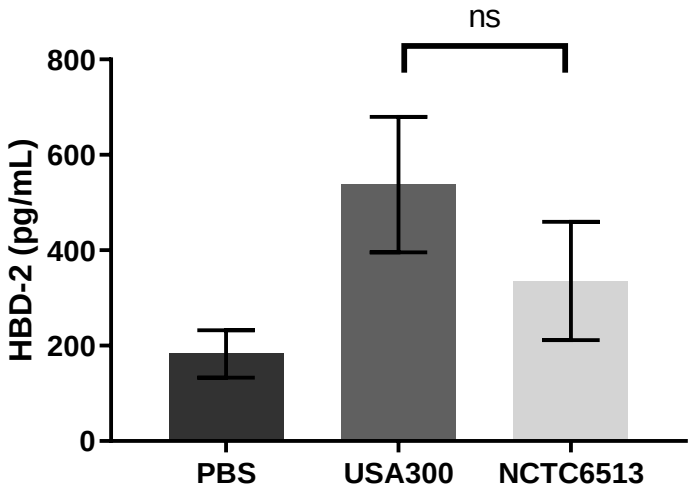
et al., 2007; Howell et al., 2006a). Also, addition of IL-4 to keratinocyte monolayers stimulated with human serum was shown to reduce HBD-2 protein production (Alase et al., 2012).

3.2. Results

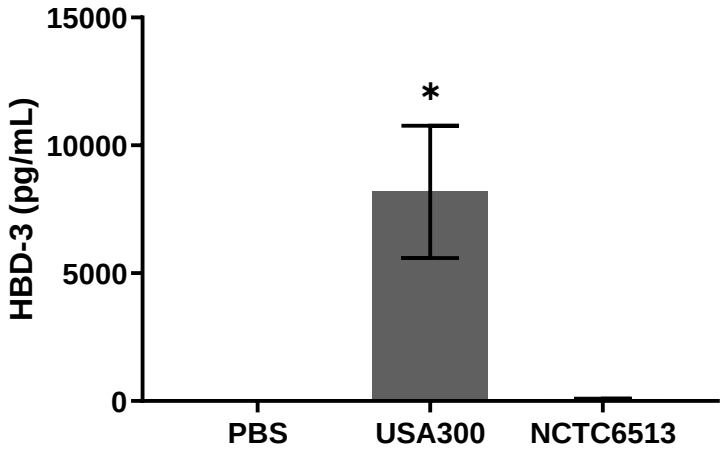
3.2.1. *S. aureus* cells induce higher HBD-3 production in 3D skin equivalent models compared to *S. epidermidis*

In order to investigate the effect of *S. aureus* and *S. epidermidis* on skin, 3D skin equivalent models were exposed to *S. aureus* USA300 and *S. epidermidis* NCTC6513 live cells. Production of HBD-2 and HBD-3 in the culture medium of the skin equivalents was then measured by ELISA[2]. The HBD-2 skin production was induced to higher levels in the presence of *S. aureus* USA300 compared to *S. epidermidis* NCTC6513 (Figure 3-1a). However, the difference was not statistically significant. By contrast, HBD-3 production was detected only in the presence of *S. aureus* USA300 (Figure 3-1b). In addition, the overall HBD-3 concentration produced after stimulation with *S. aureus* USA300 was much higher than the HBD-2 concentration (Figure 3-1).

[2]The skin equivalent experiments were performed by Dr Adewonuola Alase, post-doctoral researcher in the Wellcome Trust Brenner Building (St. James's University Hospital, Leeds, UK).



(a)



(b)

Figure 3-1: Production of HBD-2 and HBD-3 by skin equivalent models after stimulation with *S. aureus* and *S. epidermidis* cells

Skin equivalents were stimulated *S. aureus* USA300 or *S. epidermidis* NCTC6513 cells for 16 h and production of HBD-2 (a) and HBD-3 (b) was measured by ELISA. One-way ANOVA was used to determine statistical significance, n=3. Values are mean ± SEM, * $p<0.05$, ns: non-significant. Where the values were below the detection limit (PBS and NCTC6513), they were set to 62.5 pg/mL (lowest detection limit of the HBD-3 ELISA assay) to allow statistical analysis.

3.2.2. Is failure to induce HBD-3 production a general trait of *S. epidermidis* strains?

The effect of *S. aureus* and *S. epidermidis* on keratinocyte AMP production detected in skin equivalents was further investigated, using a larger collection of staphylococcal strains. As the genome of a single strain cannot be representative for the species (Fux et al., 2005), various strains were chosen, laboratory and clinical isolates. Therefore, in addition to previously characterised laboratory and clinical strains, two other *S. epidermidis* strains were used in this study, which were isolated from healthy subjects and named SE3 and SE9. Gram staining and coagulase tests (Materials and methods, 2.2.2.1) confirmed that these strains were Gram-positive and coagulase-negative, in accordance with *S. epidermidis* characteristics (Figure 3-2). To further characterise these strains, PCR amplification and DNA sequencing was performed on genomic DNA isolated from both strains. Primers specific for the *rpoB* gene were used to confirm these isolates as *S. epidermidis* (Materials and methods, 2.2.2.3) (Drancourt and Raoult, 2002).

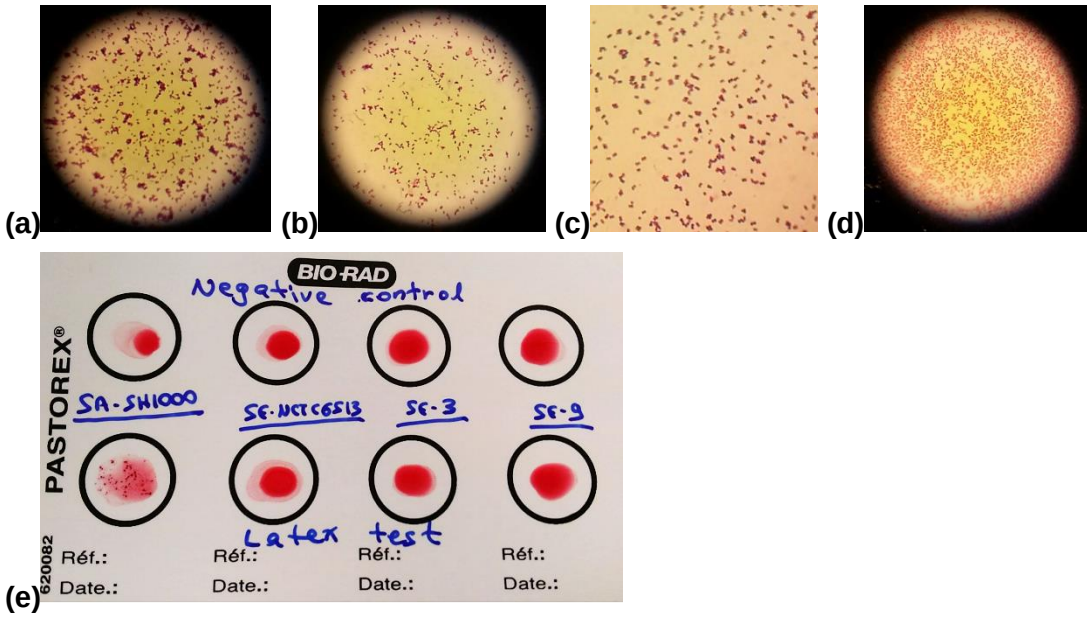


Figure 3-2: Gram staining and coagulase test of *S. epidermidis* SE3 and SE9 skin isolates

The Gram-positive *S. epidermidis* SE3 (a), *S. epidermidis* SE9 (b) and *S. aureus* SH1000 (c) were stained purple. The Gram-negative *Escherichia coli* (d) was stained red. For the coagulase test (e), the coagulase-negative *S. epidermidis* NCTC6513 and the coagulase-positive *S. aureus* SH1000 were used as controls. [(e) Full strain names from left to right: *S. aureus* SH1000, *S. epidermidis* NCTC6513, *S. epidermidis* SE3, *S. epidermidis* SE9]

3.2.3. Induction of AMPs by *S. aureus* and *S. epidermidis* cells in keratinocyte monolayers

In order to investigate HBD-3 production in skin equivalent models in more detail, further experiments were performed with keratinocyte monolayers. Compared to skin equivalent models, keratinocyte monolayers are easier to grow in a controlled environment that can be adapted to the needs of the experiments. Also, growth media and stimulants can be distributed evenly, allowing more consistent results (Edmondson et al., 2014). Monolayers of HaCaT cells were cultured in 24-well plates and stimulated independently with whole cultures of *S. aureus* (USA300, SH1000, UAMS-1, WC003 and WC006) and *S. epidermidis* strains (NCTC11047, NCTC6513, SE3, SE9 and 7765746). In accordance with the results using skin equivalents (Figure 3-1b), all *S. aureus* strains and none of the *S. epidermidis* strains induced HBD-3 production in HaCaT cells (Figure 3-3).

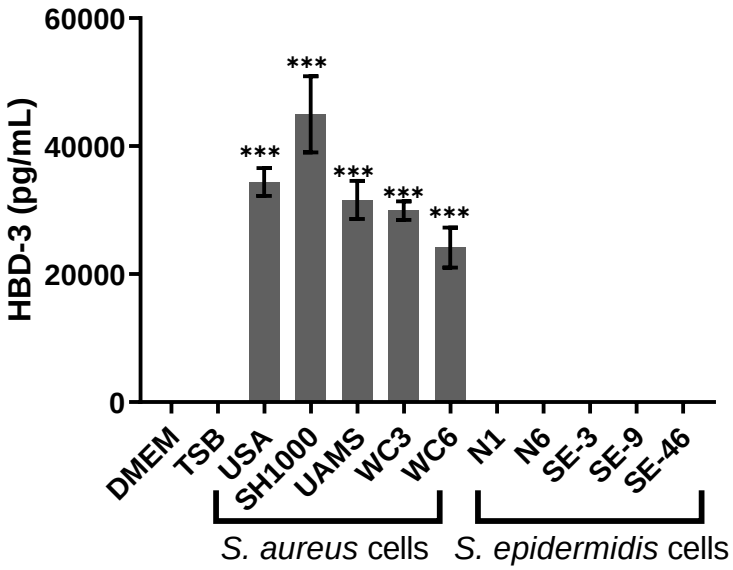


Figure 3-3: Production of HBD-3 by HaCaT cells after stimulation with *S. aureus* and *S. epidermidis* cells

HaCaT monolayers were stimulated with staphylococcal cultures for 20 h and HBD-3 production was measured by ELISA. One-way ANOVA was used to determine statistical significance, n=4. Values are mean ± SEM, *** $p<0.001$. Where the values were below the detection limit (DMEM, TSB and the *S. epidermidis* strains), they were set to 62.5 pg/mL (lowest detection limit of the HBD-3 ELISA assay) to allow statistical analysis. The full names of the x-axis labels from left to right are: DMEM, TSB, *S. aureus* USA300 (USA), *S. aureus* SH1000 (SH1000), *S. aureus* UAMS-1 (UAMS), *S. aureus* WC003 (WC3), *S. aureus* WC006 (WC6), *S. epidermidis* NCTC11047 (N1), *S. epidermidis* NCTC6513 (N6), *S. epidermidis* SE3 (SE-3), *S. epidermidis* SE9 (SE-9) and *S. epidermidis* 7765746 (SE-46).

The previous experiment showed that all *S. aureus* but none of the *S. epidermidis* strains induced HBD-3. The next step was to investigate whether *S. epidermidis* did not actively stimulate HBD-3 production, or whether it was too toxic and thereby rendered the HaCaT cells unable to respond to stimulation with *S. epidermidis* cells and produce immune molecules. For this reason, the ability of HaCaT cells to respond to stimuli by releasing immune mediators after staphylococcal stimulation was determined, by measuring the IL-8 HaCaT production after stimulation with *S. epidermidis* and *S. aureus* cells. As Figure 3-4 shows, there was no significant difference in the IL-8 production by HaCaT cells, regardless of whether they were stimulated with *S. aureus* or *S. epidermidis* cells.

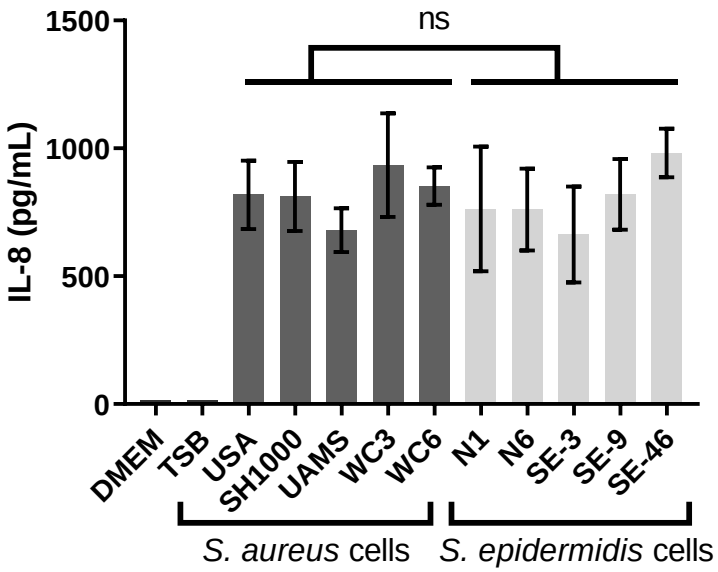


Figure 3-4: Production of IL-8 by HaCaT cells after stimulation with *S. aureus* and *S. epidermidis* cells

HaCaT monolayers were stimulated with staphylococcal cultures for 20 h and IL-8 production was measured by ELISA. One-way ANOVA was used to determine statistical significance, n=3. Values are mean ± SEM, ns: non-significant. Where the values were below the detection limit (DMEM and TSB), they were set to 15.6 pg/mL (lowest detection limit of the IL-8 ELISA assay) to allow statistical analysis. The full names of the x-axis labels are as in Figure 3-3.

In order to investigate whether the different inducing effect of *S. aureus* and *S. epidermidis* on HBD-3 keratinocyte production applies to other AMPs, this project also examined induction of LL-37, as it is a major skin AMP, along with HBDs (Kim et al., 2005). Only *S. aureus* SH1000 and to a lesser extent *S. epidermidis* SE3 induced higher LL-37 production compared to the levels present in the controls (Figure 3-5). Therefore, in contrast to the situation with HBD-3 production, the inducing effect is neither a general feature of *S. aureus* strains, nor uniformly absent from *S. epidermidis*.

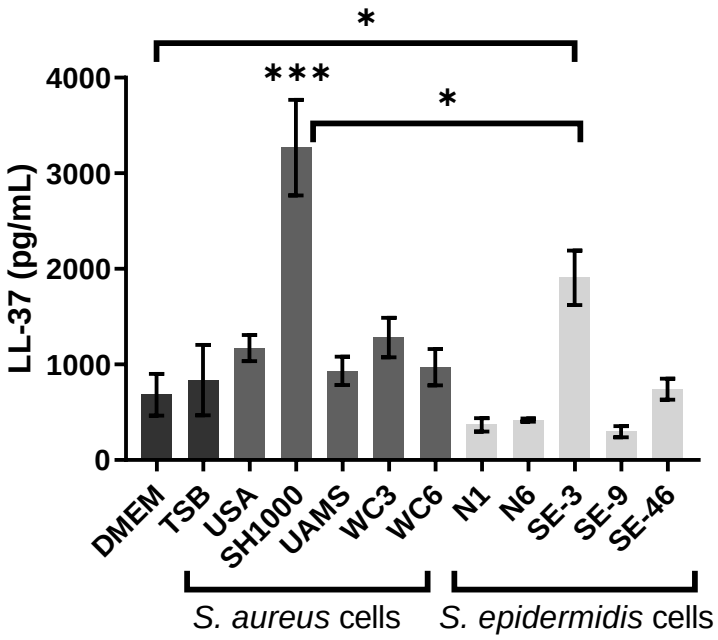


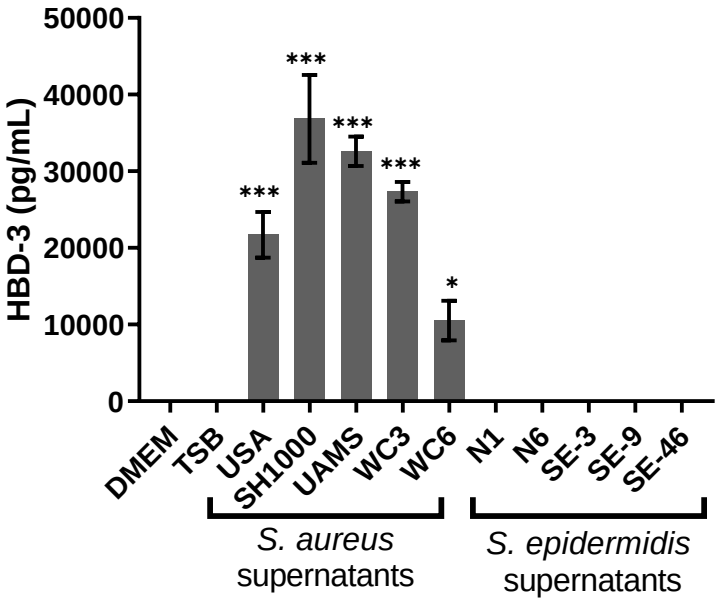
Figure 3-5: Production of LL-37 by HaCaT cells after stimulation with *S. aureus* and *S. epidermidis* cells

HaCaT monolayers were stimulated with staphylococcal cultures for 20 h and LL-37 production was measured by ELISA. One-way ANOVA was used to determine statistical significance, n=3. Values are mean ± SEM, * $p<0.05$, *** $p<0.001$. The full names of the x-axis labels are as in Figure 3-3.

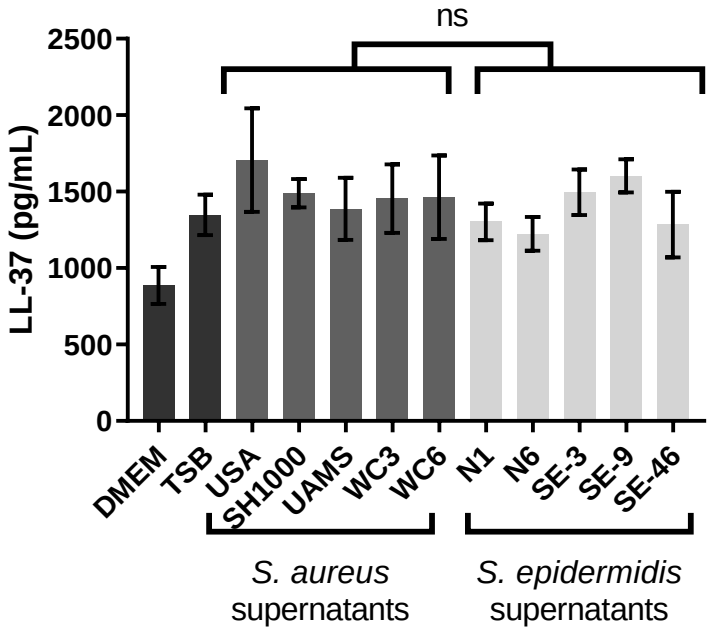
3.2.4. *S. aureus* supernatant induces higher HBD-3 production in keratinocyte monolayers compared to *S. epidermidis*

After investigating the effect of *S. aureus* and *S. epidermidis* live cells on HaCaT monolayers, it was decided to test whether the *S. aureus* factor(s) that induced AMP production were found in the growth media, in order to further plan experimental procedures to investigate these factor(s). Following growth of *S. aureus* and *S. epidermidis* cultures, bacteria were harvested and their supernatant was retained and filter-sterilised. Then, HaCaT monolayers were stimulated with filter-sterilised supernatants and HBD-2, HBD-3 and LL-37 production was measured as before (Figure 3-6). Production of HBD-3 was induced at high levels after stimulation with *S. aureus* supernatants (Figure 3-6a), similar to the results from the experiments with staphylococcal cultures (Figure 3-3) and the experiments in skin equivalents (Figure 3-1b), implying

that the *S. aureus*-inducing factor(s) driving HBD-3 production are present in the growth media. The HBD-2 levels were below the limit of detection (*data not shown*), unlike the 3D skin equivalents, where both stimulated and non-stimulated skin equivalents produced HBD-2 (Figure 3-1a). LL-37 was produced at similar levels for non-stimulated HaCaT monolayers and HaCaTs stimulated with staphylococcal supernatants (Figure 3-6b). This is in line with the results from stimulation of HaCaTs with staphylococcal cultures for all strains except *S. aureus* SH1000 and *S. epidermidis* SE3 (Figure 3-5), suggesting that the inducing factor(s) produced by SH1000 and SE3 are cell-associated, as they seem to have been removed upon filter-sterilisation of the cultures.



(a)



(b)

Figure 3-6: Production of HBD-3 and LL-37 by HaCaT cells after stimulation with *S. aureus* and *S. epidermidis* supernatants

HaCaT monolayers were stimulated with staphylococcal supernatants for 24 h and production of HBD-3 (a) and LL-37 (b) was measured by ELISA. One-way ANOVA was used to determine statistical significance, n=4 (a), n=3 (b). Values are mean ± SEM, * $p<0.05$, *** $p<0.001$, ns: non-significant. Where the values were below the detection limit [DMEM, TSB and the *S. epidermidis* strains of graph (a)], they were set to 62.5 pg/mL (lowest detection limit of the HBD-3 ELISA assay) to allow statistical analysis. The full names of the x-axis labels are as in Figure 3-3.

As the HBD-3 production after stimulation of HaCaTs with *S. epidermidis* supernatants was below the detection limit, a toxicity assay was performed, in order to rule out the possibility that *S. epidermidis* supernatants were more toxic than *S. aureus*, hence causing a reduction in HBD-3 production. The results showed that there was no statistically significant difference in the toxicity levels of *S. epidermidis* compared to *S. aureus* (Figure 3-7).

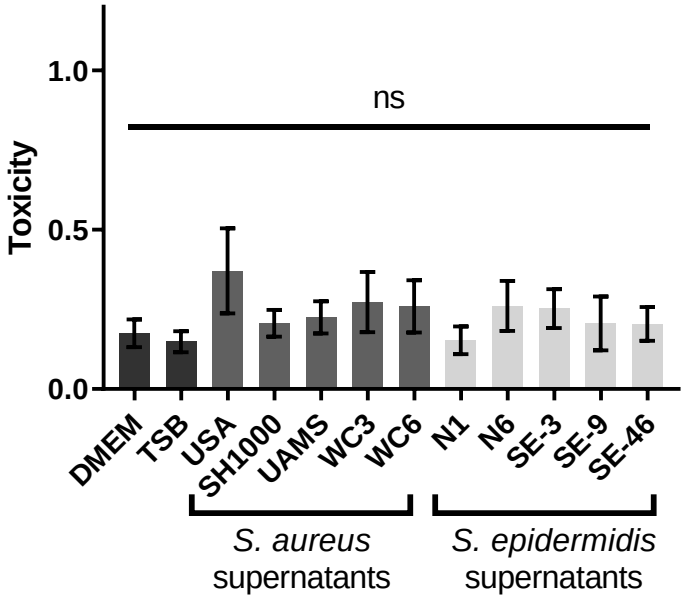


Figure 3-7: Toxicity of *S. aureus* and *S. epidermidis* supernatants towards HaCaT cell monolayers

HaCaT monolayers were stimulated with staphylococcal supernatants for 24 h. Luminescence of dead and alive HaCaT cells was determined by Toxilight. Toxicity represents the killing of HaCaT cells (ratio of dead HaCaT cells to total HaCaT cells) after stimulation. One-way ANOVA was used to determine statistical significance, n=4. Values are mean ± SEM, ns: non-significant. The full names of the x-axis labels are as in Figure 3-3.

Although the HaCaT cell line is a reliable model to study keratinocyte response (Colombo et al., 2017), the ability of *S. aureus* to induce HBD-3 production was confirmed in primary keratinocyte monolayers isolated from the epidermis of adult skin (Promocell). Keratinocytes were stimulated with filter-sterilised staphylococcal supernatants and, similar to the experiments

with HaCaT cells (Figure 3-6a), it was found that production of HBD-3 was induced only after stimulation with *S. aureus*. Although all *S. aureus* strains induced HBD-3, induction mediated by the WC006 strain was not statistically significant compared to the *S. epidermidis* strains (Figure 3-8).

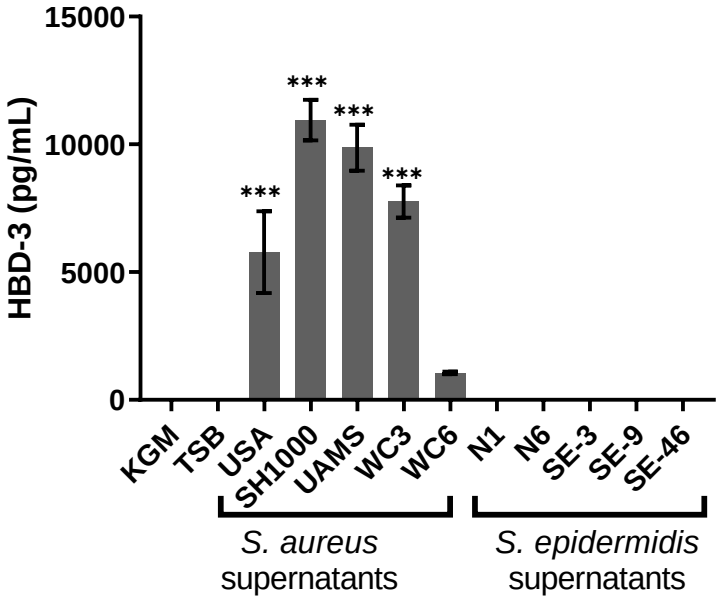


Figure 3-8: Production of HBD-3 by primary keratinocytes after stimulation with *S. aureus* and *S. epidermidis* supernatants

Primary keratinocyte monolayers were stimulated with staphylococcal supernatants for 24 h and HBD-3 production was measured by ELISA. One-way ANOVA was used to determine statistical significance, n=4. Values are mean ± SEM, *** $p<0.001$. Where the values were below the detection limit (KGM, TSB and the *S. epidermidis* strains), they were set to 62.5 pg/mL (lowest detection limit of the HBD-3 ELISA assay) to allow statistical analysis. The full names of the x-axis labels from left to right are: KGM, TSB, *S. aureus* USA300 (USA), *S. aureus* SH1000 (SH1000), *S. aureus* UAMS-1 (UAMS), *S. aureus* WC003 (WC3), *S. aureus* WC006 (WC6), *S. epidermidis* NCTC11047 (N1), *S. epidermidis* NCTC6513 (N6), *S. epidermidis* SE3 (SE-3), *S. epidermidis* SE9 (SE-9) and *S. epidermidis* 7765746 (SE-46).

In addition, there was no statistically significant difference in the toxicity levels of *S. epidermidis* compared to *S. aureus* and the controls (Figure 3-9), in accordance with the previous results with HaCaT cells (Figure 3-7).

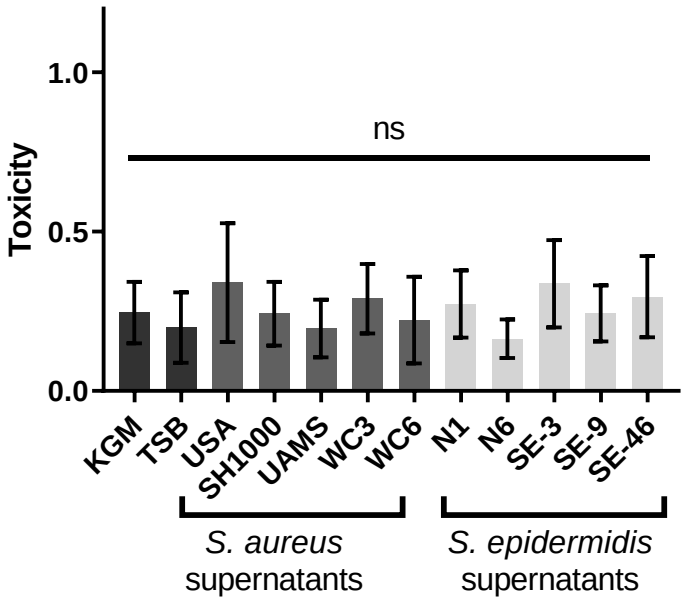


Figure 3-9: Toxicity of *S. aureus* and *S. epidermidis* supernatants towards primary keratinocytes

Primary keratinocyte monolayers were stimulated with staphylococcal supernatants for 24 h. Luminescence of dead and alive keratinocytes was determined by Toxilight. Toxicity represents the killing of keratinocytes (ratio of dead keratinocytes to total keratinocytes) after stimulation. One-way ANOVA was used to determine statistical significance, n=3. Values are mean ± SEM, ns: non-significant. The full names of the x-axis labels are as in Figure 3-8.

3.2.5. *S. epidermidis* does not actively suppress HBD-3 keratinocyte production

None of the *S. epidermidis* strains induced detectable HBD-3 production in keratinocytes or showed higher levels of toxicity compared to *S. aureus*. Whilst it was considered likely that this reflected the absence in *S. epidermidis* of the inducing factor(s) present in *S. aureus*, an alternative possibility was that *S. epidermidis* produces – in addition to the inducing factor(s) – one or more factors that inhibit HBD-3 production. In order to test this possibility, HaCaT monolayers were stimulated with a combination of *S. aureus* and *S. epidermidis* supernatants at different ratios. If the assumption about production of inhibiting factors by *S. epidermidis* was true, then combination of *S. epidermidis* and *S. aureus* supernatants should decrease HBD-3

keratinocyte production compared to stimulation by *S. aureus* supernatant only. Despite addition of different concentrations of *S. epidermidis* supernatant, there was no reduction in HBD-3 production by HaCaTs when stimulated with *S. aureus* (Figure 3-10). Therefore, it is unlikely that there is a *S. epidermidis* factor that is actively inhibiting HBD-3 production in keratinocytes.

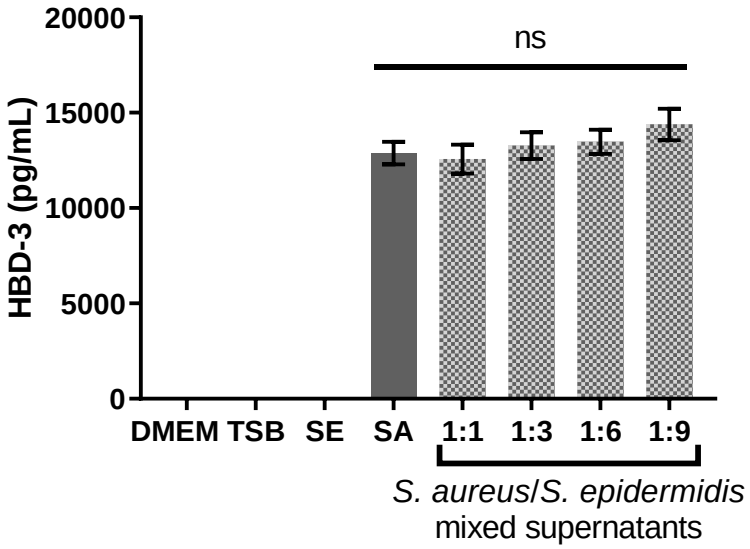


Figure 3-10: Production of HBD-3 by HaCaT cells after stimulation with combinations of *S. aureus* and *S. epidermidis* supernatants

HaCaT monolayers were stimulated with mixtures of *S. aureus* SH1000 and *S. epidermidis* NCTC6513 supernatants for 24 h. All wells contained the same ratio of TSB/DMEM and the same amount of *S. aureus* supernatant, whereas the concentration of *S. epidermidis* supernatant was increasing. A well with 9 parts of *S. epidermidis* was used as negative control (SE) and a well with just 1 part of *S. aureus* was the positive control (SA). The HBD-3 production was measured by ELISA. One-way ANOVA was used to determine statistical significance, n=4. Values are mean ± SEM, ns: non-significant. Where the values were below the detection limit (DMEM, TSB and SE), they were set to 62.5 pg/mL (lowest detection limit of the HBD-3 ELISA assay) to allow statistical analysis.

3.2.6. Some *S. aureus* strains do not induce HBD-3 in keratinocytes

The 5 *S. aureus* strains tested in the previous experiments induced HBD-3 production in keratinocytes (Figure 3-6a). In order to investigate whether this trait is universal to all *S. aureus* strains, HaCaT monolayers were stimulated with supernatants from several clinical isolates (Figure 3-11 and Table 3-1). The majority of the strains induced HBD-3, in various levels. However, the supernatants of 8 *S. aureus* strains lacked the ability to induce HBD-3 production in keratinocytes (Figure 3-11 and Table 3-1).

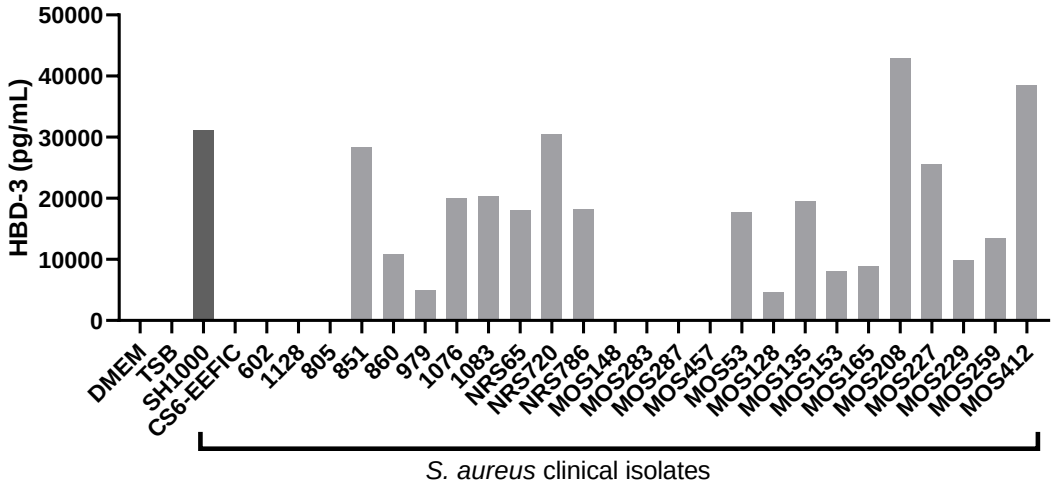


Figure 3-11: *S. aureus* strains have varying ability to induce HBD-3 in keratinocytes

HaCaT monolayers were stimulated with supernatants from *S. aureus* clinical isolates for 24 h and HBD-3 production was measured by ELISA (n=1).

Table 3-1: Supernatants from *S. aureus* clinical isolates have varying ability to induce HBD-3 in keratinocytes

The supernatants isolated from all strains in Table 3-1a induced HBD-3 production in keratinocytes. The supernatants from the strains in Table 3-1b did not exhibit such an effect.

(a) (+) HBD-3 induction			
USA300	NRS786	MOS53	MOS208
UAMS-1	851	MOS128	MOS227
WC003	860	MOS135	MOS229
WC006	979	MOS153	MOS259
NRS65	1076	MOS165	MOS412
NRS720	1083		

b) (-) HBD-3 induction	
CS6-EEFIC	MOS287
602	MOS457
1128	MOS148
805	MOS283

One potential explanation for failure of supernatants of these *S. aureus* strains to induce HBD-3 was that these strains produced a toxic component that rendered the keratinocytes unable to respond and produce immune mediators. Therefore, the toxicity of the supernatants of these *S. aureus* strains towards HaCaT cells was tested (Figure 3-12). The supernatants of *S. aureus* 1128 and 805 showed increased toxicity towards HaCaT cells (Figure 3-12), suggesting that this might be a contributory factor in the failure of these strains to induce HBD-3 production in HaCaT cells.

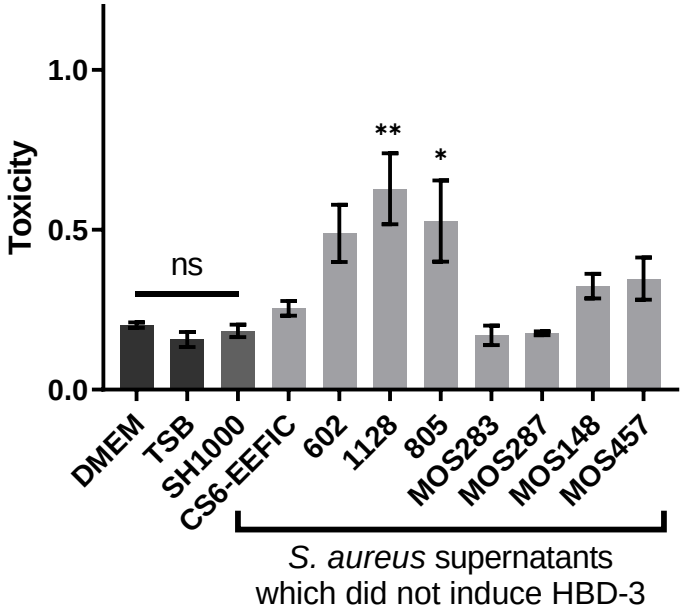


Figure 3-12: Toxicity of the *S. aureus* supernatants that did not induce HBD-3 in HaCaT cells

HaCaT monolayers were stimulated with staphylococcal supernatants for 24 h. Luminescence of dead and alive HaCaT cells was determined by Toxilight. Toxicity represents the killing of HaCaT cells (ratio of dead HaCaT cells to total HaCaT cells) after stimulation. One-way ANOVA was used to determine statistical significance, n=3. Values are mean ± SEM, * $p<0.05$, ** $p<0.01$, ns: non-significant.

The toxicity of these supernatants was confirmed visually by microscopic analysis of cultured cells (*data not shown*); incubation of HaCaT cells with *S. aureus* 1128 and 805 supernatants led to an increased number of detached HaCaT cells compared to when HaCaT cultures were incubated with other strains.

The lack of HBD-3 induction associated with these 8 strains (Table 3-1b), could imply that they either lack the inducing factor(s) or they do not release them into the growth media in sufficient quantities to trigger induction. In order to further investigate this, HaCaT monolayers were stimulated with staphylococcal cultures from these strains (Figure 3-13). As expected, *S. aureus* SH1000 induced HBD-3 production in HaCaT cells. Also, cells of *S. aureus* MOS283 and MOS287 induced significant HBD-3 production, suggesting that the HBD-3-inducing factor(s) they produce may predominantly

be associated with the cell envelope rather than released into the growth media. Moreover, CS6-EEFIC, MOS148 and MOS457 strains also showed some HBD-3 induction. However, HBD-3 production was still not induced by cells of the other 3 strains (602, 1128 and 805) (Figure 3-13).

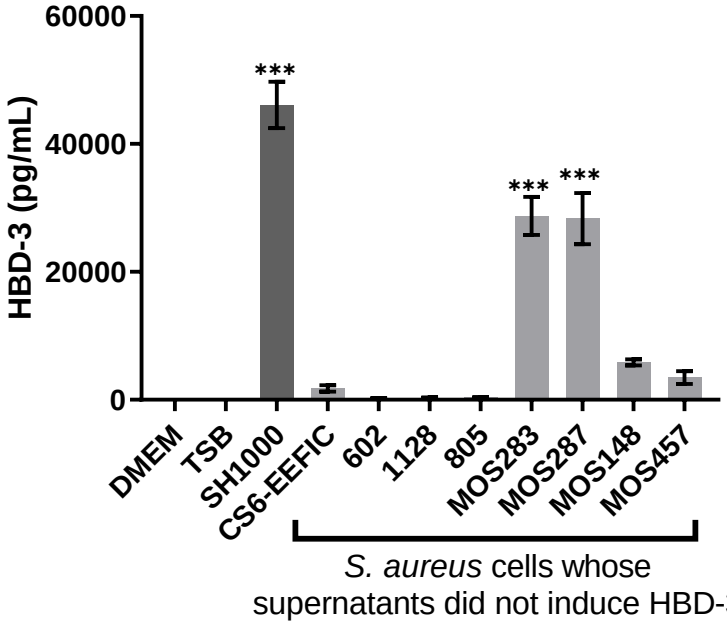


Figure 3-13: Production of HBD-3 by HaCaT cells after stimulation with the *S. aureus* cells whose supernatants failed to induce HBD-3

HaCaT monolayers were stimulated with staphylococcal cultures for 20 h and HBD-3 production was measured by ELISA. One-way ANOVA was used to determine statistical significance, n=4; Values are mean ± SEM, *** $p<0.001$. Where the values were below the detection limit (DMEM and TSB), they were set to 62.5 pg/mL (lowest detection limit of the HBD-3 ELISA assay) to allow statistical analysis.

In order to determine if these strains are able to induce production of other immune mediators in HaCaT cells, IL-8 induction was determined, essentially as described in section 3.2.3 (Figure 3-4). All strains induced IL-8 production in HaCaT cells, implying that the lack of induction in these strains is specific to HBD-3 (Figure 3-14).

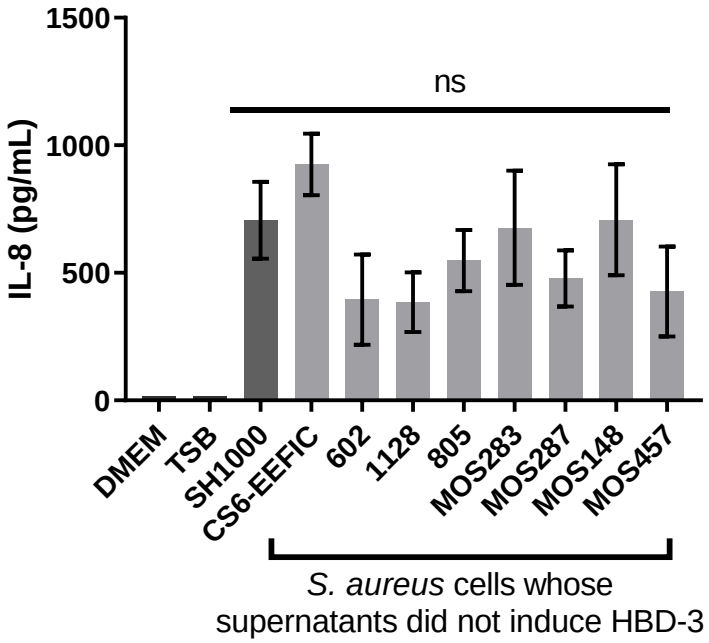


Figure 3-14: Production of IL-8 by HaCaT cells after stimulation with the *S. aureus* cells whose supernatants failed to induce HBD-3

HaCaT monolayers were stimulated with staphylococcal cultures for 20 h and IL-8 production was measured by ELISA. One-way ANOVA was used to determine statistical significance, n=3. Values are mean ± SEM, ns: non-significant. Where the values were below the detection limit (DMEM and TSB), they were set to 15.6 pg/mL (lowest detection limit of the IL-8 ELISA assay) to allow statistical analysis.

3.2.7. The Th_2 cytokine IL-4 may reduce *S. aureus*-induced HBD-3 production in keratinocytes

The previous experiments showed that *S. aureus* can induce HBD-3 production in keratinocytes (Figure 3-6a). HBD-3 is considered to be more potent against *S. aureus* compared to other AMPs (HBD-2, LL-37) (Joly et al., 2004; Midorikawa et al., 2003; Kisich et al., 2008). AMP expression in AD is lower compared to other inflammatory diseases such as psoriasis (Clausen et al., 2016) and atopic skin shows increased susceptibility to *S. aureus* (Hata and Gallo, 2008; Ong et al., 2002; Nomura et al., 2003b; Lin et al., 2007). Also, AD is characterised by a shift towards the Th_2 axis and IL-4 is a key Th_2 cytokine for AD (Brandt and Sivaprasad, 2011; Shiohara, 2011). In order to investigate the direct effect of IL-4 on the production of HBD-3 by

keratinocytes stimulated with *S. aureus*, HaCaT monolayers were treated with IL-4 (Peprotech) for 1 h, before stimulation with filter-sterilised *S. aureus* SH1000 supernatant (Figure 3-15). Production of HBD-3 was reduced to an extent when IL-4 concentration increased (Figure 3-15), which suggests that an atopic environment could have a direct effect on the ability of *S. aureus* to induce HBD-3 production in keratinocytes.

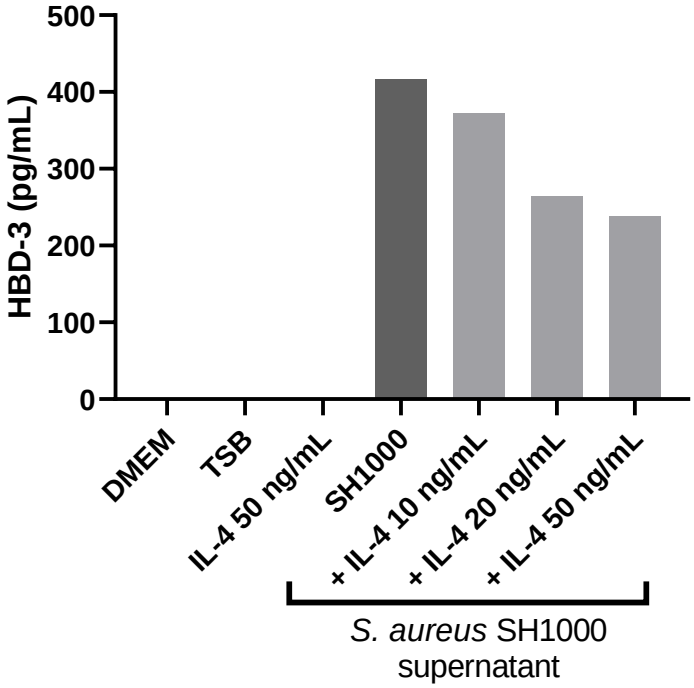


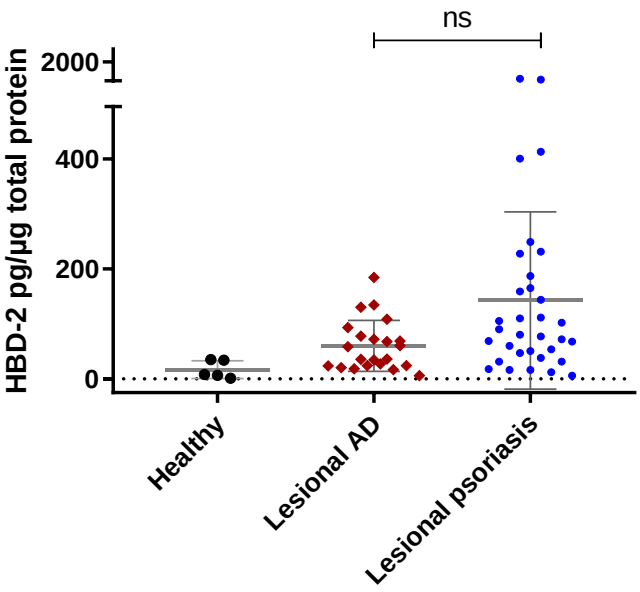
Figure 3-15: Production of HBD-3 by HaCaT cells pretreated with IL-4 and stimulated with *S. aureus* supernatant

HaCaT monolayers were pre-treated with IL-4 for 1 h and were then stimulated with SH1000 supernatant for 24 h. DMEM, TSB and IL-4 were used as negative controls. Production of HBD-3 was measured by ELISA (n=1).

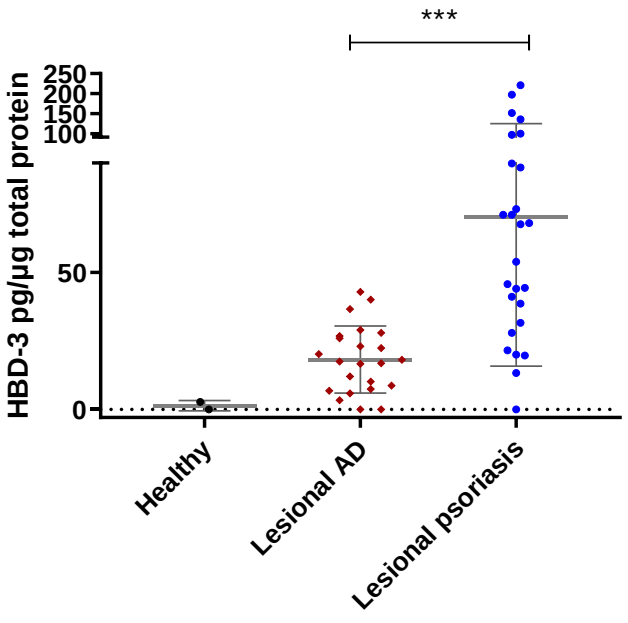
In order to further investigate changes of HBD-3 in an atopic environment, Dr Anna Berekmeri[3] measured both HBD-2 and HBD-3 in the stratum corneum of healthy people, patients with psoriasis and AD patients by tape stripping (Figure 3-16). She found that the production of HBD-3 (and not HBD-2) was

[3]Dr Anna Berekmeri is a PhD student in the University of Leeds, supervised by Dr Miriam Wittmann and Dr Martin Stacey.

significantly higher in lesional psoriatic skin compared to lesional AD skin (Figure 3-16), suggesting that HBD-3 production in AD is lower compared to other inflammatory diseases (psoriasis).



(a)



(b)

Figure 3-16: Production of HBD-2 and HBD-3 in lesional psoriatic and lesional AD skin

Production of HBD-2 and HBD-3 was measured by ELISA in tape strip samples from the stratum corneum of healthy people, patients with psoriasis and AD patients, *** $p<0.001$, ns: non-significant (work conducted by Dr Anna Berekmeri).

3.3. Discussion

In order to better understand the role of *S. aureus* in AD pathogenesis, keratinocyte response to *S. aureus* and *S. epidermidis* was investigated. Although much research has been conducted on the effect of staphylococci on AMP keratinocyte production, the results are not always consistent (Wanke et al., 2011; Ommori et al., 2013; Lai et al., 2010). Factors that may account for the different findings of this project (as well as previous projects) are different experimental conditions such as the determination of mRNA expression or AMP protein production (Vogel and Marcotte, 2012; Greenbaum et al., 2003), stimulants (cytokines, staphylococcal cells or secreted factors) (Liu et al., 2002), or the keratinocyte differentiation stage (Krisanaprakornkit et al., 2003; Liu et al., 2002).

The effect of *S. aureus* and *S. epidermidis* on AMP production in keratinocytes

To further investigate previous findings, the work in this chapter tested the effect of *S. aureus* and *S. epidermidis* on the induction of HBD-2, HBD-3 and LL-37, which are important skin AMPs that show high potency against *S. aureus* but whose levels do not seem to correspond to the degree of inflammation in AD (Hata and Gallo, 2008; Ong et al., 2002; Nomura et al., 2003b). The results showed that *S. aureus* and not *S. epidermidis* triggered HBD-3 induction in keratinocyte monolayers and skin equivalents and that the HBD-3-inducing factor(s) can usually be found in the *S. aureus* supernatant (Figure 3-1, Figure 3-3, Figure 3-6 and Figure 3-8). In line with these results, a previous study detected HBD-3 production in keratinocytes only after stimulation with *S. aureus* cells and supernatant and not *S. epidermidis* (Ommori et al., 2013), while other studies reported significantly higher HBD-3 expression after exposure of skin equivalents and keratinocyte monolayers to *S. aureus* compared to *S. epidermidis* (Haisma et al., 2013; Wanke et al., 2011). It has been shown that stimulation of skin equivalents with *S. epidermidis* cells had a much lower impact on overall gene expression in the skin equivalent, whereas *S. aureus* cells increased the expression of genes associated with cytokine production (Holland et al., 2009), supporting the theory that different bacteria may stimulate different pathways, which may

explain the different effects of *S. epidermidis* and *S. aureus* on the skin (Steubesand et al., 2009; Chung and Dale, 2004; Chung and Dale, 2008).

The skin equivalents produced only low amounts of HBD-2 after stimulation with *S. aureus* live cells and even lower following *S. epidermidis* induction (Figure 3-1). Stimulation of skin equivalents with staphylococci for a longer period of time may possibly have induced higher HBD-2 production. Nevertheless, the duration of stimulation that was chosen allowed adequate time for interactions between staphylococci and skin cells and HBD-3 was still induced by *S. aureus*, consistent with the experiments using keratinocyte monolayers (Figure 3-3, Figure 3-6 and Figure 3-8) (Vu et al., 2010; Simanski et al., 2016). Production of HBD-2 was undetectable in keratinocyte monolayers when stimulated with staphylococcal supernatants, in line with a previous study that did not find HBD-2 expression in keratinocytes exposed to *S. epidermidis* and *S. aureus* supernatants (Wanke et al., 2011). However, another study observed production of HBD-2 by keratinocytes exposed to *S. epidermidis* and *S. aureus* live cells and supernatant from *S. epidermidis* (Ommori et al., 2013). Unlike the work in this chapter, they used primary keratinocytes, different staphylococcal strains and different experimental conditions (10^8 CFU and supernatant from a 3-day staphylococcal culture) (Ommori et al., 2013). Probably, cells are more able to induce HBD-2 than supernatants and HBD-2 production may also be affected by the *S. epidermidis* strain used (Gill et al., 2005; Fux et al., 2005; Sela et al., 2018).

Regarding LL-37, only cells from *S. aureus* SH1000 and *S. epidermidis* SE3 induced significant LL-37 production (Figure 3-5 and Figure 3-6). Results are conflicting, as some researchers report no increase in LL-37 expression upon stimulation with *S. epidermidis* (Lai et al., 2010) or *S. aureus* (Menzies and Kenoyer, 2005), whereas others report upregulation by *S. aureus* (Midorikawa et al., 2003). LL-37 induction may also be influenced by the staphylococcal strains used (Gill et al., 2005; Fux et al., 2005; Sela et al., 2018), or by the fact that previous studies evaluated gene expression, unlike this project, which measured protein concentration (Vogel and Marcotte, 2012; Greenbaum et al., 2003).

Even though the findings of this chapter conflict with some previous studies that evaluated AMP mRNA expression in keratinocytes stimulated by staphylococci (Lai et al., 2010), the results shown herein are based on measurement of AMP protein production by ELISA. Increased mRNA expression does not necessarily depict increased protein production, due to several regulation stages between gene transcription and the translation of protein (Vogel and Marcotte, 2012; Greenbaum et al., 2003). Strengthening the findings in this chapter, several *S. aureus* and *S. epidermidis* strains were tested (clinical and laboratory strains, methicillin susceptible and resistant) and the staphylococcal effect on HBD-3 production was consistent in 3D skin equivalents and monolayers of HaCaTs and primary keratinocytes. As different pathways may be implicated in different AMP induction by pathogens and commensals (Sorensen et al., 2005; Lai et al., 2010; Wanke et al., 2011; Ommori et al., 2013) and *S. aureus* is a leading cause of skin infections (Tong et al., 2015; Kong et al., 2012; Otto, 2013; Bouvet et al., 2017), it seems conceivable that the skin may have evolved a *S. aureus*-specific pathway that results in increased HBD-3 production in keratinocytes. Also, HBD-3 is a potent AMP against *S. aureus* and has been found to be associated with the control of *S. aureus* colonisation (Zanger et al., 2010; Haisma et al., 2013; Kisich et al., 2008).

S. epidermidis does not actively suppress HBD-3 keratinocyte production

Further investigation aimed to determine the underlying cause in differentiation between the abilities of *S. epidermidis* and *S. aureus* to induce HBD-3. There was no difference in the survival of keratinocytes (Figure 3-7 and Figure 3-9) and in their ability to produce immune mediators (Figure 3-4) (Jiang et al., 2012) after stimulation with *S. aureus* and *S. epidermidis*. Also, *S. epidermidis* supernatant did not reduce the ability of *S. aureus* to induce HBD-3 production in keratinocytes (Figure 3-10), in line with previous research (Wanke et al., 2011). It is therefore unlikely that *S. epidermidis* produces factor(s) that can actively inhibit HBD-3 keratinocyte production, which suggests that *S. epidermidis* does not trigger HBD-3 response in keratinocytes and supports the assumption that the skin has developed mechanisms to

selectively express HBD-3 upon exposure to *S. aureus* by detecting components unique to this bacterium.

Is the induction of HBD-3 in keratinocytes a universal trait of *S. aureus*?

The work in this chapter also investigated whether the ability to induce HBD-3 production in keratinocytes is universal to all *S. aureus* strains. In addition to the 5 *S. aureus* strains that were initially tested (Figure 3-6), 26 *S. aureus* clinical isolates were screened (Figure 3-11 and Table 3-1). Supernatants from all but 8 of these *S. aureus* strains induced HBD-3 production in keratinocytes (Figure 3-11 and Table 3-1). When the ability of cells from these 8 strains to induce HBD-3 in keratinocytes was examined, it was found that cells of MOS283 and MOS287 induced significant HBD-3 production, whereas cells of CS6-EEFIC, MOS148 and MOS457 strains also showed some HBD-3 induction (Figure 3-13), suggesting that the HBD-3-inducing factor(s) they produce may predominantly be associated with the cell envelope rather than released into the growth media. Therefore, HBD-3 production was not induced by 3 out of 30 clinical isolates screened in total (Figure 3-13). The total amount of proteins-toxins produced may possibly vary among *S. aureus* strains, which would indicate that some strains are more toxic and keratinocytes may be unable to respond and produce immune mediators. Although supernatants from two of the strains that did not induce HBD-3 (1128 and 805) were found to be toxic for keratinocytes (Figure 3-12), production of other immune mediators by HaCaTs (such as IL-8) in the presence of these strains was not impaired (Figure 3-14). Previous work has found that *S. aureus* serine proteases can induce IL-8 through NF-κB activation (Rudack et al., 2009), whereas other researchers reported that proteases, such as recombinant *S. aureus* superantigen-like protein, can degrade recombinant IL-8 (Tang et al., 2019). The results of the experiment in this chapter suggest that either the proteases produced by *S. aureus* strains may degrade HBD-3 but not IL-8, or that these strains activate IL-8 through a pathway different to the HBD-3 induction pathway. Indeed, it has been reported that expression of IL-8 is mediated by NF-κB (Dejean et al., 2012; Roebuck, 1999; Mukaida et al., 1994; Huang and McCance, 2002) or EGFR (Frankart et al., 2012; Lan et al., 2013),

whereas *S. aureus* HBD-3 induction is mediated through phosphatidylinositol 3-kinase and MAPK, independently of NF-κB (Wanke et al., 2011).

S. aureus HBD-3-inducing factor(s) are released into the supernatant

The finding that *S. aureus* HBD-3-inducing factor(s) were present in the supernatant of the majority of *S. aureus* strains (Figure 3-6, Figure 3-8 and Figure 3-11) is important in order to plan further experimental procedures and facilitate the research towards the identification of these factor(s). It is not known yet whether the HBD-3-inducing factor(s) are intracellular and are released from the cells upon spontaneous lysis; cell-bound, which may be shed extracellularly during the growth cycle; or actively secreted into the supernatant. However, as washed *S. aureus* cells and filter-sterilised supernatant both induced HBD-3 keratinocyte production (Figure 3-1, Figure 3-6 and Figure 3-8), it may be hypothesised that these factor(s) are surface-associated that may be released into the supernatant and be recognised by keratinocytes. For instance, IsaB is a *S. aureus* protein which was found to be both secreted and surface-associated (Mackey-Lawrence et al., 2009).

The effect of IL-4 on the HBD-3 production in keratinocytes stimulated with *S. aureus*

S. aureus can promote Th_2 responses (Mandron et al., 2006) and IL-4 can downregulate gene expression of HBD-2 and HBD-3 in keratinocytes, induced by other cytokines (Albanesi et al., 2007; Howell et al., 2006a). Addition of IL-4 to keratinocyte monolayers stimulated with human serum was shown to reduce HBD-2 protein production (Alase et al., 2012). This project found that HBD-3 production in response to *S. aureus*-inducing factor(s) was reduced when IL-4 concentration increased in keratinocytes (Figure 3-15). Although more research is required in order to optimise the conditions of the experiment (e.g. concentrations of IL-4 and *S. aureus* supernatant and incubation times), this experiment indicates that the elevated IL-4 cytokine production of the atopic environment (Matsunaga and Yamauchi, 2016; Gandhi et al., 2017) can downregulate the levels of the HBD-3 protein produced by keratinocytes in

response to *S. aureus*. This supports the importance of HBD-3 in skin defence against *S. aureus*, as AD is characterised by a failure to effectively counter *S. aureus* infection (Baldry et al., 2018; Chiu et al., 2009; David and Cambridge, 1986) and the AMP levels in AD do not seem to correspond to the degree of inflammation (Hata and Gallo, 2008; Ong et al., 2002; Nomura et al., 2003b; Lin et al., 2007). The inadequate immune response in AD may be caused by absence of induction of HBD-3 by *S. aureus* or by inability of the immune system to initiate effective HBD-3 production.

Previous research found higher HBD-2 levels in psoriatic skin compared to AD skin and assumed that this might account for the difference in inflammation levels between psoriasis and AD (Jansen et al., 2009). However, Dr Berekmeri found that the production of HBD-3 (and not HBD-2) was significantly higher in lesional psoriatic skin compared to lesional AD skin (Figure 3-16). This finding (Figure 3-16), along with the result of the experiment of this project (Figure 3-15) and the fact that HBD-3 is more potent against *S. aureus* than HBD-2 (Schibli et al., 2002; Dhople et al., 2006; Harder et al., 2001; Jung et al., 2011), suggests a key role for HBD-3 in AD dysbiosis and a possible justification of the fact that there is less infection in psoriasis compared to AD (Nowicka and Grywalska, 2018; Christophers and Henseler, 1987; Elfatoiki et al., 2016).

In conclusion, this chapter has identified a clear difference in the effect of *S. epidermidis* and *S. aureus* on the induction of HBD-3 in keratinocytes. Taking into account the fact that *S. aureus* is a leading cause of skin infections (Tong et al., 2015; Bouvet et al., 2017) and that HBD-3 is found to be more potent against *S. aureus* compared to other AMPs (Jung et al., 2011; Ouhara et al., 2008), it is suggested that the skin may have evolved an HBD-3 pathway specific to *S. aureus*, while *S. aureus* may develop mechanisms to protect itself from this HBD-3 response. In order to further investigate the difference in the interaction of *S. epidermidis* and *S. aureus* with keratinocytes and explain why some *S. aureus* strains induce low or no HBD-3 in keratinocytes, the following chapter of the project will investigate the HBD-3-inducing factor(s) of *S. aureus*.

4. Nature of the *S. aureus* HBD-3-inducing factor(s)

4.1. Introduction

Findings described in the previous chapter indicate that there are one or more factors produced by *S. aureus* that induce HBD-3 production in keratinocytes. This chapter describes further investigations into these HBD-3-inducing *S. aureus* factor(s), by examining the effect of the Agr system on the expression of these factor(s), their heat-stability and whether they contain a proteinaceous component. Further approaches to isolate and identify these factor(s) were also attempted.

The *S. aureus* secretome refers to all proteins secreted by staphylococcal cells in the extracellular environment and represents about 30% of its proteome (Mukherjee and Mani, 2013; Agrawal et al., 2010). Various studies have tried to identify the role of *S. aureus* secreted proteins, as they play an important role in its virulence and affect the host responses to *S. aureus* colonisation (Kusch and Engelmann, 2014; Koppenol-Raab et al., 2017; Ravipaty and Reilly, 2010). As the *S. aureus* Agr system is a major regulator that controls the expression of several genes encoding surface-associated and secreted virulence factors (Wang and Tom, 2016; Boles and Horswill, 2008), it was hypothesised that it may also regulate the HBD-3-inducing factor(s). The Agr system can regulate the resistance of *S. aureus* to AMPs, e.g. through the expression of proteases that degrade AMPs (Joo and Otto, 2015; Lai et al., 2007). The expression of *agr* in *S. aureus* is influenced by the RNA polymerase factor SigB, which is itself activated by phosphoserine phosphatase RsbU. Signals that promote activation of RsbU and therefore SigB in *S. aureus* have not been fully elucidated but one trigger is believed to be environmental stress (Lauderdale et al., 2009; Pane-Farre et al., 2009; Pförtner et al., 2014). The SigB protein was found to modulate *agr* expression, downregulate the production of virulence factors such as proteases (Karlsson-Kanth et al., 2006) and promote biofilm formation and intracellular growth (Tuchscherr et al., 2015). However, the exact effects of SigB on production of virulence factors can differ, depending on the *S. aureus* strain (Pförtner et al.,

2014). Mutations of *sigB* gene in *S. aureus* SH1000 and USA300 strains were found to upregulate the Agr system, leading to increased virulence and reduced biofilm formation (Lauderdale et al., 2009; Savage et al., 2013a). In addition, mutations in *agr* have resulted in strains with an increased ability to evade the host's immune system, due to increased expression of surface proteins, enhanced biofilm formation and decreased expression of secreted virulence factors (Boles and Horswill, 2008; Savage et al., 2013a).

4.2. Results

4.2.1. The effect of *S. aureus* Agr system on the induction of HBD-3 in keratinocytes

As the *S. aureus* Agr system is a major regulator of the expression of virulence factors (Wang and Tom, 2016), it was hypothesised that it might also regulate the *S. aureus* factor(s) that induce HBD-3 production in keratinocytes. In order to investigate this, HaCaT cells were stimulated with supernatants from mutants of *S. aureus* SH1000 and USA300 strains exhibiting altered Agr function (Table 4-1 and Figure 4-1). Production of HBD-3 was reduced noticeably when HaCaT cells were stimulated with supernatants from strains exhibiting increased Agr activity (WV1 and NE1109) compared to the parental strains. However, the difference was statistically significant for WV1 only (Figure 4-1). Conversely, when HaCaT cells were exposed to supernatants from the *agr*-defective mutant NE1532, HBD-3 production was significantly higher compared to the parental strain USA300 (Savage et al., 2013a; BEI Resources, c2019). However, HBD-3 induction by the *agr*-defective variant of SH1000 (LPV1) was similar to the wild-type strain (Figure 4-1).

Table 4-1: Mutants of *S. aureus* SH1000 and USA300 that have altered Agr activity

***S. aureus* strain**	**Description**	**Mutation**	**Agr activity**	**References**
SH1000	parental strain	-	wild-type	(Horsburgh et al., 2002; O'Neill, 2010; Clarke et al., 2007)
LPV1	SH1000 variant	*agrA* loss-of-function (*agrA* missense mutation)	low	(Savage et al., 2013b)
WV1	SH1000 variant	*sigB* loss-of-function (deletion of 650-770 *sigB* nucleotides)	high	(Savage et al., 2013b)
USA300	parental strain	-	wild-type	(Azarian et al., 2016; Fey et al., 2013; Boyle-Vavra and Daum, 2007)
NE1532	USA300 mutant	transposon insertion in *agrA*	low	(Bose et al., 2013; Connolly et al., 2017; BEI Resources, c2019)
NE1109	USA300 mutant	transposon insertion in RNA polymerase SigB gene (*rpoF*)	high	(Bose et al., 2013; Karlsson-Kanth et al., 2006; Horn et al., 2018; Basu and Yap, 2017; BEI Resources, c2019)

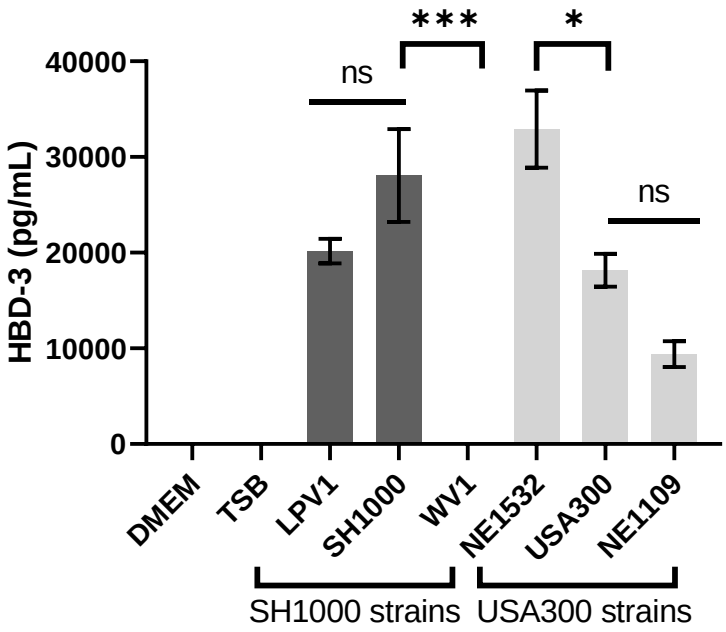


Figure 4-1: Production of HBD-3 by HaCaT cells stimulated with supernatants from *S. aureus* strains with altered Agr activity

HaCaT monolayers were stimulated with supernatants from *S. aureus* strains with altered Agr function for 24 h and HBD-3 production was measured by ELISA. One-way ANOVA was used to determine statistical significance, n=3. Values are mean ± SEM, * $p<0.05$, *** $p<0.001$, ns: non-significant. Where the values were below the detection limit (DMEM, TSB and WV1), they were set to 62.5 pg/mL (lowest detection limit of the HBD-3 ELISA assay) to allow statistical analysis.

The failure of *S. aureus* WV1 supernatant to induce HBD-3 was further explored. In order to investigate whether cells of *S. aureus* WV1 can induce HBD-3 production in keratinocytes, HaCaT monolayers were stimulated with whole cultures from *S. aureus* WV1 and SH1000 (Figure 4-2). Although *S. aureus* SH1000 cells induced HBD-3 production in HaCaT cells as expected, WV1 cells did not (Figure 4-2).

Failure of the WV1 strain to induce HBD-3 could be attributable to the increased toxicity of this strain, thereby either killing keratinocytes, or rendering them unable to respond to stimulation and produce immune mediators. Therefore, the toxicity of *S. aureus* WV1 supernatant towards HaCaT cells was compared with SH1000 and was found to be significantly increased (Figure 4-3). The toxicity of the WV1 supernatant was confirmed visually by microscopic analysis of cultured cells, which revealed an increased

number of detached HaCaT cells compared to challenge with SH1000 supernatant (*data not shown*).

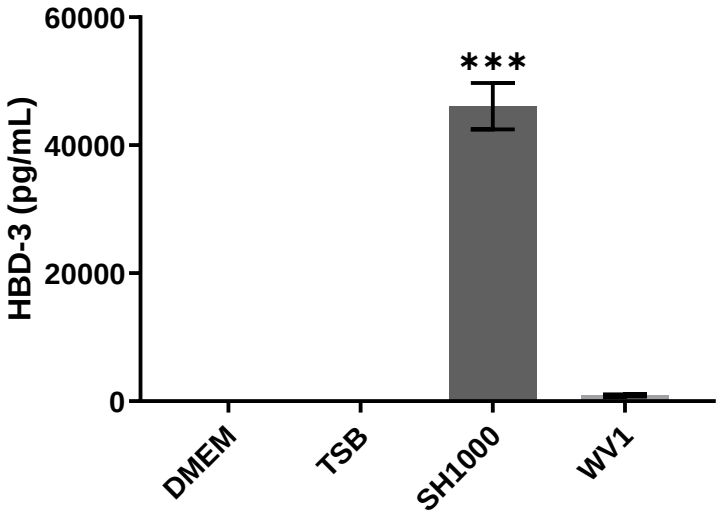


Figure 4-2: Production of HBD-3 by HaCaT cells stimulated with *S. aureus* WV1 cells

HaCaT monolayers were stimulated with cultures from *S. aureus* WV1 and SH1000 for 20 h and HBD-3 production was measured by ELISA. One-way ANOVA was used to determine statistical significance, n=4; Values are mean ± SEM, *** $p<0.001$. Where the values were below the detection limit (DMEM and TSB), they were set to 62.5 pg/mL (lowest detection limit of the HBD-3 ELISA assay) to allow statistical analysis.

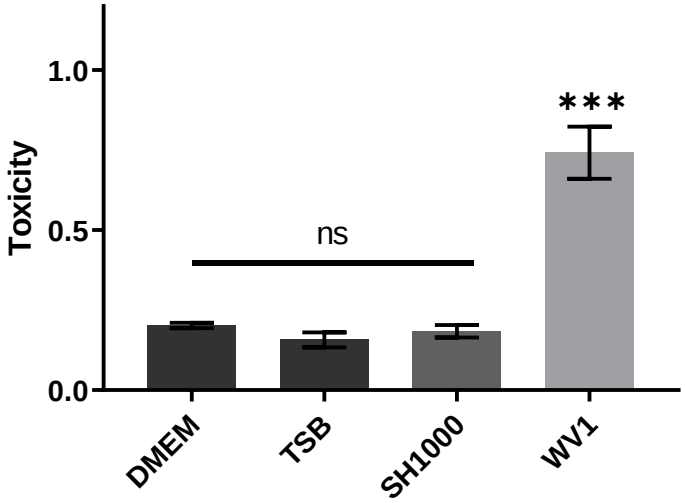


Figure 4-3: Toxicity of *S. aureus* WV1 supernatant, which did not induce HBD-3 in HaCaT cells

HaCaT monolayers were stimulated with supernatants from *S. aureus* WV1 and SH1000 for 24 h. Luminescence of dead and alive HaCaT cells was determined by Toxilight. Toxicity represents the killing of HaCaT cells (ratio of dead HaCaT cells to total HaCaT cells) after stimulation. One-way ANOVA was used to determine statistical significance, n=3. Values are mean ± SEM, *** $p<0.001$, ns: non-significant.

In order to determine whether WV1 is able to induce production of other immune mediators in HaCaT cells, IL-8 induction was determined, as described in section 3.2.3 (Figure 4-4). The results showed that WV1 strain induced IL-8 production in HaCaT cells, implying that the lack of induction in this strain is specific to HBD-3 (Figure 4-4). These results indicate that, even though HaCaT cells are not completely killed by staphylococci and are able to respond (Figure 4-4), the levels of staphylococcal toxicity (Figure 4-3) may still affect the response of HaCaT cells and the production of immune mediators.

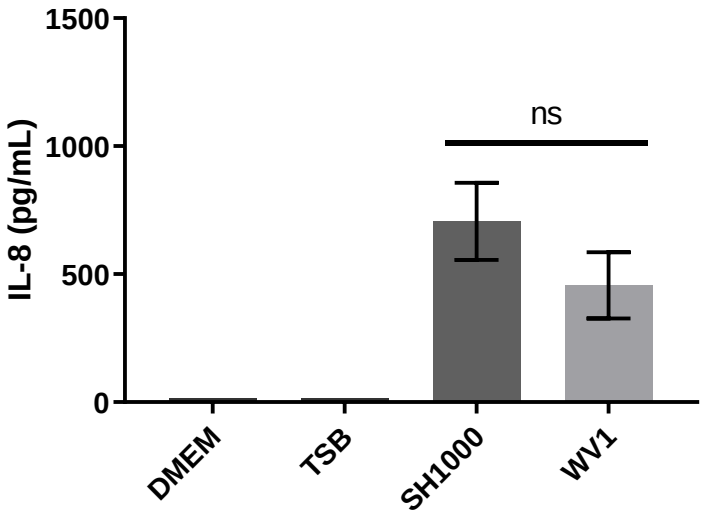


Figure 4-4: Production of IL-8 by HaCaT cells stimulated with WV1 cells

HaCaT monolayers were stimulated with cultures from *S. aureus* WV1 and SH1000 for 20 h and IL-8 production was measured by ELISA. One-way ANOVA was used to determine statistical significance, n=3; Values are mean ± SEM, ns: non-significant. Where the values were below the detection limit (DMEM and TSB), they were set to 15.6 pg/mL (lowest detection limit of the IL-8 ELISA assay) to allow statistical analysis.

The *agr* expression is associated with production of virulence factors, including proteases (Wang and Tom, 2016; Boles and Horswill, 2008). Therefore, WV1 strain shows a highly-proteolytic phenotype (Savage et al., 2013b). In addition to the elevated toxicity of WV1, it seemed plausible that the apparent lack of HBD-3 induction of this strain might in part result from this proteolytic activity destroying either the HBD-3-inducing factor(s) (if proteinaceous) or the produced HBD-3. To test this, HBD-3 keratinocyte production was determined in the presence of a protease inhibitor cocktail (PIC, Sigma-Aldrich), to block proteolytic activity (Liu et al., 2010).

First, HaCaT monolayers were pre-treated with PIC and then stimulated with supernatants from *S. aureus* SH1000 and WV1. There was again no detectable HBD-3 production by the HaCaT cells stimulated with *S. aureus* WV1, regardless of the concentration of PIC added (Figure 4-5). The positive control *S. aureus* SH1000 induced similar HBD-3 production regardless of the

PIC concentration, indicating that the PIC was not toxic to HaCaT cells (Figure 4-5).

Since pre-incubation of HaCaTs with PIC before stimulation with *S. aureus* WV1 supernatant did not result in an increase in HBD-3 production (Figure 4-5), an alternative approach was attempted. Staphylococcal cultures were grown overnight and were then incubated in the presence of PIC for 1 h. Then, HaCaT cells were stimulated with the supernatants from these cultures (Figure 4-6). No detectable HBD-3 was induced by *S. aureus* WV1, regardless of the PIC concentration (Figure 4-6).

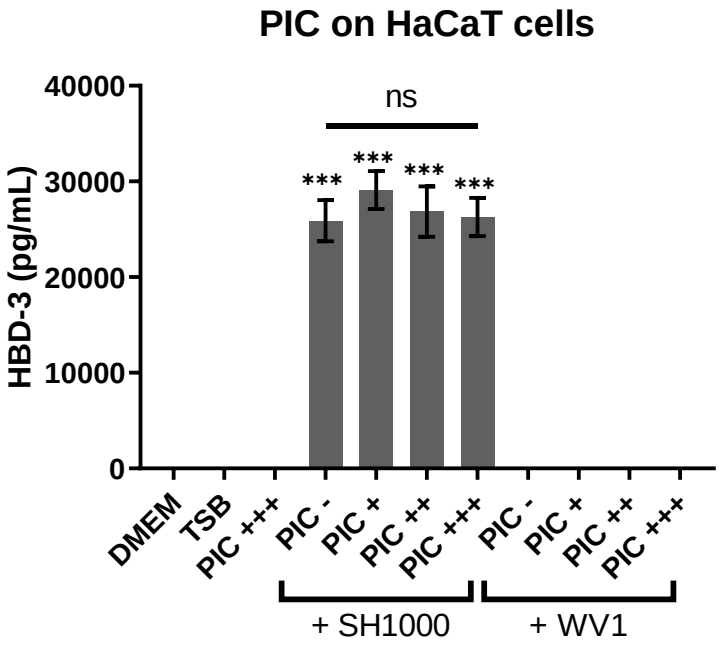


Figure 4-5: Production of HBD-3 by PIC-treated HaCaT cells stimulated with *S. aureus* supernatants

HaCaT monolayers were either left untreated (PIC -) or treated with 1 mg/mL PIC at final dilution 1:800 (1.25 µg/mL, PIC +), 1:400 (2.5 µg/mL, PIC ++) or 1:200 (5 µg/mL, PIC +++) in DMEM, according to the manufacturer's recommendation. Then, these HaCaT monolayers were stimulated with filter-sterilised supernatants from *S. aureus* SH1000 and WV1 for 24 h. Production of HBD-3 was measured by ELISA. One-way ANOVA was used to determine statistical significance, n=3. Values are mean ± SEM, *** $p<0.001$, ns: non-significant. Where the values were below the detection limit (DMEM, TSB, PIC +++ and the WV1 strains), they were set to 62.5 pg/mL (lowest detection limit of the HBD-3 ELISA assay) to allow statistical analysis.

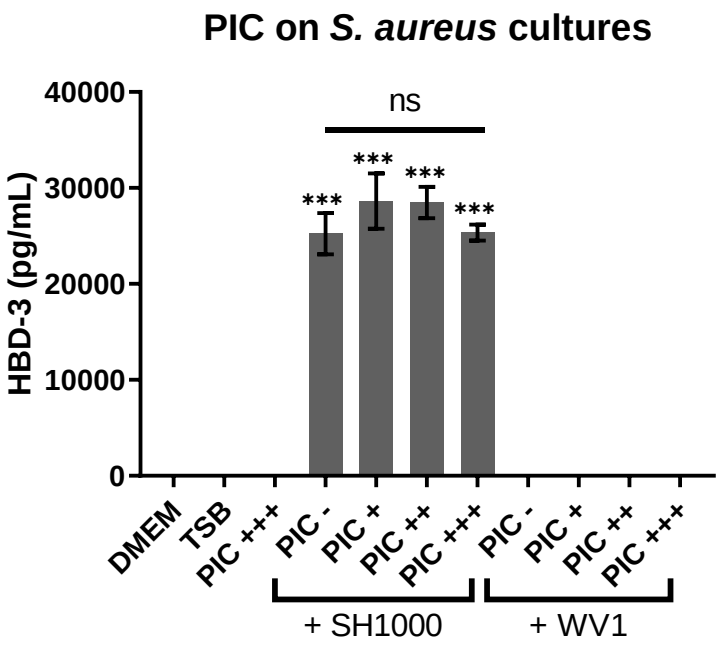


Figure 4-6: Production of HBD-3 by HaCaT cells stimulated with supernatants from PIC-treated *S. aureus* cultures

Cultures of *S. aureus* WV1 and SH1000 were grown for 17 h. Then, 1 mg/mL PIC was added to the cultures at final dilution 1:800 (1.25 µg/mL, PIC +), 1:400 (2.5 µg/mL, PIC ++) or 1:200 (5 µg/mL, PIC +++) in TSB, according to the manufacturer's recommendation and the cultures were incubated at 37°C for 1 h, with vigorous aeration. HaCaT monolayers were stimulated with filter-sterilised supernatants from these cultures for 24 h. Production of HBD-3 was measured by ELISA. One-way ANOVA was used to determine statistical significance, n=3. Values are mean ± SEM, *** $p<0.001$, ns: non-significant. Where the values were below the detection limit (DMEM, TSB, PIC +++ and the WV1 strains), they were set to 62.5 pg/mL (lowest detection limit of the HBD-3 ELISA assay) to allow statistical analysis.

4.2.2. The HBD-3-inducing *S. aureus* factor(s) are proteinaceous

The next step was to investigate the nature of the HBD-3-inducing *S. aureus* factor(s). In order to examine whether these factor(s) are proteinaceous, Proteinase K (PrK), an enzyme with proteolytic activity, was used (Ebeling et al., 1974).

First, the possibility that PrK could directly degrade HBD-3 was studied. HaCaT cells were stimulated with *S. aureus* SH1000 supernatant for 24 h and then PrK was added to the wells for a further 1 h of incubation (SH1000 + PrK) (Figure 4-7). Heat-denatured PrK (SH1000 + PrK heat) was used as a

negative control. The HBD-3 levels did not show significant reduction following addition of PrK, indicating that PrK did not degrade the HBD-3 that was already produced by HaCaT cells stimulated with SH1000 supernatant (Figure 4-7).

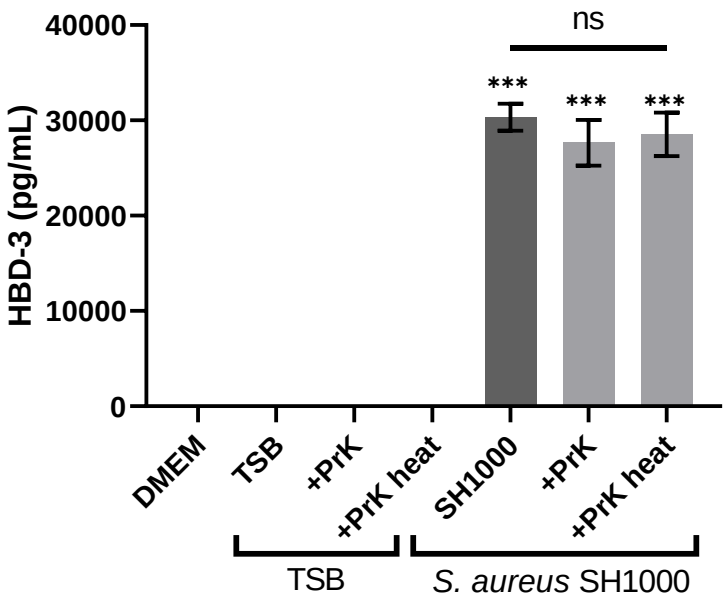


Figure 4-7: The effect of PrK upon HBD-3 stability

HaCaT cells were stimulated with *S. aureus* SH1000 supernatant for 24 h and then heat-denatured PrK (SH1000 + PrK heat) and non-heated PrK (SH1000 + PrK) was added for 1 h incubation. Production of HBD-3 was measured by ELISA. One-way ANOVA was used to determine statistical significance, n=5. Values are mean ± SEM, *** $p<0.001$, ns: non-significant. Where the values were below the detection limit (DMEM and all TSB controls), they were set to 62.5 pg/mL (lowest detection limit of the HBD-3 ELISA assay) to allow statistical analysis.

Moreover, toxicity of PrK towards HaCaT cells was investigated. The toxicity assay showed that PrK (TSB-PrK) was not toxic for the HaCaT cells (Figure 4-8). SH1000 supernatant that was heated at 100^{o}C for 10 min was also tested (SH1000 heat) to examine whether components in the supernatant may exhibit altered toxicity upon heat treatment. However, heated *S. aureus* supernatant did not show altered toxicity towards keratinocytes compared to the non-heated control (Figure 4-8).

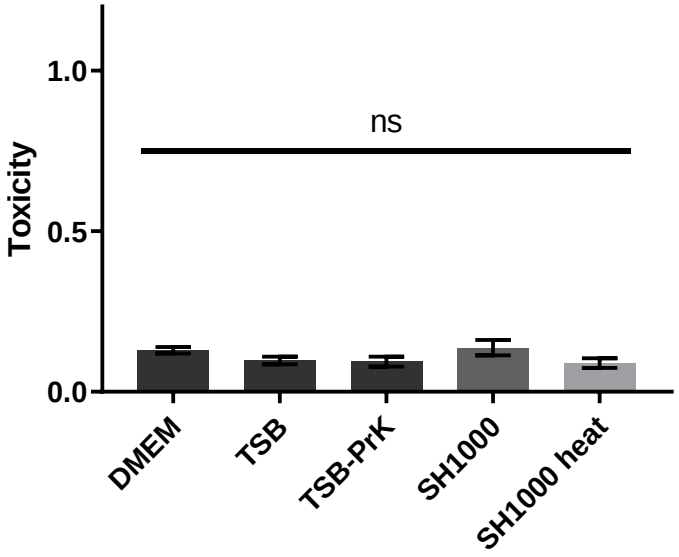


Figure 4-8: Toxicity of heated *S. aureus* supernatant and PrK towards HaCaT cells

HaCaT monolayers were stimulated for 24 h with *S. aureus* SH1000 supernatant, unheated (SH1000) or heated (SH1000 heat). Luminescence of dead and alive HaCaT cells was determined by Toxilight. Toxicity represents the killing of HaCaT cells (ratio of dead HaCaT cells to total HaCaT cells) after stimulation. One-way ANOVA was used to determine statistical significance, n=3. Values are mean ± SEM, ns: non-significant.

The above experiments showed that PrK did not degrade HBD-3 (Figure 4-7) and was not toxic towards HaCaT cells (Figure 4-8). HaCaT cells were stimulated with *S. aureus* SH1000 supernatant that was either pre-incubated with PrK or heated, in order to determine whether the HBD-3-inducing factor(s) contain a protein component and examine their heat-stability (Figure 4-9).

The results (Figure 4-9) showed that HBD-3 was undetectable when HaCaT cells were stimulated with SH1000 supernatant that was preincubated with PrK (SA-PrK). When supernatant without PrK was heated to 100°C for 10 min (SA-heat), HBD-3 was still produced, albeit at significantly reduced levels compared to when using a non-heated supernatant, suggesting that heat is not fully inactivating the HBD-3-inducing factor(s). Moreover, successful heat

inactivation of PrK at 100°C was confirmed, as SH1000 supernatant preincubated with heat-inactivated PrK (SA + PrK-heat) induced similar HBD-3 levels to the control (SA) (Figure 4-9) (Tirumurugaan et al., 2015).

Taken together, these results suggest that the HBD-3-inducing factor(s) produced by *S. aureus* are of a proteinaceous nature and heat-stable to some extent, as heating to 100°C only reduced the HBD-3 production by ~ 40%, whereas addition of PrK completely ablated it (Figure 4-9).

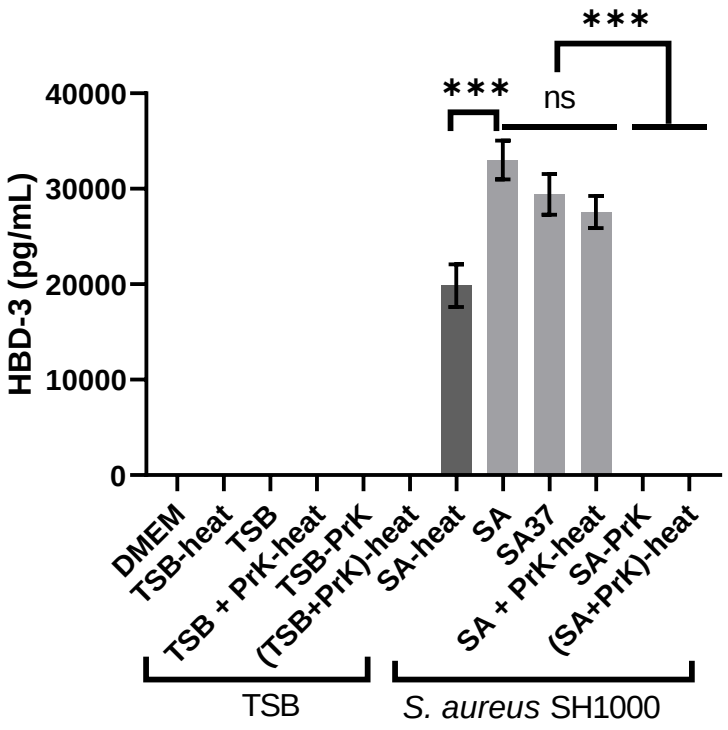


Figure 4-9: Production of HBD-3 by HaCaT cells after stimulation with *S. aureus* supernatant heated or treated with PrK

HaCaT monolayers were stimulated for 24 h with SH1000 supernatant only (SA), with heated supernatant (SA-heat) and with supernatant preincubated with heated PrK (SA + PrK-heat) or non-heated PrK (SA-PrK). As controls, an aliquot of SA-PrK was heated at 100°C for 10 min [(SA+PrK)-heat] and SA was incubated at 37°C for 1 h (SA37). Production of HBD-3 was measured by ELISA. One-way ANOVA was used to determine statistical significance, n=5. Values are mean ± SEM, *** $p<0.001$, ns: non-significant. Where the values were below the detection limit [DMEM, all TSB controls, SA-PrK and (SA+PrK)-heat], they were set to 62.5 pg/mL (lowest detection limit of the HBD-3 ELISA assay) to allow statistical analysis.

4.2.3. Fractionation of *S. aureus* supernatant with a view to identifying the HBD-3-inducing factor(s)

4.2.3.1. Ion-exchange chromatography of *S. aureus* supernatant

Given that the HBD-3-inducing factor(s) produced by *S. aureus* seem to be either protein(s) or oligopeptide(s), standard protein purification techniques were deployed in order to separate HBD-3-inducing factor(s) from other proteins present in the supernatant and allow further identification. Liquid chromatography can separate proteins found in a mixture based on their charge (ion-exchange) or their size (size-exclusion) (Coskun, 2016) and has been used to isolate *S. aureus* proteins such as SdrE (Prachi, 2013), α-hemolysin (Fiaschi et al., 2016) and *S. aureus* heme transporters (Abe et al., 2012). Ion-exchange chromatography was performed using an Äkta Purifier system (Materials and methods, 2.8.1), in order to separate staphylococcal supernatant proteins by their charge and reduce the number of candidate proteins responsible for the HBD-3 induction in keratinocytes. In order to capture the majority of proteins in the supernatant, two columns interconnected in series were used: an anion-exchange column, which binds to proteins with a net negative charge, and a cation-exchange column, which binds to proteins with a net positive charge. The columns were then uncoupled and eluted separately with linear gradients of NaCl.

Collected fractions were filter-sterilised and added to HaCaT monolayers to test their ability to induce HBD-3 production (Figure 4-10). The buffers A (20 mM MES and 50 mM NaCl, pH 5.5) and B (20 mM MES and 1 M NaCl, pH 5.5) that were used during fractionation, DMEM and TSB were the controls.

In the first experiment, every other fraction was collected, to reduce the number of samples. Fractions from both anion-exchange and cation-exchange columns induced HBD-3 in HaCaT cells (Figure 4-10, graphs a and b). In order to verify the results, a second chromatography experiment was performed. Unfortunately, the elution of the cation-exchange column failed, so only the anion-exchange column produced fractions (Figure 4-10, graph c). The peak HBD-3-inducing fractions from both exchange columns (Figure 4-10, graphs a and c) were analysed by LC-MS, in order to identify the proteins present (*see below*). A third fractionation experiment was performed, which

showed stronger HBD-3 induction by fractions from the anion-exchange column, in contrast to the first experiment (Figure 4-10, graphs d and e). The peak fractions from both columns (Figure 4-10, graphs d and e) were also analysed by LC-MS (*see below*). A fourth repeat showed higher HBD-3 induction by fractions from the cation-exchange column, whereas several fractions from the anion-exchange column induced lower levels of HBD-3 (Figure 4-10, graphs f and g). The peak fractions from the fourth experiment, along with the peak fraction from the anion-exchange column of the first fractionation (Figure 4-10, graph b) were later analysed by a newly developed LC-MS method (section 4.2.3.7, Table 4-5 and Table 4-6).

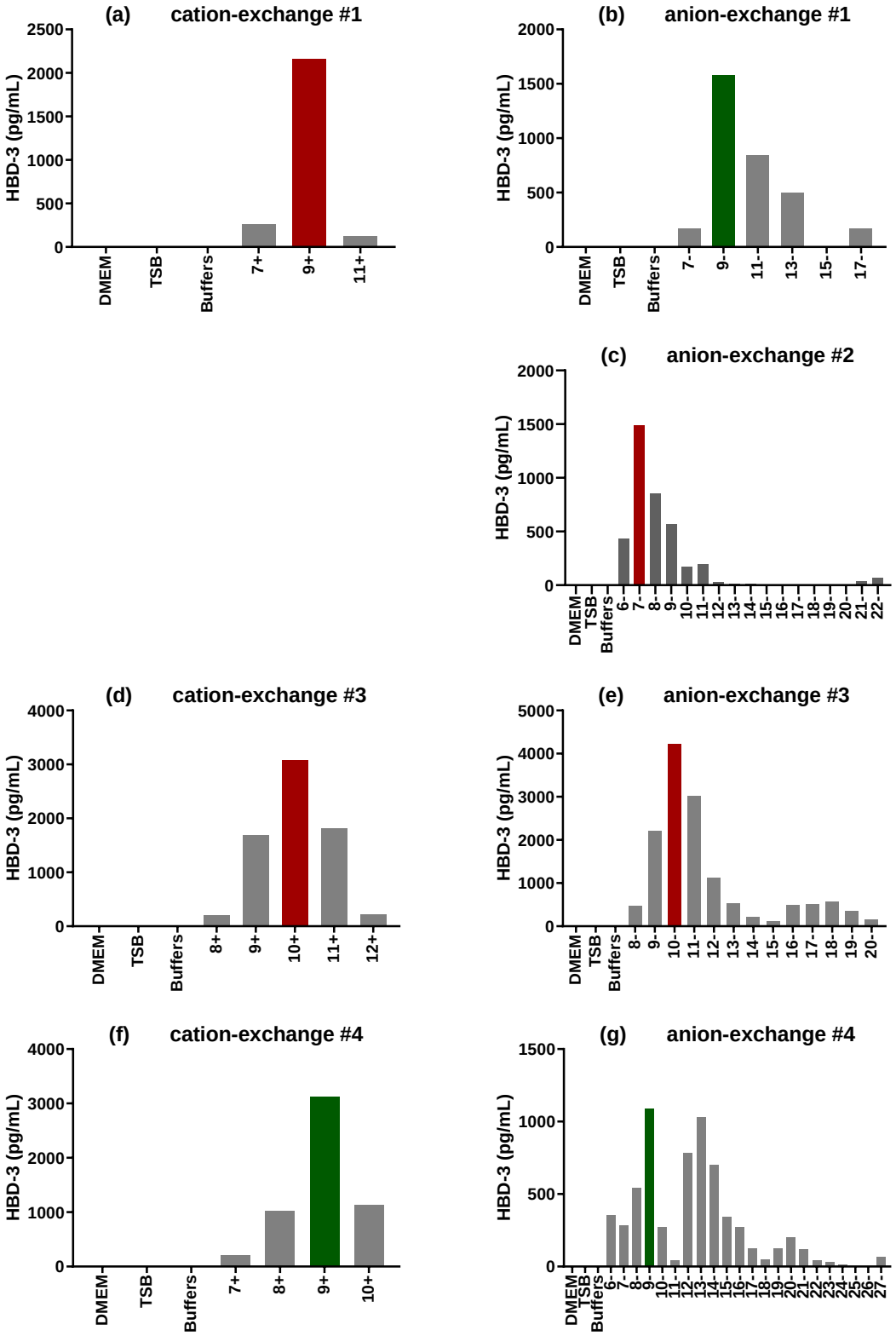


Figure 4-10: Production of HBD-3 by HaCaT cells when exposed to fractions from the ion-exchange chromatography

HaCaT monolayers were stimulated for 24 h with the fractions from the cation-exchange (a, d, f) and the anion-exchange (b, c, e, g) chromatography experiments and HBD-3 production was measured by ELISA. Peaks in red represent the fractions analysed with the initial LC-MS method and peaks in green are the samples analysed at a later time, with a newly developed LC-MS method (sections 4.2.3.3 and 4.2.3.7).

4.2.3.2. Size-exclusion chromatography

Fractions from Figure 4-10 that induced HBD-3 production in keratinocytes were analysed by SDS-PAGE. The fractions produced multiple bands, indicating the presence of numerous proteins in each fraction (*data not shown*). Size-exclusion chromatography of the HBD-3-inducing fraction 9 was performed (Figure 4-10, graph a), in an attempt to separate the proteins by size and hence further isolate the protein(s) inducing HBD-3 production. However, none of the fractions produced from the size-exclusion experiment induced HBD-3 production in HaCaT cells (*data not shown*), suggesting that the protein concentration in these fractions may not have been high enough to induce HBD-3.

4.2.3.3. Mass spectrometry

Following recovery of fractions containing the *S. aureus* HBD-3-inducing factor(s), the next step towards identification was to perform LC-MS (Materials and methods, 2.8.4). Mass spectrometry can detect peptide sequences in a mixture and match them to known proteins from a database (Pitt, 2009). The peak HBD-3-inducing fractions from two experiments from the cation-exchange column (Figure 4-10, graphs a and d) and two experiments from the anion-exchange column (Figure 4-10, graphs c and e) were analysed. The LC-MS assay was performed by the mass spectrometry group at the University of Leeds, UK and data were provided as predicted peptide sequences. As *S. epidermidis* did not induce HBD-3 production in keratinocytes (Figure 3-6 and Figure 3-8), it was assumed that *S. aureus* proteins with counterparts in *S. epidermidis* would be less likely to be implicated in HBD-3 production. Comparison of the similarity of the *S. aureus* proteins detected by LC-MS with *S. epidermidis* proteins was performed with the NCBI genomic database using the BLAST algorithm (Table 4-2 and Table 4-3) (Altschul et al., 1990; Altschul et al., 1997). Among the *S. aureus* proteins detected in these four fractions, only the IsaB protein was found in both fractions from the cation-exchange column (Table 4-2) and the SdrD protein was detected in both fractions from the anion-exchange column (Table 4-3).

Table 4-2: LC-MS detected candidate HBD-3-inducing proteins in the peak fractions produced from the cation-exchange chromatography

The following proteins were detected in fractions 9 and 10 from the cation-exchange column (Figure 4-10, graphs a and d). (Cover: percentage of peptide coverage of the total protein sequence; Pept: number of peptides that match the protein in the description; SE: percentage of similarity to *S. epidermidis* proteins; ns: no significant similarity to *S. epidermidis* proteins found)

fraction 9 from the cation-exchange column (graph a)				
Accession	**Cover (%)**	**Pept (%)**	**Description**	**SE (%)**
Q79ZW0|ISAB_STAAW	**68**	**49**	**Immunodominant staphylococcal antigen B**	**43**
Q2FZK7|ATL_STAA8	15	15	Bifunctional autolysin	67
Q99VJ0|NUC_STAAM	34	7	Thermonuclease	47
A6QG31|ISDA_STAAE	35	7	Iron-regulated surface determinant protein A	25
Q6GDN2|OATA_STAAR	13	6	O-acetyltransferase OatA	66
Q7A150|ISDE_STAAW	23	5	High-affinity heme uptake system protein IsdE	25
Q2G2B2|SASG_STAA8	3	3	Surface protein G	59
fraction 10 from the cation-exchange column (graph d)				
Accession	**Cover (%)**	**Pept (%)**	**Description**	**SE (%)**
tr|A0A1E5JB95|A0A1E5JB95_STAAU	81	45	Glycerophosphoryl diester phosphodiesterase family protein	70
tr|A0A1D4ILC4|A0A1D4ILC4_STAAU	18	13	Cell division protein FtsI [Peptidoglycan synthetase]/Transpeptidase, Penicillin binding protein transpeptidase domain	85
tr|A0A1R2DD87|A0A1R2DD87_STAAU	33	8	Uncharacterised protein	62
tr|A0A181GRB0|A0A181GRB0_STAAU	**36**	**7**	**Immunodominant antigen B**	**43**
tr|A0A0E1AE62|A0A0E1AE62_STAAU	25	4	Secretory antigen SsaA	80
tr|A0A0H2WWP4|A0A0H2WWP4_STAAC	22	4	Uncharacterised protein	57
tr|A0A077UYT7|A0A077UYT7_STAAU	29	4	Superantigen-like protein	ns

Table 4-3: LC-MS detected candidate HBD-3-inducing proteins in the peak fractions produced from the anion-exchange chromatography

The following proteins were detected in fractions 7 and 10 from the anion-exchange column (Figure 4-10, graphs c and e). (Cover: percentage of peptide coverage of the total protein sequence; Pept: number of peptides that match the protein in the description; SE: percentage of similarity to *S. epidermidis* proteins; ns: no significant similarity to *S. epidermidis* proteins found)

fraction 7 from the anion-exchange column (graph c)				
Accession	**Cover (%)**	**Pept (%)**	**Description**	**SE (%)**
Q5HFV0|DBH_STAAC	70	5	DNA-binding protein HU	99
O86488|SDRD_STAAE	**6**	**8**	**Serine-aspartate repeat-containing protein D**	**49**
Q2YWS3|G6PI_STAAB	15	6	Glucose-6-phosphate isomerase	93
fraction 10 from the anion-exchange column (graph e)				
Accession	**Cover (%)**	**Pept (%)**	**Description**	**SE (%)**
P13916|GLCA_SOYBN	5	7	Beta-conglycinin, α chain	35
P19594|2SS_SOYBN	18	5	2S albumin	ns
O86488|SDRD_STAAE	**3**	**3**	**Serine-aspartate repeat-containing protein D**	**49**
tr|A0A1D4RZS0|A0A1D4RZS0_STAAU	5	3	LPXTG-motif cell wall anchor domain-containing protein	81

4.2.3.4. *S. aureus* USA300 mutant strains from NTML

Upon identification of several candidate proteins for HBD-3 induction from the LC-MS analysis (Table 4-2 and Table 4-3), a search was performed in the NTML. This library consists of *S. aureus* USA300 strains, in which approximately 2,000 non-essential individual genes have been disrupted by the insertion of the mariner transposon *bursa aurealis* (Bose et al., 2013; BEI Resources, c2019). *S. aureus* USA300 strains that carry mutations which inactivate the genes equivalent to the candidate proteins identified by LC-MS were chosen in order to test whether any of these mutations may affect the ability of the *S. aureus* strain to induce HBD-3. Not all the detected proteins had corresponding *S. aureus* USA300 mutants in the NTML. The strains from the NTML that were tested are shown in Table 4-4.

Supernatants from these USA300 mutants were added to HaCaT monolayers and HBD-3 production was measured (Figure 4-11). There was no statistically significant difference in the induction of HBD-3 by *S. aureus* mutants compared to the wild-type strain, although HaCaT cells stimulated with NE460 and NE987 strains showed some reduction in their HBD-3 production (Figure 4-11). This suggests that either none of these proteins are implicated in the HBD-3 induction, or maybe the mutations were not sufficient to effectively inhibit the activity of the corresponding protein. These strains have insertional mutations, which would be expected to block the expression of a functional gene product (Bose et al., 2013; BEI Resources, c2019); however, sometimes the insertion may result in an altered protein, which may retain characteristics of the functional protein. Another possibility is that the synergistic effect of two or more proteins is responsible for the HBD-3 induction in keratinocytes (Figure 4-11).

Table 4-4: *S. aureus* USA300 variants from the NTML that carry mutations which inactivate the genes corresponding to the proteins detected by LC-MS

(Bose et al., 2013; BEI Resources, c2019)

***S. aureus* USA300 mutants**		
Strain Name	**Gene discription**	**Accession number**
NE24	secretory antigen precursor SsaA (*ssaA*)	SAUSA300_2503
NE33	LPXTG-motif cell wall surface anchor family protein	SAUSA300_2589
NE67	immunodominant antigen B (*isaB*)	SAUSA300_2573
NE460	autolysin (*atl*)	SAUSA300_0955
NE710	thermonuclease precursor	SAUSA300_0776
NE778	glycerophosphoryl diester phosphodiesterase (*glpQ*)	SAUSA300_0862
NE925	superantigen-like protein 7	SAUSA300_0401
NE987	secretory antigen precursor SsaA (*ssaA*)	SAUSA300_2249
NE1035	secretory antigen precursor SsaA (*ssaA*)	SAUSA300_2253
NE1241	thermonuclease (*nuc*)	SAUSA300_1222
NE1289	SdrD protein (*sdrD*)	SAUSA300_0547
NE1408	phiSLT ORF 87-like protein, putative DNA-binding protein	SAUSA300_1430
NE1544	glycerophosphoryl diester phosphodiesterase family protein	SAUSA300_1020

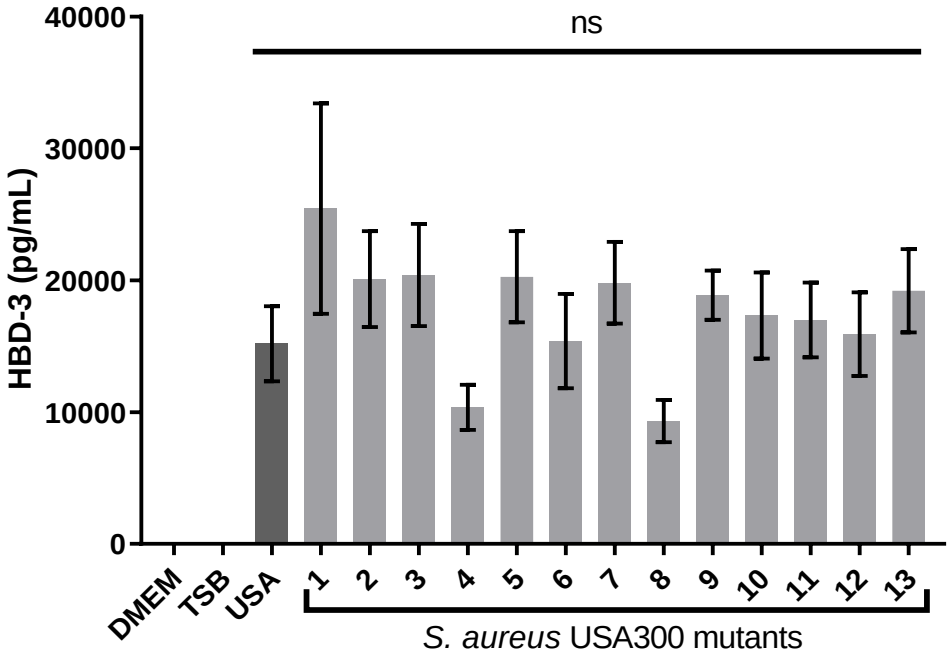


Figure 4-11: Production of HBD-3 by HaCaT cells stimulated with supernatants from *S. aureus* USA300 mutants from NMTL

HaCaT monolayers were stimulated with supernatants from *S. aureus* USA300 wild-type and mutants for 24 h and HBD-3 production was measured by ELISA. One-way ANOVA was used to determine statistical significance, n=4. Values are mean ± SEM, ns: non-significant. Where the values were below the detection limit (DMEM and TSB), they were set to 62.5 pg/mL (lowest detection limit of the HBD-3 ELISA assay) to allow statistical analysis. The full names of the x-axis labels from left to right are: DMEM, TSB, *S. aureus* USA300 (USA), NE24 (1), NE33 (2), NE67 (3), NE460 (4), NE710 (5), NE778 (6), NE925 (7), NE987 (8), NE1035 (9), NE1241 (10), NE1289 (11), NE1408 (12) and NE1544 (13) (Table 4-4).

As the experiment with the *S. aureus* USA300 mutants did not confirm any of the LC-MS candidate proteins, a different approach was taken. Although the HBD-3-inducing protein(s) could be any of the proteins identified by LC-MS, or even proteins not detected by LC-MS, the IsaB protein was selected for further investigation, as it was detected in both fractions from the cation-exchange column (Table 4-2) and the SdrD protein, which was detected in both fractions from the anion-exchange column (Table 4-3).

4.2.3.5. The SdrD protein

A purified or recombinant SdrD protein product was not available for purchase, therefore the SdrD protein needed to be produced in the laboratory. Lactococcal wild-type strains, strains with an empty vector (pMG36e) and with a vector containing the *sdrD* insert were cultured (Materials and methods, 2.7) (Askarian et al., 2017). Then, HaCaT monolayers were stimulated with cultures (~ 10^7 CFU) and filter-sterilised supernatants (at a final dilution ratio of 1:50 in DMEM) from lactococcal strains. Cultures and filter-sterilised supernatants from *S. aureus* SH1000 were used as positive control. Cultures from lactococci with an empty vector were used as a negative control, in addition to DMEM, TSB and M17 broth. There was no HBD-3 induction by lactococcal cells or supernatant, whereas the *S. aureus* SH1000 displayed induction in both cases, as expected (*data not shown*).

4.2.3.6. The IsaB protein

In order to investigate the effect of IsaB protein on keratinocytes (Materials and methods, 2.7), HaCaT monolayers were stimulated with heated IsaB (MyBiosource), as well as increasing concentrations of non-heated IsaB. As shown in Figure 4-12, HBD-3 production increased in HaCaT cells when stimulated with increasing concentrations of IsaB. Moreover, heat did not ablate the ability of IsaB to induce HBD-3 production, indicating that IsaB is heat-stable (Figure 4-12). This experiment suggests that IsaB may be a factor produced by *S. aureus* that can trigger HBD-3 production in keratinocytes.

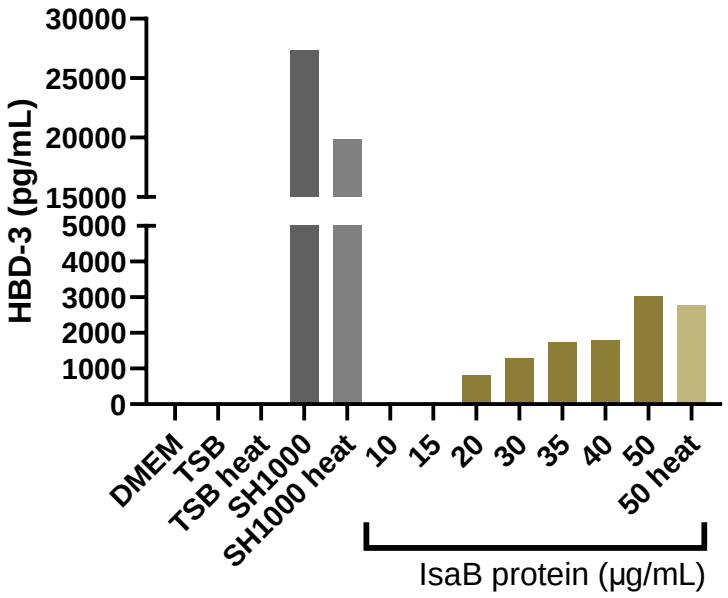


Figure 4-12: Production of HBD-3 by HaCaT cells stimulated with IsaB recombinant protein

HaCaT cells were stimulated for 24 h with increasing concentrations of IsaB protein from 10 µg/mL up to 50 µg/mL and with 50 µg/mL IsaB heated at 100°C for 10 min. *S. aureus* SH1000 supernatants heated (100°C for 10 min) and non-heated were used as positive controls. Production of HBD-3 was measured by ELISA (n=1).

4.2.3.7. S-Trap micro ultra-high recovery protocol mass spectrometry

An improved LC-MS method, which can identify proteins in the supernatant with higher sensitivity, was subsequently tested to determine if any of the components that could not be detected originally may be involved in HBD-3 induction (Appendices and supplemental material, 8.4). The peak fractions from the rest of the ion-exchange chromatography experiments were analysed by this method (Figure 4-10, graphs b, f and g) to confirm if the previously suggested candidates also appeared in these experiments. This improved method detected more proteins and more peptides from each protein (Table 4-5 and Table 4-6) compared to the previous method (Table 4-2 and Table 4-3). Similar to the results from the previous LC-MS analysis (Table 4-2 and Table 4-3), the IsaB protein was detected in the fraction from the cation-exchange column and the SdrD protein was detected in both fractions from the anion-exchange column (Table 4-5 and Table 4-6). Unfortunately, the time-frame of this project did not allow further experimental procedures.

Table 4-5: New S-Trap LC-MS detected candidate HBD-3-inducing proteins in the peak fractions produced from the anion-exchange chromatography

The following proteins were detected in fractions 9 from the anion-exchange column (Figure 4-10, graphs b and g). (Cover: percentage of peptide coverage of the total protein sequence; Pept: number of peptides that match the protein in the description; SE: percentage of similarity to *S. epidermidis* proteins; ns: no significant similarity to *S. epidermidis* proteins found)

fraction 9 from the anion-exchange column (graph b)				
Accession	**Cover (%)**	**Pept (%)**	**Description**	**SE (%)**
tr|A0A2T8C9R7|A0A2T8C9R7_STAAU	42	30	YSIRK-type signal peptide-containing protein	27
tr|A0A0H3K9U6|A0A0H3K9U6_STAAE	64	19	Ferrichrome ABC transporter lipoprotein	70
Q2G296|FTHS_STAA8	53	17	Formate-tetrahydrofolate ligase	92
O86488|SDRD_STAAE	**63**	**77**	**Serine-aspartate repeat-containing protein D**	**49**
tr|A0A0H3KH34|A0A0H3KH34_STAAE	74	19	Glutamyl-aminopeptidase	80
A6QFI1|CDR_STAAE	39	13	Coenzyme A disulfide reductase	76
tr|A0A0E1AIM5|A0A0E1AIM5_STAAU	79	15	Purine nucleoside phosphorylase DeoD-type	93
P64415|HUTH_STAAM	33	11	Histidine ammonia-lyase	84
Q5HCV3|PANB_STAAC	57	12	3-methyl-2-oxobutanoate hydroxymethyltransferase	80
tr|G0XY21|G0XY21_STAA8	22	8	Clumping factor A (Fragment)	89
tr|X5DSE7|X5DSE7_STAAU	71	8	6 7-dimethyl-8-ribityllumazine synthase	88
Q6GGT8|DBH_STAAR	64	7	DNA-binding protein HU	99
tr|A1KDX6|A1KDX6_STAAU	31	7	Immunoglobulin G binding protein A (Fragment)	37
tr|Q2FY66|Q2FY66_STAA8	17	7	Glucose-6-phosphate 1-dehydrogenase	90
tr|A0A1D4J4D7|A0A1D4J4D7_STAAU	39	7	γ-hemolysin component B	59
tr|A0A0Z0KFX7|A0A0Z0KFX7_STAAU	36	6	Putative heme-dependent peroxidase	82
tr|X5E0Y0|X5E0Y0_STAAU	24	6	GTP cyclohydrolase FolE2	90
Q6GI89|SYW_STAAR	24	6	Tryptophan-tRNA ligase	90
tr|A0A0E0VMI9|A0A0E0VMI9_STAA5	47	5	Single-stranded DNA-binding protein	86

Table 4-5 (Continued)

fraction 9 from the anion-exchange column (graph b)				
Accession	**Cover (%)**	**Pept (%)**	**Description**	**SE (%)**
tr|A0A0D1G9L3|A0A0D1G9L3_STAAU	14	5	Adenylosuccinate lyase	92
Q6GAU3|FABH_STAAS	22	4	3-oxoacyl-[acyl-carrier-protein] synthase 3	91
tr|A0A0H3KAB2|A0A0H3KAB2_STAAE	20	4	Amino-acid ABC transporter amino-acid-binding protein	82
tr|A0A122N575|A0A122N575_STAAU	12	4	2 3-bisphosphoglycerate-independent phosphoglycerate mutase	91
tr|A0A2I7Y801|A0A2I7Y801_STAAU	48	37	Hydrolase	74
Q6GHY9|DLDH_STAAR	16	3	Dihydrolipoyl dehydrogenase	96
tr|A0A2T9ULH0|A0A2T9ULH0_STAEP	26	3	Glucose-6-phosphate isomerase (Fragment)	100
tr|A0A0H3JW11|A0A0H3JW11_STAAW	17	3	Uncharacterised protein	73
tr|A0A0H2XHK0|A0A0H2XHK0_STAA3	12	3	Iron compound ABC transporter-binding protein SirA	35
fraction 9 from the anion-exchange column (graph g)				
Accession	**Cover (%)**	**Pept (%)**	**Description**	**SE (%)**
tr|A1KDX8|A1KDX8_STAAU	57	25	Immunoglobulin G binding protein A (Fragment)	40
tr|A0A0H3K9U6|A0A0H3K9U6_STAAE	29	10	Ferrichrome ABC transporter lipoprotein	70
tr|A0A0E1VN13|A0A0E1VN13_STAA3	41	9	γ-hemolysin component B	59
tr|A0A2I7Y801|A0A2I7Y801_STAAU	27	18	Fibrinogen-binding protein with hydrolase activity	74
tr|A0A0H2XGG1|A0A0H2XGG1_STAA3	20	8	6-phosphogluconate dehydrogenase decarboxylating	91
tr|A0A133PX21|A0A133PX21_STAAU	31	7	Glycerophosphodiester phosphodiesterase	43
tr|T1YE75|T1YE75_STAAU	41	6	Uncharacterised protein	43
O86488|SDRD_STAAE	**29**	**25**	**Serine-aspartate repeat-containing protein D**	**49**
tr|A0A2T8C9F8|A0A2T8C9F8_STAAU	56	3	Purine-nucleoside phosphorylase (Fragment)	97
Q2FJ56|Y569_STAA3	17	3	Putative heme-dependent peroxidase	82
tr|A0A0H3KH34|A0A0H3KH34_STAAE	12	3	Glutamyl-aminopeptidase	80

Table 4-6: New S-Trap LC-MS detected candidate HBD-3-inducing proteins in the peak fraction produced from the cation-exchange chromatography

The following proteins were detected in fraction 9 from the cation-exchange column (Figure 4-10, graph f). (Cover: percentage of peptide coverage of the total protein sequence; Pept: number of peptides that match the protein in the description; SE: percentage of similarity to *S. epidermidis* proteins; ns: no significant similarity to *S. epidermidis* proteins found)

fraction 9 from the cation-exchange column (graph f)				
Accession	**Cover (%)**	**Pept (%)**	**Description**	**SE (%)**
tr|A0A0H3JUJ6|A0A0H3JUJ6_STAAM	77	41	Glycerophosphoryl diester phosphodiesterase	70
tr|A0A142G9F1|A0A142G9F1_STAAU	30	28	N-acetylmuramoyl-L-alanine amidase	59
Q7A377|ISAB_STAAN	**68**	**26**	**Immunodominant staphylococcal antigen B**	**43**
tr|A0A0E1VN13|A0A0E1VN13_STAA3	59	18	γ-hemolysin component B	59
tr|Q2G0X9|Q2G0X9_STAA8	63	12	Uncharacterised protein	ns
tr|A0A0D1HN79|A0A0D1HN79_STAAU	61	12	Heme ABC transporter substrate-binding protein	25
tr|A1KDX8|A1KDX8_STAAU	45	11	Immunoglobulin G binding protein A (Fragment)	40
tr|A0A0U1MGQ8|A0A0U1MGQ8_STAAU	62	11	Uncharacterised protein	62
tr|A0A1D4CL80|A0A1D4CL80_STAAU	27	11	Lipoteichoic acid synthase	90
A7X659|SBI_STAA1	31	11	Immunoglobulin-binding protein Sbi	40
Q5HEI1|PHLC_STAAC	71	16	Phospholipase C	53
tr|A0A0H3KAM8|A0A0H3KAM8_STAAE	16	7	O-acetyltransferase OatA	66
tr|A0A2D1PJH6|A0A2D1PJH6_STAAU	44	7	Serine protease (Fragment)	35
tr|A0A0H2WXE4|A0A0H2WXE4_STAAC	10	7	Cell wall surface anchor family protein	52
tr|A0A0E1VIY6|A0A0E1VIY6_STAA3	39	6	Uncharacterised protein	55
tr|A0A0H2WZ01|A0A0H2WZ01_STAAC	16	6	Uncharacterised protein	ns
tr|A0A0E1VLD0|A0A0E1VLD0_STAA3	42	9	Thermonuclease	42
tr|A0A0H3KAB2|A0A0H3KAB2_STAAE	25	5	Amino-acid ABC transporter amino-acid-binding protein	82

Table 4-6 (Continued)

fraction 9 from the cation-exchange column (graph f)				
Accession	**Cover (%)**	**Pept (%)**	**Description**	**SE (%)**
tr|A0A1D4IBN5|A0A1D4IBN5_STAAU	27	4	Iron compound ABC uptake transporter substrate-binding protein	75
P52078|Y997_STAA8	21	4	Uncharacterised protein	77
Q5HJ48|LIP2_STAAC	30	14	Lipase 2	47
tr|A0A0D1JP07|A0A0D1JP07_STAAU	25	8	Leucotoxin LukD	ns
tr|A0A1D5AAC1|A0A1D5AAC1_STAAU	47	3	α/β hydrolase	69
tr|A0A0E1VJY8|A0A0E1VJY8_STAA3	27	3	Uncharacterised protein	76
tr|A0A0H3JW11|A0A0H3JW11_STAAW	16	3	Uncharacterised protein	73

Although only IsaB was detected in all fractions from the cation-exchange column (Table 4-2 and Table 4-6) and SdrD was detected in all fractions from the anion-exchange column (Table 4-3 and Table 4-5), some proteins also appeared in two or more fractions (Table 4-7). Even though none of the proteins detected by LC-MS can be excluded from the search, proteins that appear in several fractions that induced HBD-3 may be more likely to be implicated in the production of HBD-3 in keratinocytes.

Table 4-7: Proteins detected by LC-MS that are appear in two or more fractions of *S. aureus* supernatant

Protein	Fractions	Table
Thermonuclease	fractions 9 from cation-exchange column (graphs a and f)	Table 4-2 and Table 4-6
O-acetyltransferase		
Glycerophosphodiester phosphodiesterase	fraction 10 from cation-exchange column (graph d) and fraction 9 from anion-exchange column (graph g)	Table 4-2 and Table 4-5
Ferrichrome ABC transporter lipoprotein	fractions 9 from anion-exchange column (graphs b and g)	Table 4-5
6-phosphogluconate dehydrogenase decarboxylating		
Purine-nucleoside phosphorylase		
Putative heme-dependent peroxidase		
Glutamyl-aminopeptidase		
Amino-acid ABC transporter amino-acid-binding protein	fraction 9 from anion-exchange column (graph b) and fraction 9 from cation-exchange column (graph f)	Table 4-5 and Table 4-6
Iron compound ABC uptake transporter substrate-binding protein		
Hydrolase		
Immunoglobulin G binding protein A	fractions 9 from anion-exchange column (graphs b and g) and fraction 9 from cation-exchange column (graph f)	Table 4-5 and Table 4-6
γ-hemolysin component B		

4.3. Discussion

The work in the previous chapter of this thesis, as well as previous research, found that *S. aureus* supernatant contains factor(s) that can induce production of HBD-3 in keratinocytes (Figure 3-1, Figure 3-3, Figure 3-6 and Figure 3-8) (Menzies and Kenoyer, 2005; Midorikawa et al., 2003). The aim of this chapter was to further investigate and identify the *S. aureus* HBD-3-inducing factor(s), in order to examine the existence and expression patterns of these factor(s) in *S. epidermidis* and *S. aureus* that do not induce HBD-3 and to better understand the differential effect of staphylococci on HBD-3 production in keratinocytes and the role of *S. aureus* in AD pathogenesis. Other studies have also underlined that the investigation of *S. aureus* virulence factors, responsible for host inflammation, may help elucidate the role of *S. aureus* in pathogenesis and aggravation of AD (Cho et al., 2009; Ezepchuk et al., 1996; Herz et al., 1998). Although the HBD-3-inducing factor(s) could not be identified within the time-frame of this project, the available data suggest the involvement of heat-stable protein(s). Moreover, steps were taken towards detection of candidate proteins and approaches were proposed for further investigation of these *S. aureus* factor(s).

<u>The effect of the *S. aureus* Agr system on the induction of HBD-3 in keratinocytes</u>

Previous research has reported that upregulation of the Agr system leads to increased virulence (Lauderdale et al., 2009; Savage et al., 2013a) and conversely strains with an inactivation of *agr* show increased biofilm formation and decreased expression of secreted virulence factors (Boles and Horswill, 2008; Savage et al., 2013a). By contrast, this chapter showed that the strains with increased Agr activity (WV1 and NE1109) induced lower HBD-3, whereas the *agr* mutant (NE1532) induced higher HBD-3 compared to the parental strains (Figure 4-1), suggesting that reduced HBD-3 production may be associated with Agr upregulation.

Fibrinogen-binding proteins participate in *S. aureus* biofilm formation but can also induce cytokines and cause inflammatory responses (Edwards et al.,

2010; Shinji et al., 2011; McCourt et al., 2014; Reginald et al., 2011). Also, extracellular proteases have been shown to cleave virulence molecules in order to achieve autoregulation of the strain's own virulence. Therefore, virulence factors such as γ-hemolysin and PSMs may be more abundant in protease-null strains (e.g. those that have decreased Agr activity), increasing the likelihood that they would induce HBD-3 production in keratinocytes more than proteolytic strains (Kolar et al., 2013; Lindsay and Foster, 1999), supporting the findings in this work (Figure 4-1) (Savage et al., 2013a; Bose et al., 2013; BEI Resources, c2019). These results suggest that HBD-3-inducing factor(s) may be produced during the initial phases of *S. aureus* colonisation, before activation of the Agr system. Subsequently, activation of the Agr system would then change the bacterial global gene-expression profile and possibly downregulate expression of the HBD-3-inducing factor(s) or upregulate factors that degrade HBD-3 or HBD-3-inducing factor(s).

The *sigB* mutation of *S. aureus* WV1 results in increased Agr activity and a highly proteolytic secretome profile (Savage et al., 2013b; Wang and Tom, 2016; Boles and Horswill, 2008). Indeed, its supernatant was found to be toxic for keratinocytes (Figure 4-3) and WV1 strain did not induce HBD-3 production in keratinocytes (Figure 4-2), even after the addition of PIC (Figure 4-5 and Figure 4-6). Possibly the proteases of WV1 were not deactivated by the PIC. Nevertheless, although WV1 cultures did not induce HBD-3 (Figure 4-2), they induced IL-8 (Figure 4-4), suggesting that either WV1 proteases may degrade HBD-3 but not IL-8, or that these strains activate IL-8 through a pathway different to the HBD-3 one. Recognition patterns of keratinocytes may activate different immune molecules through different pathways (Dejean et al., 2012). Indeed, previous researchers reported that *S. aureus* serine proteases can induce IL-8 (Rudack et al., 2009) and that the expression of IL-8 is mediated by NF-κB (Dejean et al., 2012; Roebuck, 1999; Mukaida et al., 1994; Huang and McCance, 2002) or EGFR (Frankart et al., 2012; Lan et al., 2013), whereas *S. aureus* HBD-3 induction is mediated through phosphatidylinositol 3-kinase and MAPK, independently of NF-κB (Wanke et al., 2011). Alternative suggestions to test this idea in future work would be to stimulate keratinocytes with mixtures of *S. aureus* SH1000 and WV1 supernatant, in order to determine whether WV1 proteases can inhibit HBD-3-inducing SH1000

factor(s), or to perform *in vitro* stability assays of HBD-3 in the presence of WV1 supernatant in order to define whether WV1 proteases can directly degrade HBD-3. Analysis of HBD-3 mRNA expression by real-time reverse-transcription PCR and protein levels by ELISA or Western Blot, from both cytoplasmic and media fractions (Warters et al., 2009; Mekki et al., 2018), would then elucidate if the gene encoding HBD-3 is induced, if the protein is translated, or if it is subsequently degraded.

The HBD-3-inducing *S. aureus* factor(s) are heat-stable and proteinaceous

The fact that PrK completely abolished HBD-3 induction implies the existence of an essential proteinaceous component, responsible for this HBD-3 induction (Figure 4-9) (Daniel et al., 1996; Pinto et al., 1991; Soltis et al., 1979; Ebeling et al., 1974). Heating *S. aureus* supernatant showed only modest reduction in its ability to induce HBD-3 (Figure 4-9), indicating that at least one of the HBD-3-inducing factors is heat-stable, consistent with previous studies (Menzies and Kenoyer, 2005; Midorikawa et al., 2003).

Fractionation and characterisation of *S. aureus* supernatant

The LC-MS method identified proteins in the peak HBD-3-inducing fractions that were produced by ion-exchange chromatography (Table 4-2 and Table 4-3). The fact that none of the NTML strains with single transposon mutations tested (Table 4-4) showed a significant reduction in their HBD-3 induction (BEI Resources, c2019; Bose et al., 2013), suggests that either: none of these proteins can induce HBD-3; the mutations did not completely inhibit the activity of the corresponding protein; or HBD-3 induction in keratinocytes is a result of the synergistic effect of two or more proteins (Figure 4-11).

The IsaB protein that was detected in all fractions from the cation-exchange column (Table 4-2 and Table 4-6) and the SdrD protein that was detected in all fractions from the anion-exchange column (Table 4-3 and Table 4-5) were further analysed. The IsaB protein can be both secreted and cell-bound, which is in line with the finding of this thesis that live cells and filter-sterilised supernatant of *S. aureus* both induce HBD-3 production in keratinocytes

(Figure 3-1, Figure 3-6 and Figure 3-8) (Mackey-Lawrence et al., 2009). The IsaB protein is induced under various conditions, such as in the presence of human serum, under anaerobic conditions and during infections (Mackey-Lawrence and Jefferson, 2013). IsaB binds nucleic acids but its role in *S. aureus* virulence has not yet been elucidated (Mackey-Lawrence et al., 2009; Liu, P.-F. et al., 2015; Mackey-Lawrence and Jefferson, 2013; Fuchs et al., 2007). The SdrD protein, which belongs to the family of microbial surface components recognising adhesive matrix molecules (MSCRAMMs), is a cell-bound adhesive protein that can bind to extracellular host proteins (e.g. fibronectin, collagen) (Liu, H. et al., 2015), human squamous cells and keratinocytes *in vitro* (Askarian et al., 2016) and can increase virulence and survival of *S. aureus* (Askarian et al., 2017). Genetic variability of *sdrD* among *S. aureus* strains has been reported (Ajayi et al., 2018).

Lactococcus lactis strains expressing *sdrD* did not induce HBD-3 in keratinocytes (section 4.2.3.5). Recombinant IsaB protein induced HBD-3 only in concentrations above 20 µg/mL, which is substantially higher than the concentration of other *S. aureus* proteins released into the supernatant (e.g. α-hemolysin up to ~ 30 ng/mL and staphylococcal enterotoxin H up to ~ 700 ng/mL) (Su and Wong, 1996; Omoe et al., 2002; Monecke et al., 2014). HBD-3 was induced by IsaB in a dose-dependent manner and heat only had a modest effect on reducing its activity (Figure 4-12).

In addition to IsaB and SdrD, other proteins also appeared in two or more fractions (Table 4-7). Some proteins (e.g. amino-acid ABC transporter) were detected in fractions from both cation-exchange and anion-exchange columns (Table 4-7). Proteins are large molecules and although each protein has a defined isoelectric point, individual sites of the protein may have different charges that could allow them to potentially bind to both cation-exchange and anion-exchange columns, depending on the charge of the site of the protein that comes in contact with the column (He et al., 2002; Shire, 2015).

As *S. aureus* PSMs play a role in its virulence (Wang et al., 2007; Cheung et al., 2014), it was speculated that these proteins might induce HBD-3 in keratinocytes. However, no PSMs were detected by LC-MS. The fractions tested may have contained no PSMs or PSMs at a very low concentration.

Virulence factors detected by LC-MS worth investigating include the pore-forming proteins leukocidin D and γ-hemolysin (Nocadello et al., 2016; Vandenesch et al., 2012), the immunoglobulin-binding protein A (Falugi et al., 2013) and the fibrinogen-binding clumping factor A (Higgins et al., 2006), which are major *S. aureus* virulence factors, often implicated in skin and systemic infections (Herman-Bausier et al., 2018; Kong et al., 2016). Although many of the proteins identified in the staphylococcal supernatant are known to be involved in functions such as nutrient uptake, they can still play a role in *S. aureus* virulence. For instance, iron ABC uptake transporters are required to obtain iron, which is necessary for bacterial growth, as well as for expression of virulence factors (Dale et al., 2004; Speziali et al., 2006). Proteins of unknown function that were detected (uncharacterised proteins) are also worth studying (Table 4-2, Table 4-5 and Table 4-6).

Although testing the ability of IsaB and SdrD proteins to induce HBD-3 in keratinocytes is a sensible approach towards the identification of the *S. aureus* HBD-3-inducing factor(s), the experiments performed are not definitive. Even though the expression of genes cloned into the pMG36e vector is constitutive (Van de Guchte et al., 1989; Kim et al., 2009), it is not known if and in what concentration the SdrD protein is produced by lactococci and whether other lactococcal proteins expressed may affect the SdrD production. Purifying the SdrD protein might be preferable in order to test its effect on HBD-3 induction. With regards to the IsaB protein, as there were no reports in the literature regarding the physiologic concentration of IsaB produced by *S. aureus*, the IsaB concentrations used in this project were similar to the concentrations used in previous studies (Liu, P.-F. et al., 2015). Although it is unlikely that *S. aureus* produces IsaB in such high concentrations as those tested, the percentage of activity of the IsaB protein is unknown; therefore, the concentrations used might not correspond to the actual concentration of a fully-active protein. More research is required in order to optimise the experimental conditions. Moreover, it is possible that the synergistic action of IsaB with another *S. aureus* compound or with host molecules may increase the effect of IsaB on HBD-3 induction. Previous research has shown a synergistic effect of *S. aureus* peptidoglycan with lipopolysaccharide on the release of TNF-α and IL1-β in whole blood (Hadley et al., 2005) and with

lipoteichoic acid on the activation of NF-κB in macrophages (Schwandner et al., 1999).

In conclusion, the aim of this chapter, which was to further investigate and identify the *S. aureus* factor(s) that induce HBD-3 production in keratinocytes, was only partially achieved. Progress was made, as the results obtained showed that the HBD-3-inducing factor(s) are heat-stable and most likely proteinaceous. Candidate proteins were detected but further confirmation of these proteins is required.

5. Defining conditions to rectify staphylococcal dysbiosis: a preliminary evaluation of differing growth requirements for *S. aureus* and *S. epidermidis*

5.1. Introduction

Skin microbial dysbiosis has been found to be associated with AD (Salava and Lauerma, 2014; McPherson, 2016). The previous chapters (chapters 3 and 4) investigated the different response of keratinocytes to *S. epidermidis* and *S. aureus*, focusing on the AMP production by keratinocytes stimulated with staphylococci, since AMPs are an important skin defence mechanism against pathogens such as *S. aureus* (Midorikawa et al., 2003; Nakatsuji et al., 2017; Kong et al., 2012). Antibiotics prescribed in AD fail to restore skin microbial balance, as they act not only against pathogens but also against skin commensals (such as *S. epidermidis*), ultimately leading to reduced microbial diversity and increased *S. aureus* recolonisation (Kobayashi et al., 2015; Friedman and Goldman, 2011; Gilani et al., 2005; SanMiguel et al., 2017). This chapter sought to identify compounds that favour *S. epidermidis*, which is the most abundant skin commensal (Otto, 2009; Otto, 2012), or inhibit *S. aureus*, which is a leading cause of skin infections and is increased in the skin of the majority of AD patients (Tong et al., 2015; Kong et al., 2012; Otto, 2013; Bouvet et al., 2017; Baldry et al., 2018). This aim of this work was to contribute to the development of techniques that restore the microbial skin balance and improve the skin defence against pathogens.

Despite the molecular and genetic similarities between *S. epidermidis* and *S. aureus* (Ghebremedhin et al., 2008; Méric et al., 2015), both organisms display quite different phenotypes on human skin. *S. epidermidis* is the most abundant skin commensal (Otto, 2009; Otto, 2012) and usually causes infection only in immunocompromised people (Dong and Speer, 2014; Sabaté-Brescó et al., 2017). In contrast, *S. aureus* is much less abundant on healthy skin, is implicated in skin and systemic diseases and is found in more

than 90% of AD patients (Cogen et al., 2008; Danby, 2017; Abeck and Mempel, 1998; Baldry et al., 2018).

Known phenotypic differences between *S. aureus* and *S. epidermidis* are reflected mainly in their virulence. Although *S. epidermidis* carries genes encoding virulence factors such as PSMs, expression of cytolytic factors is lower compared to *S. aureus* (Cheung et al., 2010). Also, the most prominent virulence factor of *S. epidermidis* is biofilm formation (Sabaté-Brescó et al., 2017), whereas *S. aureus* displays a more aggressive profile, with increased expression of highly cytolytic virulence factors that trigger the immune response, such as superantigens (Bröker et al., 2016). The *coa* and *vWbp* genes, which are responsible for the coagulase-positive nature of *S. aureus* strains, have also been implicated in its pathogenicity (McAdow et al., 2012; Cheng et al., 2010), therefore also providing an explanation for the apparent lower virulence associated with coagulase-negative species (Von Eiff et al., 2002; Becker et al., 2014). Differences between the *S. epidermidis* and *S. aureus* Agr system, which plays a role in their virulence (Traber et al., 2008; Boles and Horswill, 2008), have also been reported (Vuong et al., 2003). Gene transfer between staphylococci and other bacteria may be the reason of *S. aureus* genes encoding virulence factors such as enterotoxins, not found in *S. epidermidis*, although phenotypic differences between the two species have not yet been fully elucidated (Gill et al., 2005). In addition, genes encoding factors that enhance the host immune system are expressed more in *S. epidermidis*, such as δ-toxin, which can increase the potency of host AMPs (Cogen et al., 2010a).

Although a causative relationship between the microbial dysbiosis and AD has not been proven so far (Paller et al., 2019; Lee et al., 2018), skin microbial balance is associated with AD pathogenesis (Salava and Lauerma, 2014). Imbalance of skin barrier components disrupts the skin barrier integrity and increases the susceptibility to pathogens (Agrawal and Woodfolk, 2014; Berdyshev et al., 2018; Imokawa and Ishida, 2014). Previous researchers reported imbalance of skin lipids in AD (Proksch et al., 2003; Gupta et al., 2007), including the presence of shorter chain length fatty acids and ceramides (Janssens et al., 2012; Park et al., 2012), an increase in cholesterol and a reduction in ceramide/cholesterol ratio (Di Nardo et al., 1998). An

increase in sphingomyelin can favour *S. aureus*, whereas decreased sphingosine, which is potent against *S. aureus*, is associated with increased *S. aureus* colonisation (Imokawa and Ishida, 2014; Brauweiler et al., 2014). Application of mixtures with the right proportion of ceramides, cholesterol and free fatty acids was found to improve the skin barrier integrity (Yang et al., 1995; Imokawa et al., 1989). In addition, application of a formula containing one or two ceramides and a fatty acid (behenic acid) on *ex vivo* skin preparations was found to improve the structure of stratum corneum (Berkers et al., 2018).

As *S. aureus* is the most common pathogen in AD (Baldry et al., 2018), the effect of fatty acids on *S. aureus* growth has been tested. Sphingosine, dihydrosphingosine, cis-6-hexadecenoic acid, lauric, capric and myristic acids have been shown to decrease *S. aureus* growth (Takigawa et al., 2005; Fischer et al., 2012; Bibel et al., 1992; Drake et al., 2008; Dayan and Wertz, 2011; Kelsey et al., 2006). The mode of action of fatty acids against *S. aureus* is primarily via membrane disruption. A secondary mode of action has been reported, whereby incorporation of these fatty acids into the *S. aureus* membrane can enhance host immune response (Parsons et al., 2012; Nguyen et al., 2016).

In the acidic pH of normal skin, ceramide production is optimal (pH 4.5-5.5) (Panther and Jacob, 2015; Rippke et al., 2002; Rippke et al., 2004) and colonisation of pathogens such as *S. aureus* and *Streptococcus pyogenes* is reduced (Grice and Segre, 2011; Whiting et al., 1996; Panther and Jacob, 2015). Use of moisturisers with specific acidity can improve the pH of the skin and the skin flora and reduce AD flares (Rippke et al., 2002; Lee et al., 2016; Rippke et al., 2004).

Creams containing oligosaccharides were found to increase *S. epidermidis* growth but they also increased *S. aureus* colonisation (Nodake et al., 2015; Hunt et al., 2012). This indicates the need to evaluate the effect of compounds and conditions against both *S. aureus* and *S. epidermidis*, in order to develop therapeutic schemes that target specifically *S. aureus* rather than the normal skin flora. For instance, sapienic acid was found to inhibit *S. aureus* to a

greater extent than *S. epidermidis*, due to differences in operon regulation (Moran et al., 2017).

A previous study focused on external skin application of *S. epidermidis*, rather than compounds that favour the growth of skin commensals. Although they reported improvement in skin barrier integrity, a direct association with increase of *S. epidermidis* growth could not be proven (Nodake et al., 2015). Moreover, another study noticed inhibition of *S. aureus* colonisation and improvement of skin microbial balance after application of commensal *Staphylococcus hominis* strains from healthy people on AD lesional skin (Nakatsuji et al., 2017).

Although previous research has examined the effect of various compounds and conditions on staphylococcal growth, most studies focused on the investigation of a single compound (Moran et al., 2017), or a single staphylococcal species (Kelsey et al., 2006) and the studies were performed mainly *in vivo* in healthy subjects or AD patients (Janssens et al., 2012; Di Nardo et al., 1998), or *ex vivo* in skin models (Berkers et al., 2018). This chapter involves the investigation of the growth conditions, phenotypic characterisation and susceptibility to various compounds for two *S. aureus* (USA300 and CS6-EEFIC) and two *S. epidermidis* strains (NCTC11047 and NCTC6513), in order to identify compounds that favour the growth of *S. epidermidis* over *S. aureus*. Defining conditions that rectify staphylococcal dysbiosis is a potential way to address the question as to whether dysbiosis drives AD. If AD resolves by rectifying dysbiosis, this would suggest that skin microbial dysbiosis is a key factor in AD pathogenesis.

5.2. Results

5.2.1. Characterisation of strain susceptibility to host molecules – MIC determination

In order to investigate the differing susceptibility of *S. aureus* and *S. epidermidis* to molecules present in the skin, the susceptibilities of 2 *S. aureus*

(USA300 and CS6-EEFIC) and 2 *S. epidermidis* (NCTC11047 and NCTC6513) strains to a small panel of compounds were tested using broth microdilutions and determination of MICs. Arachidonic, linoleic and γ-linolenic acids were chosen, as they are essential skin barrier components. Cis-UCA, was also chosen, as it is found in the stratum corneum, plays an important role in AD and can reduce *S. aureus* colonisation (Berbis et al., 1990; Horrobin, 1993; Kaneko et al., 2008; Miajlovic et al., 2010; Peltonen et al., 2014; Ong, 2012).

The results of the MIC determination experiments were consistent and showed no difference in the susceptibility of the staphylococcal strains to any of the molecules tested (Table 5-1). Therefore, it is unlikely that imbalance in any of these components would be the main cause of skin imbalance between *S. aureus* and *S. epidermidis* populations.

Table 5-1: MIC determination of *S. aureus* and *S. epidermidis* strains to lipid mediators

The susceptibility of four staphylococcal strains against a panel of molecules was tested. The procedure was performed twice for each agent, in duplicates. (MICs are in μg/mL)

	***S. aureus* USA300**	***S. aureus* CS6-EEFIC**	***S. epidermidis* NCTC11047**	***S. epidermidis* NCTC6513**
Arachidonic acid	64	64	64-128	128
Cis-UCA	> 256	> 256	> 256	> 256
Linoleic acid	> 512	> 512	> 512	> 512
γ-linolenic acid	64	64	64	64

5.2.2. Phenotypic characterisation

Slant cultures of *S. aureus* USA300, *S. aureus* CS6-EEFIC, *S. epidermidis* NCTC11047 and *S. epidermidis* NCTC6513 were sent to Biolog for phenotypic microarray analysis, in order to identify the factors favouring or restricting the growth of *S. epidermidis* and *S. aureus* (Biolog Inc, c2019).

Biolog investigated the metabolic pathways of the four staphylococcal strains along with ionic, osmotic and pH effects on their growth, as well as their

sensitivity to various sources (carbon, nitrogen, phosphorus, sulfur) and antimicrobials with different mechanisms of action. If the phenotype was strongly positive in a well, the cells respired actively, reducing a tetrazolium dye to display a strong colour. If the phenotype was weak or negative, respiration was slowed or halted and the result was a reduction in colour. The redox assay provided both amplification and precise quantitation of phenotypes. Incubation and recording of phenotypic data were performed automatically by the OmniLog instrument. To compare the phenotypes of two cell lines, one was recorded as a red tracing and one as a green tracing. These graphs were then overlaid by the bioinformatic software to detect differences. Areas of overlap were coloured yellow, whereas differences were highlighted as patches of red or green (Figure 5-1). The OmniLog PM software in conjunction with the OmniLog PM system and phenotype microarray panels was used by Biolog in order to analyse the data, export them in a variety of raw and processed forms, and generate reports (Biolog Inc, c2019).

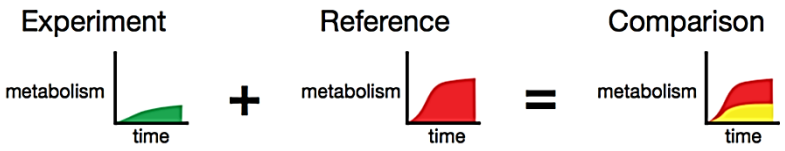


Figure 5-1: Comparison of the phenotypes of two cell lines

In order to compare the phenotypes of two cell lines, one is recorded as a red tracing and one as a green tracing. These graphs can then be overlaid and the areas of overlap are coloured yellow (Biolog Inc, c2019).

The phenotype microarray panels that were tested are available on Biolog website (Biolog Inc, c2019). The preliminary results from the phenotypic assay are shown in Appendices and supplemental material, 8.5. The compounds that favour the growth of *S. epidermidis* strains over *S. aureus* are shown in Table 5-2.

Table 5-2: Compounds that favour *S. epidermidis* growth according to the phenotypic characterisation

The following compounds were found to favour the growth of both *S. epidermidis* strains tested over both *S. aureus* strains. The underlined compounds were purchased to verify the results from the phenotypic characterisation with MICs.

Compounds that favour *S. epidermidis* growth over *S. aureus*			
L-alanine	D-alanine	Met-Asp	Leu-Met
D-aspartic acid	L-citrulline	Ala-Ala	Ala-Arg
L-homoserine	Leu-Ala	Val-Leu	Leu-Leu
Ala-Phe	Phe-Ala	Leu-Pro	Leu-Asp
Met-Leu	Ala-Lys	Met-Met	Gly-Phe
Ala-Leu	Ala-Pro	Ala-Met	Paromomycin
Ala-Thr	Arg-Ala	Sodium m-arsenite	Boric acid
Asp-Leu	Gly-Leu		

In order to verify these results, broth microdilutions and determination of MICs were performed for the underlined compounds of Table 5-2, using the four staphylococcal strains (*S. aureus* USA300, *S. aureus* CS6-EEFIC, *S. epidermidis* NCTC11047 and *S. epidermidis* NCTC6513). The culture medium that was used by Biolog was prepared, according to the information that was available on the website. This formula contained 100 mM NaCl, 30 mM triethanolamine HCl (pH 7.1), 5 mM NH_4Cl, 2 mM NaH_2PO_4, 0.25 mM Na_2SO_4, 0.05 mM $MgCl_2$, 1 mM KCl and 0.01% (v/v) tetrazolium violet. Where a nitrogen source was tested, NH_4Cl was omitted from the medium. However, media prepared in the laboratory failed to show staphylococcal growth. As an alternative, MHB-II was used and several concentrations were tested (Appendices and supplemental material, 8.5). Unfortunately, there was no difference in the susceptibility of *S. aureus* and *S. epidermidis* to any of the compounds tested.

5.3. Discussion

The aim of this chapter was to investigate differences in *S. aureus* and *S. epidermidis* growth conditions (Otto, 2009; Laborel-Préneron et al., 2015; Kong et al., 2012; Otto, 2012; Nowicka and Grywalska, 2018). The ultimate goal was to identify compounds that can contribute to improvement of the skin microbial balance and skin barrier integrity, which are dysregulated in AD (Leung and Bieber, 2003; Hata and Gallo, 2008; Wollina, 2017; Salava and Lauerma, 2014) and evaluate whether restoration of the skin flora can improve AD. Although the phenotypic characterisation identified compounds that favour the growth of *S. epidermidis* over *S. aureus*, subsequent laboratory experiments were unable to reproduce these differences in susceptibility of *S. aureus* and *S. epidermidis* to host molecules and amino-acids. The fact that these results could not be reproduced under similar but not identical experimental conditions indicates the phenotypic similarities of *S. aureus* and *S. epidermidis* and the need for deeper investigation of differences in gene regulation.

Staphylococcal susceptibility to host molecules

The susceptibility of *S. aureus* and *S. epidermidis* to skin lipids and cis-UCA was tested using broth microdilutions and determination of MICs. Although it has been reported that cis-UCA can reduce *S. aureus* colonisation (Kaneko et al., 2008; Miajlovic et al., 2010), previous work failed to show the killing capacity of cis-UCA against *S. aureus* (Rinaldi et al., 2006). Regarding skin lipids, the susceptibility of *S. aureus* to cis-9, cis-12 linoleic acid and trans-10, cis-12 conjugated (bio-converted) linoleic acid has been tested, with MIC values ranging between 50-100 μg/mL unlike this thesis, which found MIC values > 512 μg/mL. However, these experiments used TSB for bacterial growth, which does not conform to CLSI standards (Cockerill et al., 2012) and ethanol as a solvent, which may be toxic for staphylococci (Kelsey et al., 2006). In this thesis, sterile water was used as a solvent and MHB-II was used for bacterial growth, in accordance with the CLSI standards (Cockerill et al., 2012), which strengthens the validity of the results. Other work has shown that bio-converted linoleic acid displays an MIC value of 1500 μg/mL with *S.*

aureus, when the compound is dissolved in DMSO and Luria-Bertani media is used (Young Shin et al., 2005), which is again not in line with the CLSI standards (Cockerill et al., 2012). Although the experimental conditions, the form of the compound and its solvent, as well as the *S. aureus* strain used may affect the outcome, it seems that these compounds are not candidates for selective *S. aureus* killing, as both *S. aureus* and *S. epidermidis* showed similar resistance to skin lipids and cis-UCA (Table 5-1).

.

Phenotypic characterisation

Given that the compounds tested with broth microdilutions and determination of MICs did not show any difference in *S. aureus* versus *S. epidermidis* growth, a phenotypic assay was performed, which identified compounds that may show preferential growth of *S. epidermidis* over *S. aureus* (Table 5-2) (Biolog Inc, c2019). Most of these compounds were amino-acids (Table 5-2). Previous analyses of staphylococcal susceptibility to amino-acids is limited. One study reported that alanine can increase susceptibility of *S. aureus* to nisin (Chen et al., 2012). Another study found that L-aspartic acid and L-cysteine inhibited *S. aureus* biofilm formation (Yang et al., 2015). The medium used by Biolog was not sufficient for growth in subsequent tests, as further information such as the pH of the medium, temperature, incubation times, concentrations of the tested compounds and solvents used were not available. Therefore, different growth media were used but the results could not be reproduced. This may in part be attributed to the similarity of the *S. aureus* and *S. epidermidis* genome (Méric et al., 2015; Ghebremedhin et al., 2008), which results in many common phenotypic characteristics, such as growth conditions and suggests the need for investigation of the conditions that differently affect staphylococcal gene expression, colonisation and virulence.

Suggestions for further research

Enhancement or inhibition of staphylococcal growth by a compound may be optimal under specific conditions. It may be preferable to perform growth curves instead of MICs, in order to monitor the effect of a compound on

staphylococcal growth over time. Also, testing various conditions such as different pH, temperatures or concentrations of a compound may be useful in order to identify whether molecules such as skin lipids or compounds identified by phenotypic characterisation can enhance *S. epidermidis* growth. Using minimal media might also show a more profound difference between *S. epidermidis* and *S. aureus* growth in the presence of these compounds. As the killing potency of compounds can be affected by time and concentration (Knapp and Melly, 1986), another suggestion is to perform competition assays, in which both *S. epidermidis* and *S. aureus* are exposed together to a compound and use appropriate controls in order to evaluate staphylococcal growth with and without the presence of the compound. This experiment could also test whether a compound can increase the ability of one strain to dominate over the other.

Also, if there are indications that a compound has a beneficial effect for *S. epidermidis* or an inhibitory effect for *S. aureus*, it may be preferable to test this effect on 3D skin equivalent models that mimic healthy and AD skin, which could provide further information regarding staphylococcal growth and skin barrier integrity. The use of 3D skin equivalent models in order to study the effect of skin microbiome on the skin barrier has been proposed in the past and has several benefits (Popov et al., 2014; Rademacher et al., 2018). A skin equivalent is a simulation of the skin structure, with a differentiated and stratified epidermal layer. The environment can be easily modified according to study objectives and no major ethical issues arise (Rademacher et al., 2018). Previous research has also used skin equivalents in order to study the colonisation and interaction of *S. epidermidis* with skin (Holland et al., 2008), as well as the effect of *S. aureus* virulence factors on skin molecules, such as tight junction proteins (Popov et al., 2014; Basler et al., 2017).

In conclusion, there is a lot of ongoing research on methods to restore skin microbial balance and skin barrier integrity and improve AD. Some researchers investigated compounds such as skin lipids (Fischer et al., 2012; Takigawa et al., 2005), or conditions such as the pH (Lee et al., 2016), whereas others applied external *S. epidermidis* onto the skin (Nodake et al.,

2015) to enhance skin commensal bacteria. This project took into account the fact that *S. epidermidis* and *S. aureus* have many similarities (Méric et al., 2015) and the need to selectively enhance *S. epidermidis* and inhibit *S. aureus* growth and investigated compounds that favour *S. epidermidis* over *S. aureus* with phenotypic characterisation and MIC determination. Although compounds that favour *S. epidermidis* over *S. aureus* growth under some conditions *in vitro* were detected, further research is needed in order to confirm their effect and further test them *in vivo*. Defining conditions that rectify staphylococcal dysbiosis may help elucidate whether dysbiosis drives AD. If AD improves by restoring the skin microbial balance, this would suggest that skin microbial dysbiosis is a key factor in AD pathogenesis.

6. Conclusions

A diverse array of factors has been implicated in the pathogenesis of AD, including genetic predisposition, environmental factors, skin barrier integrity, immune response and the skin microbiome (McPherson, 2016; Thomsen, 2014). However, it has not yet been established whether any of these factors is the driving cause of AD and which factors might be secondary causes or indeed consequences of AD (McPherson, 2016; Salava and Lauerma, 2014; Thomsen, 2014). A deeper understanding of the association of these factors with AD, including the role of microbial dysbiosis in the immune response and inflammation of the skin, will contribute to the development of more targeted and effective treatments.

This project investigated the differential effect of *S. aureus* and *S. epidermidis* on keratinocytes, in order to clarify how the skin reacts to commensals and bacteria commonly involved in infection (Otto, 2009; Otto, 2012; Bouvet et al., 2017). It also attempted to identify compounds that favour the growth of *S. epidermidis* over *S. aureus*, based on the assumption that enhancing skin commensals and rectifying the skin microbial balance could improve AD, which would indicate that the skin microbiome might be a key factor in AD pathogenesis.

The results of the thesis showed a clear difference in the effect of *S. aureus* and *S. epidermidis* on HBD-3 production in keratinocytes, as HBD-3 was uniquely induced by *S. aureus* (Figure 3-1, Figure 3-3, Figure 3-6 and Figure 3-8). By contrast, induction of HBD-2 and LL-37 was similar for *S. epidermidis* and *S. aureus* (Figure 3-1, Figure 3-5 and Figure 3-6). The normal skin flora consists mainly of *S. epidermidis* (Prescott et al., 2017; Otto, 2009; Otto, 2012), whereas *S. aureus* is a leading cause of skin infections (Tong et al., 2015; Kong et al., 2012; Otto, 2013; Bouvet et al., 2017). HBD-3 is considered to be more potent against *S. aureus* compared to other AMPs (Joly et al., 2004; Midorikawa et al., 2003; Kisich et al., 2008). It therefore appears that the skin has evolved not only an effective strategy for defending itself against *S. aureus* (HBD-3 production), but also a means of triggering this response specifically to non-commensal staphylococci by detecting a cell component unique to *S. aureus*.

Identifying the *S. aureus* factor(s) that induce HBD-3 production in keratinocytes is of great importance in order to further investigate the mechanism of the different skin response to commensals and bacteria commonly involved in infection, such as *S. aureus* (Tong et al., 2015; Kong et al., 2012; Otto, 2013; Bouvet et al., 2017). Moreover, given the fact that *S. aureus* is the most common pathogen in AD (Baldry et al., 2018), it will help to explain the changes and defects of the pathways that result in reduced HBD-3 production in AD and to better understand why many AD patients inadequately control *S. aureus* infections. This knowledge could in turn contribute to the development of approaches to restore these defects and amplify HBD-3 expression, with a view to increasing skin defence and reducing infection.

All *S. epidermidis* strains tested failed to induce HBD-3 (Figure 3-3, Figure 3-6 and Figure 3-8). Results presented showed that *S. epidermidis* is unlikely to produce factor(s) that actively inhibit HBD-3 keratinocyte production (Figure 3-10), further pointing to the existence of *S. aureus*-specific HBD-3-inducing factor(s). It is not yet known whether there are one or more *S. aureus* factors that induce HBD-3 in keratinocytes. This project found that there is at least one essential proteinaceous factor that acts as an inducer of HBD-3 production (Figure 4-9) and detected candidate inducer proteins in the *S. aureus* supernatant using LC-MS (Table 4-2, Table 4-3, Table 4-5 and Table 4-6). Further tests to identify the HBD-3-inducing proteins were promising but not conclusive and more research is required in order to identify the *S. aureus* HBD-3-inducing factor(s). The ion-exchange chromatography successfully produced fractions of *S. aureus* supernatant. The improved LC-MS method that was utilised in the latter stages of the project should assist in this endeavour, since it proved capable of identifying a considerably greater number of proteins in the fractions tested. However, this also created the need to reduce the number of proteins contained in each fraction, e.g. by optimising the experimental conditions and increasing the protein concentration in the fractions to allow a second fractionation step, or by heating the *S. aureus* supernatant before fractionation to remove heat-unstable proteins, since the HBD-3-inducing factor(s) were found to be heat-stable (Figure 4-9).

This project also found that the *S. aureus agr* mutant strain (NE1532) induced higher HBD-3 in keratinocytes, whereas *S. aureus* strains with increased Agr activity (WV1 and NE1109) induced lower HBD-3 compared to the parental strains (Figure 4-1). These results suggest that HBD-3 may be mainly expressed during the initial colonisation stage of *S. aureus*, when the Agr system is inactive, whereas subsequent Agr activation, which increases the expression of host-damaging factors (Wang and Tom, 2016; Boles and Horswill, 2008), may possibly downregulate the expression of HBD-3-inducing factors or activate factors that degrade HBD-3 or the HBD-3-inducing factor(s). This observation proposes that the skin may have developed a means of activating the potent anti-*S. aureus* HBD-3 (Joly et al., 2004; Midorikawa et al., 2003; Kisich et al., 2008) in response to *S. aureus* components produced in the early stages of staphylococcal colonisation (Wang and Tom, 2016; Jenkins et al., 2015), to prevent further skin damage. It may be suggested that subsequent activation of the *S. aureus* Agr system after establishment of colonisation (Le and Otto, 2015), can possibly downregulate the HBD-3-inducing factor(s), to allow the bacterium to counter this HBD-3 response, evade the skin defence and spread to other host sites.

In order to investigate whether the ability to induce HBD-3 production in keratinocytes is universal to all *S. aureus* strains, 30 clinical isolates were screened in total. The majority of *S. aureus* strains induced production of HBD-3 in keratinocytes (Figure 3-6 and Figure 3-11), though 3 out of 30 clinical strains tested (1128, 805 and 602) did not (Figure 3-13). The *S. aureus* CS6-EEFIC strain induced very low HBD-3 compared to the rest of the strains (Figure 3-13). Failure to induce is probably due to the absence of the HBD-3-inducing factor(s) but could potentially also result from the degradation of HBD-3. These strains are all successful AD isolates (O'Neill et al., 2004; O'Neill et al., 2007a; O'Neill et al., 2007b). Previous research speculated that their success could be attributed to their toxin profile and antibiotic resistance (Stobberingh et al., 2012; O'Neill et al., 2007a; O'Neill et al., 2007b). However, the results presented in this thesis suggest that these strains are successful AD clones due to their ability to evade the main anti-*S. aureus* HBD-3 response in the skin (Figure 3-13 and Figure 3-14) (Dejean et al., 2012; Wanke et al., 2011). Although other AD isolates induced HBD-3, a

considerable proportion failed to induce (4 out of a total of 9 AD isolates tested) (Figure 3-11). Such strains are expected to be more likely to cause AD, since they can evade important host immune mechanisms (HBD-3 production).

Expression of HBD-3 is generally increased in AD compared to healthy skin but the increase is lower compared to other inflammatory diseases (e.g. psoriasis), due to increased Th_2 cytokine levels (Clausen et al., 2018; Harder et al., 2010; Clausen et al., 2016; Jin et al., 2014; Schonthaler et al., 2013). Previous research has found that other AMPs (HBD-2, psoriasin) are increased in lesional AD skin compared to healthy skin, whereas HBD-3 is found mainly in non-lesional AD skin (Clausen et al., 2018). Moreover, keratinocytes of AD patients have shown reduced ability to mobilise HBD-3 upon *S. aureus* stimulation compared to keratinocytes from healthy skin (Kisich et al., 2008). These findings suggest that HBD-3 may play an important role in AD, with reduced HBD-3 resulting in increased disease severity. This project found that HBD-3 production in response to *S. aureus* HBD-3-inducing factor(s) was reduced when IL-4 concentration increased in keratinocytes (Figure 3-15). Although this is a preliminary observation which requires further research, it is in line with the findings of this thesis regarding the role of HBD-3 in skin defence against *S. aureus*, as AD is characterised by failure to effectively counter *S. aureus* infection (Baldry et al., 2018; Chiu et al., 2009; David and Cambridge, 1986) and the AMP levels in AD do not seem to correspond to the degree of inflammation (Hata and Gallo, 2008; Ong et al., 2002; Nomura et al., 2003b; Lin et al., 2007). The inadequate immune response in AD may be caused by the absence of induction of HBD-3 by *S. aureus* or by the inability of the immune system to initiate effective HBD-3 expression.

This thesis proposed two approaches in order to correct dysbiosis, restore the normal skin flora and improve the skin defence against pathogens. The first approach refers to amplifying the host immune response and mainly the HBD-3 response against *S. aureus*, as analysed above. The second approach refers to favouring the growth of skin commensals. Phenotypic characterisation (Morandi et al., 2009) is a sensible method in order to study phenotypic differences between *S. epidermidis*, which is the most abundant

skin commensal (Prescott et al., 2017; Otto, 2009; Otto, 2012) and *S. aureus*, which is commonly implicated in skin infections (Tong et al., 2015; Kong et al., 2012; Otto, 2013; Bouvet et al., 2017) and identify compounds that favour colonisation of *S. epidermidis* over *S. aureus* (Table 5-2). This could contribute to the development of therapeutic techniques that enhance the growth of skin commensals and restore the skin microbial flora.

Overall, the data in this thesis have shown that *S. aureus* and *S. epidermidis* have different effects on the production of HBD-3 by keratinocytes and suggest that the skin may have developed mechanisms to selectively express HBD-3 upon exposure to *S. aureus* by detecting components unique to this bacterium. This may indicate the skin's ability to selectively initiate immune response against non-commensals by detecting specific components.

After identification of the HBD-3-inducing factor(s) of *S. aureus*, exogenous introduction of the encoding gene(s) to *S. epidermidis* would further confirm the function. Another suggestion for future research is the construction of *S. aureus* mutant strains with deletion of the gene encoding the HBD-3-inducing factor(s) or development of an inhibitor of the protein product(s) (Bae and Schneewind, 2006; Wright and Nair, 2012). This will help to examine whether more than one molecule can induce HBD-3 and if there is a main HBD-3-inducing factor along with other secondary factors. Pathways of HBD-3 induction by external pathogens have been described (Sorensen et al., 2005; Lai et al., 2010; Wanke et al., 2011). Identification of the *S. aureus* HBD-3-inducing factor(s) and expression patterns may help to define whether *S. aureus* strains that do not induce HBD-3: do not produce the corresponding factor(s); produce factors that may degrade HBD-3 or the HBD-3-inducing factor(s) in order to evade the host's immune system; or are too toxic and render keratinocytes unable to respond. This may explain why some *S. aureus* strains are more likely to be implicated in AD and other inflammatory diseases (Moriwaki et al., 2019) and why the skin reacts differently to commensal bacteria, such as *S. epidermidis*.

The skin microbiome plays an important role in skin defence (Prescott et al., 2017; Zipperer et al., 2016; Nakatsuji et al., 2017; Hannigan and Grice, 2013). If disruption of the skin microbial balance is proven to be able to trigger AD, or

if restoration of the skin flora can improve the symptoms, then this will further verify the key role of the skin microbiome in skin defence and AD pathogenesis. This will help to develop more effective therapeutic approaches for AD, such as cream preparations containing analogues of the HBD-3-inducing factor(s) in order to boost the HBD-3 production and the skin immune response, or compounds that favour the growth of skin commensals (*S. epidermidis*) at the expense of *S. aureus*, in order to enhance skin microbial balance and avoid the overuse of antibiotics and the side-effects of current treatments (Nowicki et al., 2015; Megna et al., 2016; Hong et al., 2011). The ultimate aim is to improve the quality of life of patients suffering from AD (McPherson, 2016) and potentially other diseases characterised by skin microbial imbalance, such as immunodeficiencies and diabetes mellitus (Oh et al., 2013; Thimmappaiah Jagadeesh et al., 2017).

7. References

Abe, R., Caaveiro, J.M., Kozuka-Hata, H., Oyama, M. and Tsumoto, K. 2012. Mapping ultra-weak protein-protein interactions between heme transporters of Staphylococcus aureus. *J Biol Chem.* **287**(20), pp.16477-16487.

Abeck, D. and Mempel, M. 1998. Staphylococcus aureus colonization in atopic dermatitis and its therapeutic implications. *British Journal of Dermatology.* **139**, pp.13-16.

Abiko, Y., Nishimura, M., Kusano, K., Yamazaki, M., Arakawa, T., Takuma, T. and Kaku, T. 2003. Upregulated expression of human β defensin-1 and -3 mRNA during differentiation of keratinocyte immortalized cell lines, HaCaT and PHK16-0b. *Journal of Dermatological Science.* **31**(3), pp.225-228.

Absolon, C.M., Cottrell, D., Eldridge, S.M. and Glover, M.T. 2003. Psychological disturbance in atopic eczema: the extent of the problem in school-aged children. *British Journal of Dermatology.* **137**(2), pp.241-245.

Abtin, A., Eckhart, L., Gläser, R., Gmeiner, R., Mildner, M. and Tschachler, E. 2010. The Antimicrobial Heterodimer S100A8/S100A9 (Calprotectin) Is Upregulated by Bacterial Flagellin in Human Epidermal Keratinocytes. *Journal of Investigative Dermatology.* **130**(10), pp.2423-2430.

Adams, S., O'Neill, D.W. and Bhardwaj, N. 2005. Recent advances in dendritic cell biology. *J Clin Immunol.* **25**(3), pp.177-188.

Agarwal, U.S., Besarwal, R.K., Gupta, R., Agarwal, P. and Napalia, S. 2014. Hand eczema. *Indian journal of dermatology.* **59**(3), pp.213-224.

Agrawal, G.K., Jwa, N.S., Lebrun, M.H., Job, D. and Rakwal, R. 2010. Plant secretome: unlocking secrets of the secreted proteins. *Proteomics.* **10**(4), pp.799-827.

Agrawal, R. and Woodfolk, J.A. 2014. Skin Barrier Defects in Atopic Dermatitis. *Current allergy and asthma reports.* **14**(5), pp.433-433.

Ahmad-Nejad, P., Mrabet-Dahbi, S., Breuer, K., Klotz, M., Werfel, T., Herz, U., Heeg, K., Neumaier, M. and Renz, H. 2004. The toll-like receptor 2 R753Q polymorphism defines a subgroup of patients with atopic dermatitis having severe phenotype. *J Allergy Clin Immunol.* **113**(3), pp.565-567.

Ahn, A.C. and Kaptchuk, T.J. 2011. Spatial Anisotropy Analyses of Subcutaneous Tissue Layer: Potential Insights into its Biomechanical Characteristics. *Journal of anatomy.* **219**(4), pp.515-524.

Ajayi, C., Åberg, E., Askarian, F., Sollid, J.U.E., Johannessen, M. and Hanssen, A.-M. 2018. Genetic variability in the sdrD gene in Staphylococcus aureus from healthy nasal carriers. *BMC Microbiology.* **18**(1), p34.

Alase, A., Seltmann, J., Werfel, T. and Wittmann, M. 2012. Interleukin-33 modulates the expression of human β-defensin 2 in human primary keratinocytes and may influence the susceptibility to bacterial superinfection in acute atopic dermatitis. *British Journal of Dermatology.* **167**(6), pp.1386-1389.

Albanesi, C., Fairchild, H.R., Madonna, S., Scarponi, C., De Pità, O., Leung, D.Y.M. and Howell, M.D. 2007. IL-4 and IL-13 Negatively Regulate TNF-α- and IFN-γ-

Induced β-Defensin Expression through STAT-6, Suppressor of Cytokine Signaling (SOCS)-1, and SOCS-3. *The Journal of Immunology.* **179**(2), pp.984-992.

Alowami, S., Qing, G., Emberley, E., Snell, L. and Watson, P.H. 2003. Psoriasin (S100A7) expression is altered during skin tumorigenesis. *BMC Dermatology.* **3**(1), pp.1-7.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. 1990. Basic local alignment search tool. *J Mol Biol.* **215**(3), pp.403-410.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**.

Aly, R., Maibach, H.I. and Shinefield, H.R. 1977. MIcrobial flora of atopic dermatitis. *Archives of Dermatology.* **113**(6), pp.780-782.

Amano, W., Nakajima, S., Kunugi, H., Numata, Y., Kitoh, A., Egawa, G., Dainichi, T., Honda, T., Otsuka, A., Kimoto, Y., Yamamoto, Y., Tanimoto, A., Matsushita, M., Miyachi, Y. and Kabashima, K. 2015. The Janus kinase inhibitor JTE-052 improves skin barrier function through suppressing signal transducer and activator of transcription 3 signaling. *J Allergy Clin Immunol.* **136**(3), pp.667-677.e667.

Amarbayasgalan, T., Takahashi, H., Dekio, I. and Morita, E. 2013. Interleukin-8 content in the stratum corneum as an indicator of the severity of inflammation in the lesions of atopic dermatitis. *Int Arch Allergy Immunol.* **160**(1), pp.63-74.

Anderson, J.M. and Van Itallie, C.M. 2009. Physiology and Function of the Tight Junction. *Cold Spring Harbor Perspectives in Biology.* **1**(2), pp.a002584-a002584.

Aochi, S., Tsuji, K., Sakaguchi, M., Huh, N., Tsuda, T., Yamanishi, K., Komine, M. and Iwatsuki, K. 2011. Markedly elevated serum levels of calcium-binding S100A8/A9 proteins in psoriatic arthritis are due to activated monocytes/macrophages. *Journal of the American Academy of Dermatology.* **64**(5), pp.879-887.

Arciola, C.R., Bustanji, Y., Conti, M., Campoccia, D., Baldassarri, L., Samorì, B. and Montanaro, L. 2003. Staphylococcus epidermidis–fibronectin binding and its inhibition by heparin. *Biomaterials.* **24**(18), pp.3013-3019.

Arikawa, J., Ishibashi, M., Kawashima, M., Takagi, Y., Ichikawa, Y. and Imokawa, G. 2002. Decreased Levels of Sphingosine, a Natural Antimicrobial Agent, may be Associated with Vulnerability of the Stratum Corneum from Patients with Atopic Dermatitis to Colonization by Staphylococcus aureus. *Journal of Investigative Dermatology.* **119**(2), pp.433-439.

Arrecubieta, C., Lee, M.-H., Macey, A., Foster, T.J. and Lowy, F.D. 2007. SdrF, a Staphylococcus epidermidis Surface Protein, Binds Type I Collagen. *Journal of Biological Chemistry.* **282**(26), pp.18767-18776.

Askarian, F., Ajayi, C., Hanssen, A.-M., van Sorge, N.M., Pettersen, I., Diep, D.B., Sollid, J.U.E. and Johannessen, M. 2016. The interaction between Staphylococcus aureus SdrD and desmoglein 1 is important for adhesion to host cells. *Scientific Reports.* **6**, p22134.

Askarian, F., Uchiyama, S., Valderrama, J.A., Ajayi, C., Sollid, J.U., van Sorge, N.M., Nizet, V., van Strijp, J.A. and Johannessen, M. 2017. Serine-Aspartate Repeat Protein D Increases Staphylococcus aureus Virulence and Survival in Blood. *Infect Immun.* **85**(1).

Atherton, D.J. 2003. Topical corticosteroids in atopic dermatitis. *BMJ (Clinical research ed.).* **327**(7421), pp.942-943.

Aubry-Damon, H., Soussy, C.-J. and Courvalin, P. 1998. Characterization of Mutations in the rpoB Gene That Confer Rifampin Resistance in Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy.* **42**(10), pp.2590-2594.

Azarian, T., Daum, R.S., Petty, L.A., Steinbeck, J.L., Yin, Z., Nolan, D., Boyle-Vavra, S., Hanage, W.P., Salemi, M. and David, M.Z. 2016. Intrahost Evolution of Methicillin-Resistant Staphylococcus aureus USA300 Among Individuals With Reoccurring Skin and Soft-Tissue Infections. *J Infect Dis.* **214**(6), pp.895-905.

Büttner, H., Mack, D. and Rohde, H. 2015. Structural basis of Staphylococcus epidermidis biofilm formation: mechanisms and molecular interactions. *Frontiers in cellular and infection microbiology.* **5**.

Bae, T. and Schneewind, O. 2006. Allelic replacement in Staphylococcus aureus with inducible counter-selection. *Plasmid.* **55**(1), pp.58-63.

Baker, B.S. 2006. The role of microorganisms in atopic dermatitis. *Clinical and Experimental Immunology.* **144**(1), pp.1-9.

Baldry, M., Nakamura, Y., Nakagawa, S., Frees, D., Matsue, H., Nunez, G. and Ingmer, H. 2018. Application of an agr-Specific Antivirulence Compound as Therapy for Staphylococcus aureus-Induced Inflammatory Skin Disease. *J Infect Dis.* **218**(6), pp.1009-1013.

Banner, M.A., Cunniffe, J.G., Macintosh, R.L., Foster, T.J., Rohde, H., Mack, D., Hoyes, E., Derrick, J., Upton, M. and Handley, P.S. 2007. Localized tufts of fibrils on Staphylococcus epidermidis NCTC 11047 are comprised of the accumulation-associated protein. *Journal of bacteriology.* **189**(7), pp.2793-2804.

Bao, L., Alexander, J.B., Zhang, H., Shen, K. and Chan, L.S. 2016. Interleukin-4 Downregulation of Involucrin Expression in Human Epidermal Keratinocytes Involves Stat6 Sequestration of the Coactivator CREB-Binding Protein. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research.* **36**(6), pp.374-381.

Barker, J.N., Jones, M.L., Mitra, R.S., Crockett-Torabe, E., Fantone, J.C., Kunkel, S.L., Warren, J.S., Dixit, V.M. and Nickoloff, B.J. 1991. Modulation of keratinocyte-derived interleukin-8 which is chemotactic for neutrophils and T lymphocytes. *The American Journal of Pathology.* **139**(4), pp.869-876.

Barnetson, R.S.C. and Rogers, M. 2002. Childhood atopic eczema. *BMJ : British Medical Journal.* **324**(7350), pp.1376-1379.

Basler, K., Galliano, M.F., Bergmann, S., Rohde, H., Wladykowski, E., Vidal, Y.S.S., Guiraud, B., Houdek, P., Schuring, G., Volksdorf, T., Caruana, A., Bessou-Touya, S., Schneider, S.W., Duplan, H. and Brandner, J.M. 2017. Biphasic influence of Staphylococcus aureus on human epidermal tight junctions. *Ann N Y Acad Sci.* **1405**(1), pp.53-70.

Basu, A. and Yap, M.-N.F. 2017. Disassembly of the Staphylococcus aureus hibernating 100S ribosome by an evolutionarily conserved GTPase. *Proceedings of the National Academy of Sciences of the United States of America.* **114**(39), pp.E8165-E8173.

Bath-Hextall, F.J., Birnie, A.J., Ravenscroft, J.C. and Williams, H.C. 2010. Interventions to reduce Staphylococcus aureus in the management of atopic eczema: an updated Cochrane review. *British Journal of Dermatology.* **163**, pp.12-26.

Baviera, G., Maiello, N. and Galli, E. 2015. Staphylococcus Aureus and Atopic Dermatitis: Which Came First, the Chicken or the Egg? *European Medical Journal of Dermatology.* **3**(1), pp.92-97.

Beaumont, P.E., Li, H.-N. and Davidson, D.J. 2013. LL-37: An Immunomodulatory Antimicrobial Host Defence Peptide. In: Hiemstra, P.S. and Zaat, S.A.J. eds. *Antimicrobial Peptides and Innate Immunity.* Basel: Springer Basel, pp.97-121.

Bechinger, B. and Gorr, S.U. 2017. Antimicrobial Peptides: Mechanisms of Action and Resistance. *J Dent Res.* **96**(3), pp.254-260.

Becker, K., Friedrich, A.W., Lubritz, G., Weilert, M., Peters, G. and Von Eiff, C. 2003. Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of Staphylococcus aureus isolated from blood and nasal specimens. *J Clin Microbiol.* **41**(4), pp.1434-1439.

Becker, K., Heilmann, C. and Peters, G. 2014. Coagulase-Negative Staphylococci. *Clinical Microbiology Reviews.* **27**(4), pp.870-926.

Becknell, B. and Spencer, J.D. 2016. A Review of Ribonuclease 7's Structure, Regulation, and Contributions to Host Defense. *International journal of molecular sciences.* **17**(3), pp.423-423.

BEI Resources. c2019. *Network on Antimicrobial Resistance in Staphylococcus aureus (NARSA). National Institute of Allergy and Infectious Diseases.* [Online]. Available from: https://www.beiresources.org/Catalog/BEIMutantBacteria/NR-46775.aspx

Bekker, L.G., Freeman, S., Murray, P.J., Ryffel, B. and Kaplan, G. 2001. TNF-alpha controls intracellular mycobacterial growth by both inducible nitric oxide synthase-dependent and inducible nitric oxide synthase-independent pathways. *J Immunol.* **166**(11), pp.6728-6734.

Belizário, J.E., Faintuch, J. and Garay-Malpartida, M. 2018. Gut Microbiome Dysbiosis and Immunometabolism: New Frontiers for Treatment of Metabolic Diseases. *Mediators of Inflammation.* **2018**, pp.1-12.

Berardesca, E. and Maibach, H.I. 1988. Racial differences in sodium lauryl sulphate induced cutaneous irritation: black and white. *Contact Dermatitis.* **18**(2), pp.65-70.

Berbis, P., Hesse, S. and Privat, Y. 1990. Essential fatty acids and the skin. *Allerg Immunol (Paris).* **22**(6), pp.225-231.

Berdyshev, E., Goleva, E., Bronova, I., Dyjack, N., Rios, C., Jung, J., Taylor, P., Jeong, M., Hall, C.F., Richers, B.N., Norquest, K.A., Zheng, T., Seibold, M.A. and Leung, D.Y. 2018. Lipid abnormalities in atopic skin are driven by type 2 cytokines. *JCI insight.* **3**(4), pe98006.

Berekmeri, A., Latzko, A., Alase, A., Macleod, T., Ainscough, J.S., Laws, P., Goodfield, M., Wright, A., Helliwell, P., Edward, S., Brown, G.D., Reid, D.M., Wenzel, J., Stacey, M. and Wittmann, M. 2018. Detection of IL-36gamma through noninvasive tape stripping reliably discriminates psoriasis from atopic eczema. *J Allergy Clin Immunol.* **142**(3), pp.988-991.e984.

Berkers, T., Visscher, D., Gooris, G.S. and Bouwstra, J.A. 2018. Topically Applied Ceramides Interact with the Stratum Corneum Lipid Matrix in Compromised Ex Vivo Skin. *Pharmaceutical research.* **35**(3), pp.48-48.

Bernard, F.X., Morel, F., Camus, M., Pedretti, N., Barrault, C., Garnier, J. and Lecron, J.C. 2012. Keratinocytes under Fire of Proinflammatory Cytokines: Bona Fide Innate Immune Cells Involved in the Physiopathology of Chronic Atopic Dermatitis and Psoriasis. *J Allergy (Cairo).* **2012**, pp.718-725.

Berube, B.J., Sampedro, G.R., Otto, M. and Bubeck Wardenburg, J. 2014. The psmα locus regulates production of Staphylococcus aureus alpha-toxin during infection. *Infection and immunity.* **82**(8), pp.3350-3358.

Bibel, D.J., Aly, R. and Shinefield, H.R. 1992. Antimicrobial Activity of Sphingosines. *Journal of Investigative Dermatology.* **98**(3), pp.269-273.

Bieber, T. 2010. Atopic Dermatitis. *Annals of Dermatology.* **22**(2), pp.125-137.

Biedermann, T., Skabytska, Y., Kaesler, S. and Volz, T. 2015. Regulation of T cell immunity in atopic dermatitis by microbes: The Yin and Yang of cutaneous inflammation. *Frontiers in Immunology.* **6**, p353.

Bierbaum, G., Gotz, F., Peschel, A., Kupke, T., van de Kamp, M. and Sahl, H.G. 1996. The biosynthesis of the lantibiotics epidermin, gallidermin, Pep5 and epilancin K7. *Antonie van Leeuwenhoek.* **69**(2), pp.119-127.

Biolog Inc. c2019. *Phenotype MicroArrays for Microbial Cells, Hayward CA.* [Online]. Available from: https://biolog.com/products-portfolio-overview/phenotype-microarrays-for-microbial-cells/

Bitoun, E., Chavanas, S., Irvine, A.D., Lonie, L., Bodemer, C., Paradisi, M., Hamel-Teillac, D., Ansai, S., Mitsuhashi, Y., Taieb, A., de Prost, Y., Zambruno, G., Harper, J.I. and Hovnanian, A. 2002. Netherton syndrome: disease expression and spectrum of SPINK5 mutations in 21 families. *J Invest Dermatol.* **118**(2), pp.352-361.

Błażewicz, I., Jaśkiewicz, M., Bauer, M., Piechowicz, L., Nowicki, R.J., Kamysz, W. and Barańska-Rybak, W. 2017. Decolonization of Staphylococcus aureus in patients with atopic dermatitis: a reason for increasing resistance to antibiotics? *Advances in Dermatology and Allergology.* **34**(6), pp.553-560.

Bochner, B.R. 2001. Phenotype MicroArrays for High-Throughput Phenotypic Testing and Assay of Gene Function. *Genome Research.* **11**(7), pp.1246-1255.

Bochner, B.R. 2009. Global phenotypic characterization of bacteria. *FEMS Microbiol Rev.* **33**(1), pp.191-205.

Boer, M., Duchnik, E., Maleszka, R. and Marchlewicz, M. 2016. Structural and biophysical characteristics of human skin in maintaining proper epidermal barrier function. *Advances in Dermatology and Allergology/Postępy Dermatologii i Alergologii.* **33**(1), pp.1-5.

Boguniewicz, M. and Leung, D.Y.M. 2006. 10. Atopic dermatitis. *Journal of Allergy and Clinical Immunology.* **117**(2, Supplement 2), pp.S475-S480.

Boles, B.R. and Horswill, A.R. 2008. agr-Mediated Dispersal of Staphylococcus aureus Biofilms. *PLOS Pathogens.* **4**(4), pe1000052.

Bose, J.L., Fey, P.D. and Bayles, K.W. 2013. Genetic tools to enhance the study of gene function and regulation in Staphylococcus aureus. *Appl Environ Microbiol.* **79**(7), pp.2218-2224.

Bouvet, C., Gjoni, S., Zenelaj, B., Lipsky, B.A., Hakko, E. and Uçkay, I. 2017. Staphylococcus aureus soft tissue infection may increase the risk of subsequent staphylococcal soft tissue infections. *International Journal of Infectious Diseases.* **60**, pp.44-48.

Boyle-Vavra, S. and Daum, R.S. 2007. Community-acquired methicillin-resistant Staphylococcus aureus: the role of Panton-Valentine leukocidin. *Lab Invest.* **87**(1), pp.3-9.

Brüssow, H. 2016. Turning the inside out: the microbiology of atopic dermatitis. *Environmental Microbiology.* **18**(7), pp.2089-2102.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry.* **72**(1), pp.248-254.

Brandner, J., Zorn-Kruppa, M., Yoshida, T., Moll, I., Beck, L. and De Benedetto, A. 2015. Epidermal tight junctions in health and disease. **3**(1-2), pe974451.

Brandt, E.B. and Sivaprasad, U. 2011. Th2 Cytokines and Atopic Dermatitis. *Journal of clinical & cellular immunology.* **2**(3), p110.

Brandtzaeg, P., Gabrielsen, T.-Ø., Dale, I., Müller, F., Steinbakk, M. and Fagerhol, M.K. 1995. The Leucocyte Protein L1 (Calprotectin): A Putative Nonspecific Defence Factor at Epithelial Surfaces. In: Mestecky, J., et al. eds. *Advances in Mucosal Immunology: Part A.* Boston, MA: Springer US, pp.201-206.

Brauweiler, A.M., Goleva, E. and Leung, D.Y.M. 2014. Th2 cytokines increase Staphylococcus aureus alpha toxin-induced keratinocyte death through the signal transducer and activator of transcription 6 (STAT6). *J Invest Dermatol.* **134**(8), pp.2114-2121.

Brauweiler, A.M., Goleva, E. and Leung, D.Y.M. 2016. Interferon-γ Protects from Staphylococcal Alpha Toxin-Induced Keratinocyte Death through Apolipoprotein L1. *Journal of Investigative Dermatology.* **136**(3), pp.658-664.

Brennan, M.P., Loughman, A., Devocelle, M., Arasu, S., Chubb, A.J., Foster, T.J. and Cox, D. 2009. Elucidating the role of Staphylococcus epidermidis serine-aspartate repeat protein G in platelet activation. *J Thromb Haemost.* **7**(8), pp.1364-1372.

Breuer, K., Häussler, S., Kapp, A. and Werfel, T. 2002. Staphylococcus aureus: colonizing features and influence of an antibacterial treatment in adults with atopic dermatitis. *British Journal of Dermatology.* **147**(1), pp.55-61.

Breuer, K., Kohlrautz, V., Kempe, K., Kapp, A. and Werfel, T. 2004. Staphylococcal alpha toxin induces IFN-γ in skin and blood derived T cells of patients with atopic dermatitis. *Journal of Allergy and Clinical Immunology.* **113**(2), pS96.

Breuer, K., Wittmann, M., Bosche, B., Kapp, A. and Werfel, T. 2000. Severe atopic dermatitis is associated with sensitization to staphylococcal enterotoxin B (SEB). *Allergy.* **55**(6), pp.551-555.

Breuer, K., Wittmann, M., Kempe, K., Kapp, A., Mai, U., Dittrich-Breiholz, O., Kracht, M., Mrabet-Dahbi, S. and Werfel, T. 2005. Alpha-toxin is produced by skin colonizing

Staphylococcus aureus and induces a T helper type 1 response in atopic dermatitis. *Clinical & Experimental Allergy.* **35**(8), pp.1088-1095.

Bridgett, C., Norén, P. and Staughton, R.C.D. 1996. *Atopic Skin Disease: A manual for practitioners.* Petersfield: Wrightson Biomedical.

Bröker, B.M., Mrochen, D. and Péton, V. 2016. The T Cell Response to Staphylococcus aureus. *Pathogens (Basel, Switzerland).* **5**(1), p31.

Broome, A.-M., Ryan, D. and Eckert, R.L. 2003. S100 Protein Subcellular Localization During Epidermal Differentiation and Psoriasis. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society.* **51**(5), pp.675-685.

Brown, S.J. and Irwin-Mclean, W.H. 2009. Eczema Genetics: Current State of Knowledge and Future Goals. *Journal of Investigative Dermatology.* **129**(3), pp.543-552.

Brunner, P.M. and Guttman-Yassky, E. 2019. Racial differences in atopic dermatitis. *Annals of Allergy, Asthma & Immunology.* **122**(5), pp.449-455.

Brunner, P.M., Guttman-Yassky, E. and Leung, D.Y. 2017. The immunology of atopic dermatitis and its reversibility with broad-spectrum and targeted therapies. *J Allergy Clin Immunol.* **139**(4s), pp.S65-s76.

Bunikowski, R., Mielke, M.E., Skarabis, H., Worm, M., Anagnostopoulos, I., Kolde, G., Wahn, U. and Renz, H. 2000. Evidence for a disease-promoting effect of Staphylococcus aureus-derived exotoxins in atopic dermatitis. *J Allergy Clin Immunol.* **105**(4), pp.814-819.

Burian, M. and Schittek, B. 2015. The secrets of dermcidin action. *Int J Med Microbiol.* **305**(2), pp.283-286.

Byrd, A.L., Belkaid, Y. and Segre, J.A. 2018. The human skin microbiome. *Nature Reviews Microbiology.* **16**(3), pp.143-155.

Cacioppo, J.T., Berntson, G.G., Malarkey, W.B., Kiecolt-Glaser, J.K., Sheridan, J.F., Poehlmann, K.M., Burleson, M.H., Ernst, J.M., Hawkley, L.C. and Glaser, R. 1998. Autonomic, neuroendocrine, and immune responses to psychological stress: the reactivity hypothesis. *Ann N Y Acad Sci.* **840**, pp.664-673.

Calder, P.C. 2006. Abnormal fatty acid profiles occur in atopic dermatitis but what do they mean? *Clin Exp Allergy.* **36**(2), pp.138-141.

Calder, P.C. and Burdge, G.C. 2012. Chapter 1 - Fatty acids. In: Nicolaou, A. and Kokotos, G. eds. *Bioactive Lipids.* Woodhead Publishing, pp.1-36.

Calder, P.C. and Miles, E.A. 2000. Fatty acids and atopic disease. *Pediatr Allergy Immunol.* **11**, pp.29–36.

Cameron, M. and Kelvin, D. 2013. Cytokines, Chemokines and Their Receptors. *Madame Curie Bioscience Database. Austin (TX): Landes Bioscience.*

Campbell, D.E. and Kemp, A.S. 1997. Proliferation and production of interferon-gamma (IFN-gamma) and IL-4 in response to Staphylococcus aureus and Staphylococcal superantigen in childhood atopic dermatitis. *Clinical and experimental immunology.* **107**(2), pp.392-397.

Candi, E., Oddi, S., Terrinoni, A., Paradisi, A., Ranalli, M., Finazzi-Agró, A. and Melino, G. 2001. Transglutaminase 5 Cross-links Loricrin, Involucrin, and Small Proline-rich Proteins in Vitro. *Journal of Biological Chemistry.* **276**(37), pp.35014-35023.

Candi, E., Schmidt, R. and Melino, G. 2005. The cornified envelope: a model of cell death in the skin. *Nat Rev Mol Cell Biol.* **6**(4), pp.328-340.

Carding, S., Verbeke, K., Vipond, D.T., Corfe, B.M. and Owen, L.J. 2015. Dysbiosis of the gut microbiota in disease. *Microbial ecology in health and disease.* **26**, pp.26191-26191.

Carnes, E.C., Lopez, D.M., Donegan, N.P., Cheung, A., Gresham, H., Timmins, G.S. and Brinker, C.J. 2010. Confinement-induced quorum sensing of individual Staphylococcus aureus bacteria. *Nature chemical biology.* **6**(1), pp.41-45.

Carr, W.W. 2013. Topical calcineurin inhibitors for atopic dermatitis: review and treatment recommendations. *Paediatr Drugs.* **15**(4), pp.303-310.

Cevikbas, F., Wang, X., Akiyama, T., Kempkes, C., Savinko, T., Antal, A., Kukova, G., Buhl, T., Ikoma, A., Buddenkotte, J., Soumelis, V., Feld, M., Alenius, H., Dillon, S.R., Carstens, E., Homey, B., Basbaum, A. and Steinhoff, M. 2014. A sensory neuron-expressed IL-31 receptor mediates T helper cell-dependent itch: Involvement of TRPV1 and TRPA1. *J Allergy Clin Immunol.* **133**(2), pp.448-460.

Chambers, H.F. and Deleo, F.R. 2009. Waves of resistance: Staphylococcus aureus in the antibiotic era. *Nat Rev Microbiol.* **7**(9), pp.629-641.

Chan, A. and Mauro, T. 2011. Acidification in the epidermis and the role of secretory phospholipases. *Dermatoendocrinol.* **3**(2), pp.84-90.

Chapkin, R.S., Ziboh, V.A., Marcelo, C.L. and Voorhees, J.J. 1986. Metabolism of essential fatty acids by human epidermal enzyme preparations: evidence of chain elongation. *Journal of Lipid Research.* **27**(9), pp.945-954.

Chavakis, T., Hussain, M., Kanse, S.M., Peters, G., Bretzel, R.G., Flock, J.-I., Herrmann, M. and Preissner, K.T. 2002. Staphylococcus aureus extracellular adherence protein serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes. *Nat Med.* **8**(7), pp.687-693.

Chen, L.-C., Chiang, W.-D., Chen, W.-C., Chen, H.-H., Huang, Y.-W., Chen, W.-J. and Lin, S.-B. 2012. Influence of alanine uptake on Staphylococcus aureus surface charge and its susceptibility to two cationic antibacterial agents, nisin and low molecular weight chitosan. *Food Chemistry.* **135**(4), pp.2397-2403.

Chen, L., Lin, S.X., Agha-Majzoub, R., Overbergh, L., Mathieu, C. and Chan, L.S. 2006. CCL27 is a critical factor for the development of atopic dermatitis in the keratin-14 IL-4 transgenic mouse model. *Int Immunol.* **18**(8), pp.1233-1242.

Chen, X., Niyonsaba, F., Ushio, H., Okuda, D., Nagaoka, I., Ikeda, S., Okumura, K. and Ogawa, H. 2005. Synergistic effect of antibacterial agents human β-defensins, cathelicidin LL-37 and lysozyme against Staphylococcus aureus and Escherichia coli. *Journal of Dermatological Science.* **40**(2), pp.123-132.

Cheng, A.G., McAdow, M., Kim, H.K., Bae, T., Missiakas, D.M. and Schneewind, O. 2010. Contribution of Coagulases towards Staphylococcus aureus Disease and Protective Immunity. *PLOS Pathogens.* **6**(8), pe1001036.

Cheung, G.Y., Rigby, K., Wang, R., Queck, S.Y., Braughton, K.R., Whitney, A.R., Teintze, M., DeLeo, F.R. and Otto, M. 2010. Staphylococcus epidermidis strategies to avoid killing by human neutrophils. *PLoS Pathog.* **6**(10), pe1001133.

Cheung, G.Y.C., Joo, H.-S., Chatterjee, S.S. and Otto, M. 2014. Phenol-soluble modulins – critical determinants of staphylococcal virulence. *FEMS Microbiology Reviews.* **38**(4), pp.698-719.

Chieosilapatham, P., Ogawa, H. and Niyonsaba, F. 2017. Current insights into the role of human beta-defensins in atopic dermatitis. *Clin Exp Immunol.* **190**(2), pp.155-166.

Chiu, L.S., Chow, V.C.Y., Ling, J.M.L. and Hon, K.L. 2010. Staphylococcus aureus Carriage in the Anterior Nares of Close Contacts of Patients With Atopic Dermatitis. *Archives of Dermatology.* **146**(7).

Chiu, L.S., Ho, M.S.L., Hsu, L.Y. and Tang, M.B.Y. 2009. Prevalence and molecular characteristics of Staphylococcus aureus isolates colonizing patients with atopic dermatitis and their close contacts in Singapore. *British Journal of Dermatology.* **160**(5), pp.965-971.

Cho, J.W., Cho, S.Y. and Lee, K.S. 2009. Roles of SEA-expressing Staphylococcus aureus, isolated from an atopic dermatitis patient, on expressions of human beta-defensin-2 and inflammatory cytokines in HaCaT cells. *Int J Mol Med.* **23**(3), pp.331-335.

Cho, S.H., Strickland, I., Boguniewicz, M. and Leung, D.Y.M. 2001. Fibronectin and fibrinogen contribute to the enhanced binding of Staphylococcus aureus to atopic skin. *Journal of Allergy and Clinical Immunology.* **108**(2), pp.269-274.

Christophers, E. and Henseler, T. 1987. Contrasting disease patterns in psoriasis and atopic dermatitis. *Arch Dermatol Res.* **279 Suppl**, pp.S48-51.

Chung, W.O. and Dale, B.A. 2004. Innate immune response of oral and foreskin keratinocytes: utilization of different signaling pathways by various bacterial species. *Infect Immun.* **72**(1), pp.352-358.

Chung, W.O. and Dale, B.A. 2008. Differential utilization of nuclear factor-kappaB signaling pathways for gingival epithelial cell responses to oral commensal and pathogenic bacteria. *Oral Microbiol Immunol.* **23**(2), pp.119-126.

Clark, R. and Kupper, T. 2005. Old Meets New: The Interaction Between Innate and Adaptive Immunity. *Journal of Investigative Dermatology.* **125**(4), pp.629-637.

Clarke, S.R., Mohamed, R., Bian, L., Routh, A.F., Kokai-Kun, J.F., Mond, J.J., Tarkowski, A. and Foster, S.J. 2007. The Staphylococcus aureus Surface Protein IsdA Mediates Resistance to Innate Defenses of Human Skin. *Cell Host Microbe.* **1**(3), pp.199-212.

Clausen, M.-L., Slotved, H.C., Krogfelt, K.A. and Agner, T. 2018. Measurements of AMPs in stratum corneum of atopic dermatitis and healthy skin–tape stripping technique. *Scientific Reports.* **8**(1).

Clausen, M.-L., Slotved, H.C., Krogfelt, K.A., Andersen, P.S. and Agner, T. 2016. In vivo expression of antimicrobial peptides in atopic dermatitis. *Experimental Dermatology.* **25**(1), pp.3-9.

Coates, M., Blanchard, S. and MacLeod, A.S. 2018. Innate antimicrobial immunity in the skin: A protective barrier against bacteria, viruses, and fungi. *PLOS Pathogens.* **14**(12), pe1007353.

Coates, R., Moran, J. and Horsburgh, M.J. 2014. Staphylococci: colonizers and pathogens of human skin. *Future Microbiol.* **9**(1), pp.75-91.

Cockerill, F.R., Wickler, M.A., Alder, J., Dudlay, M.N., Eliopoulos, G.M., Ferraro, M.J., Hardy, D.J., Andhecht, D.W., Hindler, J.A., Patel, J.B., Powel, M., Swenson, J.M., Thomprron, J.B., Traczewski, M.M., Turnidge, J.A., Weinstein, M.P. and Zimmer, B.L. 2012. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. 11th edition. CLSI standard (M07-A9). *Wayne: Clinical and Laboratory Standards Institute.*

Coderch, L., Lopez, O., de la Maza, A. and Parra, J.L. 2003. Ceramides and skin function. *Am J Clin Dermatol.* **4**(2), pp.107-129.

Cogen, A.L., Nizet, V. and Gallo, R.L. 2008. Skin microbiota: a source of disease or defence? *The British journal of dermatology.* **158**(3), pp.442-455.

Cogen, A.L., Yamasaki, K., Muto, J., Sanchez, K.M., Crotty Alexander, L., Tanios, J., Lai, Y., Kim, J.E., Nizet, V. and Gallo, R.L. 2010a. Staphylococcus epidermidis Antimicrobial δ-Toxin (Phenol-Soluble Modulin-γ) Cooperates with Host Antimicrobial Peptides to Kill Group A Streptococcus. *PLoS ONE.* **5**(1), pe8557.

Cogen, A.L., Yamasaki, K., Sanchez, K.M., Dorschner, R.A., Lai, Y., MacLeod, D.T., Torpey, J.W., Otto, M., Nizet, V., Kim, J.E. and Gallo, R.L. 2010b. Selective antimicrobial action is provided by phenol-soluble modulins derived from Staphylococcus epidermidis, a normal resident of the skin. *The Journal of investigative dermatology.* **130**(1), pp.192-200.

Colombo, I., Sangiovanni, E., Maggio, R., Mattozzi, C., Zava, S., Corbett, Y., Fumagalli, M., Carlino, C., Corsetto, P.A., Scaccabarozzi, D., Calvieri, S., Gismondi, A., Taramelli, D. and Dell'Agli, M. 2017. HaCaT Cells as a Reliable In Vitro Differentiation Model to Dissect the Inflammatory/Repair Response of Human Keratinocytes. *Mediators of Inflammation.* **2017**, pp.1-12.

Connolly, J., Boldock, E., Prince, L.R., Renshaw, S.A., Whyte, M.K. and Foster, S.J. 2017. Identification of Staphylococcus aureus Factors Required for Pathogenicity and Growth in Human Blood. *Infect Immun.* **85**(11).

Cookson, W.O.C.M. and Moffatt, M.F. 2002. The genetics of atopic dermatitis. *Journal of Allergy and Clinical Immunology.* **2**(5), pp.383-387.

Coondoo, A. 2011. Cytokines in dermatology - a basic overview. *Indian journal of dermatology.* **56**(4), pp.368-374.

Corbin, B.D., Seeley, E.H., Raab, A., Feldmann, J., Miller, M.R., Torres, V.J., Anderson, K.L., Dattilo, B.M., Dunman, P.M., Gerads, R., Caprioli, R.M., Nacken, W., Chazin, W.J. and Skaar, E.P. 2008. Metal Chelation and Inhibition of Bacterial Growth in Tissue Abscesses. *Science.* **319**(5865), pp.962-965.

Cork, M.J., Danby, S.G., Vasilopoulos, Y., Hadgraft, J., Lane, M.E., Moustafa, M., Guy, R.H., MacGowan, A.L., Tazi-Ahnini, R. and Ward, S.J. 2009. Epidermal Barrier Dysfunction in Atopic Dermatitis. *J Invest Dermatol.* **129**(8), pp.1892-1908.

Correale, C.E., Walker, C., Murphy, L. and Craig, T.J. 1999. Atopic dermatitis: a review of diagnosis and treatment. *Am Fam Physician.* **60**(4), pp.1191-1198, 1209-1110.

Coskun, O. 2016. Separation techniques: Chromatography. *Northern clinics of Istanbul.* **3**(2), pp.156-160.

Costello, E.K., Lauber, C.L., Hamady, M., Fierer, N., Gordon, J.I. and Knight, R. 2009. Bacterial Community Variation in Human Body Habitats Across Space and Time. *Science.* **326**(5960), pp.1694-1697.

Cowland, J.B., Johnsen, A.H. and Borregaard, N. 1995. hCAP-18, a cathelin/pro-bactenecin-like protein of human neutrophil specific granules. *FEBS Lett.* **368**(1), pp.173-176.

Cox, K.L., Devanarayan, V., Kriauciunas, A., Manetta, J., Montrose, C. and Sittampalam, S. 2004. Immunoassay Methods. In: Sittampalam, G.S., et al. eds. *Assay Guidance Manual.* Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences.

Crouch, S.P.M., Kozlowski, R., Slater, K.J. and Fletcher, J. 1993. The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *Journal of Immunological Methods.* **160**(1), pp.81-88.

Cunden, L.S., Brophy, M.B., Rodriguez, G.E., Flaxman, H.A. and Nolan, E.M. 2017. Biochemical and Functional Evaluation of the Intramolecular Disulfide Bonds in the Zinc-Chelating Antimicrobial Protein Human S100A7 (Psoriasin). *Biochemistry.* **56**(43), pp.5726-5738.

Dahl, M.V. 2012. Stem cells and the skin. *Journal of Cosmetic Dermatology.* **11**(4), pp.297-306.

Dale, S.E., Doherty-Kirby, A., Lajoie, G. and Heinrichs, D.E. 2004. Role of Siderophore Biosynthesis in Virulence of Staphylococcus aureus: Identification and Characterization of Genes Involved in Production of a Siderophore. *Infection and Immunity.* **72**(1), pp.29-37.

Danby, S.G. 2017. Staphylococcus aureus in atopic dermatitis: a diverse problem? *British Journal of Dermatology.* **177**(5), pp.1164-1164.

Daniel, R.M., Dines, M. and Petach, H.H. 1996. The denaturation and degradation of stable enzymes at high temperatures. *Biochemical Journal.* **317**(Pt 1), pp.1-11.

Dastgheyb, S.S., Villaruz, A.E., Le, K.Y., Tan, V.Y., Duong, A.C., Chatterjee, S.S., Cheung, G.Y.C., Joo, H.-S., Hickok, N.J. and Otto, M. 2015. Role of Phenol-Soluble Modulins in Formation of Staphylococcus aureus Biofilms in Synovial Fluid. *Infection and Immunity.* **83**(7), pp.2966-2975.

David, T.J. and Cambridge, G.C. 1986. Bacterial infection and atopic eczema. *Archives of disease in childhood.* **61**(1), pp.20-23.

Dayan, N. and Wertz, P.W. 2011. *Innate Immune System of Skin and Oral Mucosa: Properties and Impact in Pharmaceutics, Cosmetics, and Personal Care Products.* Wiley.

De Benedetto, A., Agnihothri, R., McGirt, L.Y., Bankova, L.G. and Beck, L.A. 2009. Atopic Dermatitis: A Disease Caused by Innate Immune Defects? *Journal of Investigative Dermatology.* **129**(1), pp.14-30.

De Goffau, M.C., Yang, X., Van Dijl, J.M. and Harmsen, H.J.M. 2009. Bacterial pleomorphism and competition in a relative humidity gradient. *Environmental Microbiology.* **11**(4), pp.809-822.

De Haas, C.J.C., Veldkamp, K.E., Peschel, A., Weerkamp, F., Van Wamel, W.J.B., Heezius, E.C.J.M., Poppelier, M.J.J.G., Van Kessel, K.P.M. and van Strijp, J.A.G. 2004. Chemotaxis Inhibitory Protein of Staphylococcus aureus, a Bacterial Antiinflammatory Agent. *The Journal of Experimental Medicine.* **199**(5), pp.687-695.

De Palma, G., Nadal, I., Medina, M., Donat, E., Ribes-Koninckx, C., Calabuig, M. and Sanz, Y. 2010. Intestinal dysbiosis and reduced immunoglobulin-coated bacteria associated with coeliac disease in children. *BMC Microbiol.* **10**, p63.

Dejean, E., Foisseau, M., Lagarrigue, F., Lamant, L., Prade, N., Marfak, A., Delsol, G., Giuriato, S., Gaits-Iacovoni, F. and Meggetto, F. 2012. ALK+ALCLs induce cutaneous, HMGB-1-dependent IL-8/CXCL8 production by keratinocytes through NF- B activation. *Blood.* **119**(20), pp.4698-4707.

Deleuran, M., Hvid, M., Kemp, K., Christensen, G.B., Deleuran, B. and Vestergaard, C. 2012. IL-25 induces both inflammation and skin barrier dysfunction in atopic dermatitis. *Chem Immunol Allergy.* **96**, pp.45-49.

Desbonnet, L., Garrett, L., Clarke, G., Kiely, B., Cryan, J.F. and Dinan, T.G. 2010. Effects of the probiotic Bifidobacterium infantis in the maternal separation model of depression. *Neuroscience.* **170**(4), pp.1179-1188.

Dethlefsen, L., Huse, S., Sogin, M.L. and Relman, D.A. 2008. The Pervasive Effects of an Antibiotic on the Human Gut Microbiota, as Revealed by Deep 16S rRNA Sequencing. *PLoS Biology.* **6**(11), pe280.

Dhople, V., Krukemeyer, A. and Ramamoorthy, A. 2006. The human beta-defensin-3, an antibacterial peptide with multiple biological functions. *Biochimica et Biophysica Acta (BBA) - Biomembranes.* **1758**(9), pp.1499-1512.

Di Domenico, E.G., Cavallo, I., Bordignon, V., Prignano, G., Sperduti, I., Gurtner, A., Trento, E., Toma, L., Pimpinelli, F., Capitanio, B. and Ensoli, F. 2018. Inflammatory cytokines and biofilm production sustain Staphylococcus aureus outgrowth and persistence: a pivotal interplay in the pathogenesis of Atopic Dermatitis. *Scientific reports.* **8**(1), pp.9573-9573.

Di Nardo, A., Wertz, P., Giannetti, A. and Seidenari, S. 1998. Ceramide and cholesterol composition of the skin of patients with atopic dermatitis. *Acta Derm Venereol.* **78**(1), pp.27-30.

Di, Z.-H., Ma, L., Qi, R.-Q., Sun, X.-D., Huo, W., Zhang, L., Lyu, Y.-N., Hong, Y.-X., Chen, H.-D. and Gao, X.-H. 2016. T Helper 1 and T Helper 2 Cytokines Differentially Modulate Expression of Filaggrin and its Processing Proteases in Human Keratinocytes. *Chinese Medical Journal.* **129**(3), pp.295-303.

Dinarello, C.A. 2007. Historical insights into cytokines. **37**(S1), pp.S34-S45.

Dinulos, J.G.H., Mentele, L., Fredericks, L.P., Dale, B.A. and Darmstadt, G.L. 2003. Keratinocyte Expression of Human β Defensin 2 following Bacterial Infection: Role in Cutaneous Host Defense. *Clinical and Diagnostic Laboratory Immunology.* **10**(1), pp.161-166.

Dong, Y. and Speer, C.P. 2014. The role of Staphylococcus epidermidis in neonatal sepsis: Guarding angel or pathogenic devil? *International Journal of Medical Microbiology.* **304**(5), pp.513-520.

Dorschner, R.A., Pestonjamasp, V.K., Tamakuwala, S., Ohtake, T., Rudisill, J., Nizet, V., Agerberth, B., Gudmundsson, G.H. and Gallo, R.L. 2001. Cutaneous Injury

Induces the Release of Cathelicidin Anti-Microbial Peptides Active Against Group A Streptococcus. *Journal of Investigative Dermatology.* **117**(1), pp.91-97.

Drake, D.R., Brogden, K.A., Dawson, D.V. and Wertz, P.W. 2008. Thematic Review Series: Skin Lipids. Antimicrobial lipids at the skin surface. *Journal of Lipid Research.* **49**(1), pp.4-11.

Drancourt, M. and Raoult, D. 2002. rpoB gene sequence-based identification of Staphylococcus species. *J Clin Microbiol.* **40**(4), pp.1333-1338.

Duclohier, H. 1994. Anion pores from magainins and related defensive peptides. *Toxicology.* **87**(1-3), pp.175-188.

Eady, R.A. and Pope, F.M. 2004. *Rook's Textbook of Dermatology. Chapter 3: Anatomy and Organization of Human Skin.* 7 ed. Blackwell Publishing.

Ebeling, W., Hennrich, N., Klockow, M., Metz, H., Orth, H.D. and Lang, H. 1974. Proteinase K from Tritirachium album Limber. *European Journal of Biochemistry.* **47**(1), pp.91-97.

Eberlein-Konig, B., Schafer, T., Huss-Marp, J., Darsow, U., Mohrenschlager, M., Herbert, O., Abeck, D., Kramer, U., Behrendt, H. and Ring, J. 2000. Skin surface pH, stratum corneum hydration, trans-epidermal water loss and skin roughness related to atopic eczema and skin dryness in a population of primary school children. *Acta Derm Venereol.* **80**(3), pp.188-191.

Eckert, R.L., Efimova, T., Dashti, S.R., Balasubramanian, S., Deucher, A., Crish, J.F., Sturniolo, M. and Bone, F. 2002. Keratinocyte Survival, Differentiation, and Death: Many Roads Lead to Mitogen-Activated Protein Kinase. *Journal of Investigative Dermatology Symposium Proceedings.* **7**(1), pp.36-40.

Eckhart, L., Lippens, S., Tschachler, E. and Declercq, W. 2013. Cell death by cornification. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research.* **1833**(12), pp.3471-3480.

Edmondson, R., Broglie, J.J., Adcock, A.F. and Yang, L. 2014. Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. *Assay Drug Dev Technol.* **12**(4), pp.207-218.

Edwards, A.M., Potts, J.R., Josefsson, E. and Massey, R.C. 2010. Staphylococcus aureus Host Cell Invasion and Virulence in Sepsis Is Facilitated by the Multiple Repeats within FnBPA. *PLoS Pathogens.* **6**(6), pe1000964.

Elfatoiki, F.Z., El Azhari, M., El Kettani, A., Serhier, Z., Othmani, M.B., Timinouni, M., Benchikhi, H., Chiheb, S. and Fellah, H. 2016. Psoriasis and Staphylococcus aureus skin colonization in Moroccan patients. *Pan Afr Med J.* **23**, p33.

Elias, P.M. 1983. Epidermal Lipids, Barrier Function, and Desquamation. *Journal of Investigative Dermatology.* **80**, pp.S44-S49.

Elias, P.M. 2007. The skin barrier as an innate immune element. *Seminars in Immunopathology.* **29**(1), pp.3-14.

Elias, P.M. 2008. Skin Barrier Function. *Current allergy and asthma reports.* **8**(4), pp.299-305.

Elias, P.M., Hatano, Y. and Williams, M.L. 2008. Basis for the barrier abnormality in atopic dermatitis: Outside-inside-outside pathogenic mechanisms. *The Journal of allergy and clinical immunology.* **121**(6), pp.1337-1343.

Eyerich, K. and Eyerich, S. 2015. Th22 cells in allergic disease. *Allergo journal international.* **24**(1), pp.1-7.

Eyerich, K., Pennino, D., Scarponi, C., Foerster, S., Nasorri, F., Behrendt, H., Ring, J., Traidl-Hoffmann, C., Albanesi, C. and Cavani, A. 2009. IL-17 in atopic eczema: Linking allergen-specific adaptive and microbial-triggered innate immune response. *Journal of Allergy and Clinical Immunology.* **123**(1), pp.59-66.e54.

Eyerich, S., Eyerich, K., Traidl-Hoffmann, C. and Biedermann, T. 2018. Cutaneous Barriers and Skin Immunity: Differentiating A Connected Network. *Trends Immunol.* **39**(4), pp.315-327.

Ezepchuk, Y.V., Leung, D.Y., Middleton, M.H., Bina, P., Reiser, R. and Norris, D.A. 1996. Staphylococcal toxins and protein A differentially induce cytotoxicity and release of tumor necrosis factor-alpha from human keratinocytes. *J Invest Dermatol.* **107**(4), pp.603-609.

Falconer, A., Ikram, M., Bissett, C.E., Cerio, R., Quinn, A.G. and Ali, R.S. 2001. Expression of the Peptide Antibiotics Human β Defensin-1 and Human β Defensin-2 in Normal Human Skin. *Journal of Investigative Dermatology.* **117**(1), pp.106-111.

Falugi, F., Kim, H.K., Missiakas, D.M. and Schneewind, O. 2013. Role of Protein A in the Evasion of Host Adaptive Immune Responses by Staphylococcus aureus. *MBio.* **4**(5), pp.e00575-00513-e00575.

Fennessy, M., Coupland, S., Popay, J. and Naysmith, K. 2000. The Epidemiology and Experience of Atopic Eczema during Childhood: A Discussion Paper on the Implications of Current Knowledge for Health Care, Public Health Policy and Research. *Journal of Epidemiology and Community Health.* **54**(8), pp.581-589.

Ferreira, V., Borba, H., Bonetti, A., Leonart, L. and Pontarolo, R. 2019. Cytokines and Interferons: Types and Functions. IntechOpen.

Fey, P.D., Endres, J.L., Yajjala, V.K., Widhelm, T.J., Boissy, R.J., Bose, J.L. and Bayles, K.W. 2013. A Genetic Resource for Rapid and Comprehensive Phenotype Screening of Nonessential Staphylococcus aureus Genes. *mBio.* **4**(1).

Fiaschi, L., Di Palo, B., Scarselli, M., Pozzi, C., Tomaszewski, K., Galletti, B., Nardi-Dei, V., Arcidiacono, L., Mishra, R.P., Mori, E., Pallaoro, M., Falugi, F., Torre, A., Fontana, M.R., Soriani, M., Bubeck Wardenburg, J., Grandi, G., Rappuoli, R., Ferlenghi, I. and Bagnoli, F. 2016. Auto-Assembling Detoxified Staphylococcus aureus Alpha-Hemolysin Mimicking the Wild-Type Cytolytic Toxin. *Clin Vaccine Immunol.* **23**(6), pp.442-450.

Findley, K. and Grice, E.A. 2014. The Skin Microbiome: A Focus on Pathogens and Their Association with Skin Disease. *PLoS pathogens.* **10**(11), pe1004436.

Fischer, C.L., Drake, D.R., Dawson, D.V., Blanchette, D.R., Brogden, K.A. and Wertz, P.W. 2012. Antibacterial Activity of Sphingoid Bases and Fatty Acids against Gram-Positive and Gram-Negative Bacteria. *Antimicrob Agents Chemother.* **56**(3), pp.1157-1161.

Flaherty, D.K. 2012. Chapter 2 - Innate Immunity. *Immunology for Pharmacy.* Saint Louis: Mosby, pp.15-22.

Foster, T.J. 2005. Immune evasion by staphylococci. *Nat Rev Micro.* **3**(12), pp.948-958.

Frankart, A., Coquette, A., Schroeder, K.-R. and Poumay, Y. 2012. Studies of cell signaling in a reconstructed human epidermis exposed to sensitizers: IL-8 synthesis and release depend on EGFR activation. *Archives of Dermatological Research.* **304**(4), pp.289-303.

Fraser, T.P. and John, D. 2016. *Streptococcal Superantigens: Biological properties and potential role in disease.* Oklahoma City: University of Oklahoma Health Sciences Center.

Friedman, B.-C. and Goldman, R.D. 2011. Anti-staphylococcal treatment in dermatitis. *Canadian Family Physician.* **57**(6), pp.669-671.

Frohm, M., Agerberth, B., Ahangari, G., Ståhle-Bäckdahl, M., Lidén, S., Wigzell, H. and Gudmundsson, G.H. 1997. The Expression of the Gene Coding for the Antibacterial Peptide LL-37 Is Induced in Human Keratinocytes during Inflammatory Disorders. *Journal of Biological Chemistry.* **272**(24), pp.15258-15263.

Frye, M., Bargon, J. and Gropp, R. 2001. Expression of human beta-defensin-1 promotes differentiation of keratinocytes. *J Mol Med (Berl).* **79**(5-6), pp.275-282.

Fu, T., Keiser, E., Linos, E., Rotatori, R.M., Sainani, K., Lingala, B., Lane, A.T., Schneider, L. and Tang, J.Y. 2014. Eczema and sensitization to common allergens in the United States: a multiethnic, population-based study. *Pediatr Dermatol.* **31**(1), pp.21-26.

Fuchs, E. 1993. Epidermal differentiation and keratin gene expression. *J Cell Sci Suppl.* **17**, pp.197-208.

Fuchs, S., Pane-Farre, J., Kohler, C., Hecker, M. and Engelmann, S. 2007. Anaerobic gene expression in Staphylococcus aureus. *J Bacteriol.* **189**(11), pp.4275-4289.

Fux, C.A., Shirtliff, M., Stoodley, P. and Costerton, J.W. 2005. Can laboratory reference strains mirror "real-world" pathogenesis? *Trends Microbiol.* **13**(2), pp.58-63.

Gaitanis, G., Velegraki, A., Mayser, P. and Bassukas, I.D. 2013. Skin diseases associated with Malassezia yeasts: Facts and controversies. *Clinics in Dermatology.* **31**(4), pp.455-463.

Gambichler, T., Skrygan, M., Tomi, N.S., Altmeyer, P. and Kreuter, A. 2006. Changes of antimicrobial peptide mRNA expression in atopic eczema following phototherapy. *Br J Dermatol.* **155**(6), pp.1275-1278.

Gandhi, N.A., Pirozzi, G. and Graham, N.M.H. 2017. Commonality of the IL-4/IL-13 pathway in atopic diseases. *Expert Rev Clin Immunol.* **13**(5), pp.425-437.

Ganz, T. 2003. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol.* **3**(9), pp.710-720.

Ganz, T. and Lehrer, R.I. 1997. Antimicrobial peptides of leukocytes. *Curr Opin Hematol.* **4**(1), pp.53-58.

Gao, Z., Perez-Perez, G.I., Chen, Y. and Blaser, M.J. 2010. Quantitation of Major Human Cutaneous Bacterial and Fungal Populations. *Journal of Clinical Microbiology.* **48**(10), pp.3575-3581.

Garreis, F., Gottschalt, M., Schlorf, T., Gläser, R., Harder, J., Worlitzsch, D. and Paulsen, F.P. 2011. Expression and Regulation of Antimicrobial Peptide Psoriasin

(S100A7) at the Ocular Surface and in the Lacrimal Apparatus. *Investigative Ophthalmology & Visual Science.* **52**(7), pp.4914-4922.

Gavrilova, T. 2018. Immune Dysregulation in the Pathogenesis of Atopic Dermatitis. *Dermatitis.* **29**(2), pp.57-62.

Geoghegan, J.A., Irvine, A.D. and Foster, T.J. 2018. Staphylococcus aureus and Atopic Dermatitis: A Complex and Evolving Relationship. *Trends Microbiol.* **26**(6), pp.484-497.

Ghebremedhin, B., Layer, F., König, W. and König, B. 2008. Genetic Classification and Distinguishing of Staphylococcus Species Based on Different Partial gap, 16S rRNA, hsp60, rpoB, sodA, and tuf Gene Sequences. *Journal of Clinical Microbiology.* **46**(3), p1019.

Giam, Y.C., Hebert, A.A., Dizon, M.V., Van Bever, H., Tiongco-Recto, M., Kim, K.-H., Soebono, H., Munasir, Z., Diana, I.A. and Luk, D.C.K. 2016. A review on the role of moisturizers for atopic dermatitis. *Asia Pacific allergy.* **6**(2), pp.120-128.

Giese, B., Glowinski, F., Paprotka, K., Dittmann, S., Steiner, T., Sinha, B. and Fraunholz, M.J. 2011. Expression of delta-toxin by Staphylococcus aureus mediates escape from phago-endosomes of human epithelial and endothelial cells in the presence of beta-toxin. *Cell Microbiol.* **13**(2), pp.316-329.

Gilani, S.J.K., Gonzalez, M., Hussain, I., Finlay, A.Y. and Patel, G.K. 2005. Staphylococcus aureus re-colonization in atopic dermatitis: beyond the skin. *Clinical and Experimental Dermatology.* **30**(1), pp.10-13.

Gilbert, S.F. 2000. The Epidermis and the Origin of Cutaneous Structures. *Developmental Biology. 6th edition. Sunderland.*

Gill, S.R., Fouts, D.E., Archer, G.L., Mongodin, E.F., Deboy, R.T., Ravel, J., Paulsen, I.T., Kolonay, J.F., Brinkac, L., Beanan, M., Dodson, R.J., Daugherty, S.C., Madupu, R., Angiuoli, S.V., Durkin, A.S., Haft, D.H., Vamathevan, J., Khouri, H., Utterback, T., Lee, C., Dimitrov, G., Jiang, L., Qin, H., Weidman, J., Tran, K., Kang, K., Hance, I.R., Nelson, K.E. and Fraser, C.M. 2005. Insights on Evolution of Virulence and Resistance from the Complete Genome Analysis of an Early Methicillin-Resistant Staphylococcus aureus Strain and a Biofilm-Producing Methicillin-Resistant Staphylococcus epidermidis Strain. *Journal of Bacteriology.* **187**(7), pp.2426-2438.

Girolomoni, G., Abeni, D., Masini, C., Sera, F., Ayala, F., Belloni-fortina, A., Bonifazi, E., Fabbri, P., Gelmetti, C., Monfrecola, G., Peserico, A., Seidenari, S. and Giannetti, A. 2003. The epidemiology of atopic dermatitis in Italian schoolchildren. *Allergy.* **58**(5), pp.420-425.

Gittler, J.K., Shemer, A., Suarez-Farinas, M., Fuentes-Duculan, J., Gulewicz, K.J., Wang, C.Q.F., Mitsui, H., Cardinale, I., Strong, C.D., Krueger, J.G. and Guttman-Yassky, E. 2012. Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. *Journal of Allergy and Clinical Immunology.* **130**(6), pp.1344-1354.

Gläser, R., Harder, J., Lange, H., Bartels, J., Christophers, E. and Schröder, J.M. 2005. Antimicrobial psoriasin (S100A7) protects human skin from Escherichia coli infection. *Nat Immunol.* **6**(1), pp.57-64.

Gläser, R., Meyer-Hoffert, U., Harder, J., Cordes, J., Wittersheim, M., Kobliakova, J., Folster-Holst, R., Proksch, E., Schröder, J.M. and Schwarz, T. 2009. The antimicrobial protein psoriasin (S100A7) is upregulated in atopic dermatitis and after experimental skin barrier disruption. *J Invest Dermatol.* **129**(3), pp.641-649.

Glatz, M., Bosshard, P. and Schmid-Grendelmeier, P. 2017. The Role of Fungi in Atopic Dermatitis. *Immunology and Allergy Clinics of North America.* **37**(1), pp.63-74.

Gong, J.Q., Lin, L., Lin, T., Hao, F., Zeng, F.Q., Bi, Z.G., Yi, D. and Zhao, B. 2006. Skin colonization by Staphylococcus aureus in patients with eczema and atopic dermatitis and relevant combined topical therapy: a double-blind multicentre randomized controlled trial. *British Journal of Dermatology.* **155**(4), pp.680-687.

Gonzalezmariscal, L. 2003. Tight junction proteins. *Progress in Biophysics and Molecular Biology.* **81**(1), pp.1-44.

Greenbaum, D., Colangelo, C., Williams, K. and Gerstein, M. 2003. Comparing protein abundance and mRNA expression levels on agenomic scale. *Genome Biology.* **4**(9), p117.

Grice, E.A., Kong, H.H., Conlan, S., Deming, C.B., Davis, J., Young, A.C., Bouffard, G.G., Blakesley, R.W., Murray, P.R., Green, E.D., Turner, M.L. and Segre, J.A. 2009. Topographical and temporal diversity of the human skin microbiome. *Science.* **324**(5931), pp.1190-1192.

Grice, E.A. and Segre, J.A. 2011. The skin microbiome. *Nature reviews. Microbiology.* **9**(4), pp.244-253.

Gruber, R., Börnchen, C., Rose, K., Daubmann, A., Volksdorf, T., Wladykowski, E., Vidal-y-Sy, S., Peters, E.M., Danso, M., Bouwstra, J.A., Hennies, H.C., Moll, I., Schmuth, M. and Brandner, J.M. 2015. Diverse Regulation of Claudin-1 and Claudin-4 in Atopic Dermatitis. *The American Journal of Pathology.* **185**(10), pp.2777-2789.

Gschwandtner, M., Mildner, M., Mlitz, V., Gruber, F., Eckhart, L., Werfel, T., Gutzmer, R., Elias, P.M. and Tschachler, E. 2013. Histamine suppresses epidermal keratinocyte differentiation and impairs skin barrier function in a human skin model. *Allergy.* **68**(1), pp.37-47.

Gupta, J., Grube, E., Lucky, A., Sheth, A., Assa'ad, A. and Khurana Hershey, G.K. 2007. Transepidermal Water Loss As A Biological Marker In Atopic Dermatitis In Children. *Journal of Allergy and Clinical Immunology.* **119**(1), pS280.

Gurtner, G.C., Werner, S., Barrandon, Y. and Longaker, M.T. 2008. Wound repair and regeneration. *Nature.* **453**(7193), pp.314-321.

Gutowska-Owsiak, D., Schaupp, A.L., Salimi, M., Selvakumar, T.A., McPherson, T., Taylor, S. and Ogg, G.S. 2012. IL-17 downregulates filaggrin and affects keratinocyte expression of genes associated with cellular adhesion. *Experimental Dermatology.* **21**(2), pp.104-110.

Gutowska-Owsiak, D., Schaupp, A.L., Salimi, M., Taylor, S. and Ogg, G.S. 2011. Interleukin-22 downregulates filaggrin expression and affects expression of profilaggrin processing enzymes. *British Journal of Dermatology.* **165**(3), pp.492-498.

Hadley, J.S., Wang, J.E., Foster, S.J., Thiemermann, C. and Hinds, C.J. 2005. Peptidoglycan of Staphylococcus aureus Upregulates Monocyte Expression of CD14, Toll-Like Receptor 2 (TLR2), and TLR4 in Human Blood: Possible Implications for Priming of Lipopolysaccharide Signaling. *Infection and Immunity.* **73**(11), pp.7613-7619.

Haisma, E.M., Rietveld, M.H., De Breij, A., Van Dissel, J.T., El Ghalbzouri, A. and Nibbering, P.H. 2013. Inflammatory and Antimicrobial Responses to Methicillin-Resistant Staphylococcus aureus in an In Vitro Wound Infection Model. *PLoS ONE.* **8**(12), pe82800.

Hanel, K.H., Cornelissen, C., Luscher, B. and Baron, J.M. 2013. Cytokines and the skin barrier. *Int J Mol Sci.* **14**(4), pp.6720-6745.

Hanifin, J. and Rajka, G. 1980. Diagnostic features of atopic dermatitis. *Acta Dermatovenerologica (Stockholm).* pp.44–47.

Hannigan, G.D. and Grice, E.A. 2013. Microbial Ecology of the Skin in the Era of Metagenomics and Molecular Microbiology. *Cold Spring Harb Perspect Med.* **3**(12), pp.a015362-a015362.

Hansson, C., Eriksson, C. and Alenius, G.-M. 2014. S-Calprotectin (S100A8/S100A9): A Potential Marker of Inflammation in Patients with Psoriatic Arthritis. *Journal of Immunology Research.* **2014**, p5.

Harada, K., Saito, M., Sugita, T. and Tsuboi, R. 2015. Malassezia species and their associated skin diseases. *Journal of Dermatology.* **42**(3), pp.250-257.

Harder, J., Bartels, J., Christophers, E. and Schröder, J.M. 1997. A peptide antibiotic from human skin. *Nature.* **387**(6636), pp.861-861.

Harder, J., Bartels, J., Christophers, E. and Schröder, J.M. 2001. Isolation and Characterization of Human β-Defensin-3, a Novel Human Inducible Peptide Antibiotic. *Journal of Biological Chemistry.* **276**(8), pp.5707-5713.

Harder, J., Dressel, S., Wittersheim, M., Cordes, J., Meyer-Hoffert, U., Mrowietz, U., Folster-Holst, R., Proksch, E., Schröder, J.M., Schwarz, T. and Gläser, R. 2010. Enhanced Expression and Secretion of Antimicrobial Peptides in Atopic Dermatitis and after Superficial Skin Injury. *J Invest Dermatol.* **130**(5), pp.1355-1364.

Harder, J., Meyer-Hoffert, U., Teran, L.M., Schwichtenberg, L., Bartels, J., Maune, S. and Schröder, J.M. 2000. Mucoid Pseudomonas aeruginosa, TNF- α , and IL-1 β , but Not IL-6, Induce Human β -Defensin-2 in Respiratory Epithelia. *American Journal of Respiratory Cell and Molecular Biology.* **22**(6), pp.714-721.

Harder, J., Meyer-Hoffert, U., Wehkamp, K., Schwichtenberg, L. and Schröder, J.M. 2004. Differential Gene Induction of Human β-Defensins (hBD-1, -2, -3, and -4) in Keratinocytes Is Inhibited by Retinoic Acid. *Journal of Investigative Dermatology.* **123**(3), pp.522-529.

Harder, J. and Schröder, J.M. 2002. RNase 7, a novel innate immune defense antimicrobial protein of healthy human skin. *J Biol Chem.* **277**(48), pp.46779-46784.

Harder, J. and Schröder, J.M. 2005. Psoriatic scales: a promising source for the isolation of human skin-derived antimicrobial proteins. *Journal of Leukocyte Biology.* **77**(4), pp.476-486.

Harkins, C.P., Holden, M.T.G. and Irvine, A.D. 2019. Antimicrobial resistance in atopic dermatitis: Need for an urgent rethink. *Annals of Allergy, Asthma & Immunology.* **122**(3), pp.236-240.

Harkins, C.P., McAleer, M.A., Bennett, D., McHugh, M., Fleury, O.M., Pettigrew, K.A., Oravcova, K., Parkhill, J., Proby, C.M., Dawe, R.S., Geoghegan, J.A., Irvine, A.D. and Holden, M.T.G. 2018. The widespread use of topical antimicrobials enriches for resistance in Staphylococcus aureus isolated from patients with atopic dermatitis. *Br J Dermatol.* **179**(4), pp.951-958.

Harrop, J., Chinn, S., Verlato, G., Olivieri, M., Norback, D., Wjst, M., Janson, C., Zock, J.P., Leynaert, B., Gislason, D., Ponzio, M., Villani, S., Carosso, A., Svanes, C.,

Heinrich, J. and Jarvis, D. 2007. Eczema, atopy and allergen exposure in adults: a population-based study. *Clinical And Experimental Allergy.* **37**(4), pp.526-535.

Hart, P.H., Townley, S.L., Grimbaldeston, M.A., Khalil, Z. and Finlay-Jones, J.J. 2002. Mast cells, neuropeptides, histamine, and prostaglandins in UV-induced systemic immunosuppression. *Methods.* **28**(1), pp.79-89.

Hata, T.R. and Gallo, R.L. 2008. Antimicrobial Peptides, Skin Infections and Atopic Dermatitis. *Seminars in cutaneous medicine and surgery.* **27**(2), pp.144-150.

Hatta, I., Ohta, N., Inoue, K. and Yagi, N. 2006. Coexistence of two domains in intercellular lipid matrix of stratum corneum. *Biochimica et Biophysica Acta (BBA) - Biomembranes.* **1758**(11), pp.1830-1836.

Hatta, M., Tabri, F., Djawad, K., Wahab, S. and Patellongi, I. 2016. The Relationship Between Serum Level of Interleukin-10 and State of the Disease with Atopic Dermatitis and Helminth in Children. *International Journal of Immunology.* **4**(6), pp.73-77.

He, Y.Y., Garvie, C.W., Elworthy, S., Manfield, I.W., McNally, T., Lawrenson, I.D., Phillips, S.E. and Stockley, P.G. 2002. Structural and functional studies of an intermediate on the pathway to operator binding by Escherichia coli MetJ. *J Mol Biol.* **320**(1), pp.39-53.

Heilborn, J.D., Nilsson, M.F., Kratz, G., Weber, G., Sorensen, O., Borregaard, N. and Stahle-Backdahl, M. 2003. The cathelicidin anti-microbial peptide LL-37 is involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium. *J Invest Dermatol.* **120**(3), pp.379-389.

Herd, R.M., Tidman, M.J., Prescott, R.J. and Hunter, J.A. 1996. Prevalence of atopic eczema in the community: the Lothian Atopic Dermatitis study. *The British journal of dermatology.* **135**(1), pp.18-19.

Herman-Bausier, P., Labate, C., Towell, A.M., Derclaye, S., Geoghegan, J.A. and Dufrêne, Y.F. 2018. Staphylococcus aureus clumping factor A is a force-sensitive molecular switch that activates bacterial adhesion. *Proceedings of the National Academy of Sciences of the United States of America.* **115**(21), pp.5564-5569.

Hertz, C.J., Wu, Q., Porter, E.M., Zhang, Y.J., Weismüller, K.-H., Godowski, P.J., Ganz, T., Randell, S.H. and Modlin, R.L. 2003. Activation of Toll-Like Receptor 2 on Human Tracheobronchial Epithelial Cells Induces the Antimicrobial Peptide Human β Defensin-2. *The Journal of Immunology.* **171**(12), pp.6820-6826.

Herz, U., Bunikowski, R. and Renz, H. 1998. Role of T cells in atopic dermatitis. New aspects on the dynamics of cytokine production and the contribution of bacterial superantigens. *Int Arch Allergy Immunol.* **115**(3), pp.179-190.

Higaki, S., Morohashi, M., Yamagishi, T. and Hasegawa, Y. 1999. Comparative study of staphylococci from the skin of atopic dermatitis patients and from healthy subjects. *International Journal of Dermatology.* **38**(4), pp.265-269.

Higgins, J., Loughman, A., van Kessel, K.P., van Strijp, J.A. and Foster, T.J. 2006. Clumping factor A of Staphylococcus aureus inhibits phagocytosis by human polymorphonuclear leucocytes. *FEMS Microbiol Lett.* **258**(2), pp.290-296.

Hijnen, D., Knol, E.F., Gent, Y.Y., Giovannone, B., Beijn, S.J.P., Kupper, T.S., Bruijnzeel-Koomen, C.A.F.M. and Clark, R.A. 2013. CD8(+) T cells in the lesional skin of atopic dermatitis and psoriasis patients are an important source of IFN-γ, IL-13, IL-17, and IL-22. *The Journal of investigative dermatology.* **133**(4), pp.973-979.

Hoeger, P.H. 2004. Antimicrobial susceptibility of skin-colonizing S. aureus strains in children with atopic dermatitis. *Pediatric Allergy and Immunology.* **15**(5), pp.474-477.

Holland, D.B., Bojar, R.A., Farrar, M.D. and Holland, K.T. 2009. Differential innate immune responses of a living skin equivalent model colonized by Staphylococcus epidermidis or Staphylococcus aureus. *FEMS Microbiology Letters.* **290**(2), pp.149-155.

Holland, D.B., Bojar, R.A., Jeremy, A.H., Ingham, E. and Holland, K.T. 2008. Microbial colonization of an in vitro model of a tissue engineered human skin equivalent--a novel approach. *FEMS Microbiol Lett.* **279**(1), pp.110-115.

Homey, B., Steinhoff, M., Ruzicka, T. and Leung, D.Y.M. 2006. Cytokines and chemokines orchestrate atopic skin inflammation. *Journal of Allergy and Clinical Immunology.* **118**(1), pp.178-189.

Homey, B., Wang, W., Soto, H., Buchanan, M.E., Wiesenborn, A., Catron, D., Müller, A., McClanahan, T.K., Dieu-Nosjean, M.-C., Orozco, R., Ruzicka, T., Lehmann, P., Oldham, E. and Zlotnik, A. 2000. Cutting Edge: The Orphan Chemokine Receptor G Protein-Coupled Receptor-2 (GPR-2, CCR10) Binds the Skin-Associated Chemokine CCL27 (CTACK/ALP/ILC). *The Journal of Immunology.* **164**(7), pp.3465-3470.

Homey, B. and Zlotnik, A. 1999. Chemokines in allergy. *Current opinion in immunology.* **11**(6), pp.626-634.

Hon, K.L. and Leung, A.K. 2013. Use of ceramides and related products for childhood-onset eczema. *Recent Pat Inflamm Allergy Drug Discov.* **7**(1), pp.12-19.

Hon, K.L., Leung, A.K.C. and Barankin, B. 2013. Barrier Repair Therapy in Atopic Dermatitis: An Overview. *American Journal of Clinical Dermatology.* **14**(5), pp.389-399.

Hong, J., Buddenkotte, J., Berger, T.G. and Steinhoff, M. 2011. Management of itch in atopic dermatitis. *Seminars in cutaneous medicine and surgery.* **30**(2), pp.71-86.

Horn, J., Klepsch, M., Manger, M., Wolz, C., Rudel, T. and Fraunholz, M. 2018. Long Noncoding RNA SSR42 Controls Staphylococcus aureus Alpha-Toxin Transcription in Response to Environmental Stimuli. *Journal of bacteriology.* **200**(22), pp.e00252-00218.

Horrobin, D.F. 1993. Fatty acid metabolism in health and disease: the role of Δ-6-desaturase. *The American Journal of Clinical Nutrition.* **57**(5), pp.732S-737S.

Horrobin, D.F. and Manku, M.S. 1990. *Clinical biochemistry of essentiall fatty acids. In: Horrobin DF, ed. Omega-6 essential fatty acids – pathophysiology and roles in clinical medicine.* New York: Wiley Liss.

Horsburgh, M.J., Aish, J.L., White, I.J., Shaw, L., Lithgow, J.K. and Foster, S.J. 2002. σB modulates virulence determinant expression and stress resistance: characterization of a functional rsbU strain derived from Staphylococcus aureus 8325-4. *JBacteriol.* **184**.

Howell, M., Kim, B.E., Gao, P.S., Grant, A.V., Boguniewicz, M., DeBenedetto, A., Schneider, L., Beck, L.A., Barnes, K.C. and Leung, D.Y.M. 2009. Cytokine modulation of atopic dermatitis filaggrin skin expression (Reprinted from J Allergy Clin Immunol vol 120, pg 150-5, 2007). *Journal of Allergy and Clinical Immunology.* **124**(3), pp.R7-R12.

Howell, M.D., Boguniewicz, M., Pastore, S., Novak, N., Bieber, T., Girolomoni, G. and Leung, D.Y. 2006a. Mechanism of HBD-3 deficiency in atopic dermatitis. *Clin Immunol.* **121**(3), pp.332-338.

Howell, M.D., Gallo, R.L., Boguniewicz, M., Jones, J.F., Wong, C., Streib, J.E. and Leung, D.Y.M. 2006b. Cytokine Milieu of Atopic Dermatitis Skin Subverts the Innate Immune Response to Vaccinia Virus. *Immunity.* **24**(3), pp.341-348.

Howell, M.D., Novak, N., Bieber, T., Pastore, S., Girolomoni, G., Boguniewicz, M., Streib, J., Wong, C., Gallo, R.L. and Leung, D.Y.M. 2005. Interleukin-10 Downregulates Anti-Microbial Peptide Expression in Atopic Dermatitis. *Journal of Investigative Dermatology.* **125**(4), pp.738-745.

Hsiao, E.Y., McBride, S.W., Hsien, S., Sharon, G., Hyde, E.R., McCue, T., Codelli, J.A., Chow, J., Reisman, S.E., Petrosino, J.F., Patterson, P.H. and Mazmanian, S.K. 2013. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell.* **155**(7), pp.1451-1463.

Huang, J.T., Abrams, M., Tlougan, B., Rademaker, A. and Paller, A.S. 2009. Treatment of Staphylococcus aureus Colonization in Atopic Dermatitis Decreases Disease Severity. *Pediatrics.* **123**(5), pp.e808-e814.

Huang, S.M. and McCance, D.J. 2002. Downregulation of the Interleukin-8 Promoter by Human Papillomavirus Type 16 E6 and E7 through Effects on CREB Binding Protein/p300 and P/CAF. *Journal of Virology.* **76**(17), pp.8710-8721.

Huffnagle, G.B. and Noverr, M.C. 2013. The emerging world of the fungal microbiome. *Trends in Microbiology.* **21**(7), pp.334-341.

Hunt, K.M., Preuss, J., Nissan, C., Davlin, C.A., Williams, J.E., Shafii, B., Richardson, A.D., McGuire, M.K., Bode, L. and McGuire, M.A. 2012. Human Milk Oligosaccharides Promote the Growth of Staphylococci. *Appl Environ Microbiol.* **78**(14), pp.4763-4770.

Imokawa, G., Abe, A., Jin, K., Higaki, Y., Kawashima, M. and Hidano, A. 1991. Decreased level of ceramides in stratum corneum of atopic dermatitis: an etiologic factor in atopic dry skin? *Journal of Investigative Dermatology.* **96**(4), pp.523-526.

Imokawa, G., Akasaki, S., Minematsu, Y. and Kawai, M. 1989. Importance of intercellular lipids in water-retention properties of the stratum corneum: induction and recovery study of surfactant dry skin. *Archives of Dermatological Research.* **281**(1), pp.45-51.

Imokawa, G. and Ishida, K. 2014. Role of Ceramide in the Barrier Function of the Stratum Corneum, Implications for the Pathogenesis of Atopic Dermatitis. *J Clin Exp Dermatol Res.* **5**(1), p206.

Inngjerdingen, M., Damaj, B. and Maghazachi, A.A. 2000. Human NK cells express CC chemokine receptors 4 and 8 and respond to thymus and activation-regulated chemokine, macrophage-derived chemokine, and I-309. *J Immunol.* **164**(8), pp.4048-4054.

Inthasin, N., Wongprompitak, P., Boonwong, C. and Ekpo, P. 2018. Role of Toll-like receptor 2 in mediating the production of cytokines and human beta-defensins in oral mucosal epithelial cell response to Leptospiral infection. *Asian Pac J Allergy Immunol.* (doi: 10.12932/AP-100518-0308).

Iozumi, K., Hoganson, G.E., Pennella, R., Everett, M.A. and Fuller, B.B. 1993. Role of Tyrosinase as the Determinant of Pigmentation in Cultured Human Melanocytes. *Journal of Investigative Dermatology.* **100**(6), pp.806-811.

Iwasaki, A. and Medzhitov, R. 2004. Toll-like receptor control of the adaptive immune responses. *Nat Immunol.* **5**(10), pp.987-995.

Iwase, T., Uehara, Y., Shinji, H., Tajima, A., Seo, H., Takada, K., Agata, T. and Mizunoe, Y. 2010. Staphylococcus epidermidis Esp inhibits Staphylococcus aureus biofilm formation and nasal colonization. *Nature.* **465**(7296), pp.346-349.

Iwatsuki, K., Yamasaki, O., Morizane, S. and Oono, T. 2006. Staphylococcal cutaneous infections: Invasion, evasion and aggression. *Journal of Dermatological Science.* **42**(3), pp.203-214.

James, W.D., Berger, T. and Elston, D.M. 2005. *Andrews' Diseases of the Skin: Clinical Dermatology.* 10 ed. Saunders.

Jansen, P.A.M., Rodijk-Olthuis, D., Hollox, E.J., Kamsteeg, M., Tjabringa, G.S., de Jongh, G.J., van Vlijmen-Willems, I.M.J.J., Bergboer, J.G.M., van Rossum, M.M., de Jong, E.M.G.J., den Heijer, M., Evers, A.W.M., Bergers, M., Armour, J.A.L., Zeeuwen, P.L.J.M. and Schalkwijk, J. 2009. β-Defensin-2 Protein Is a Serum Biomarker for Disease Activity in Psoriasis and Reaches Biologically Relevant Concentrations in Lesional Skin. *PLoS ONE.* **4**(3), pe4725.

Janssens, M., van Smeden, J., Gooris, G.S., Bras, W., Portale, G., Caspers, P.J., Vreeken, R.J., Hankemeier, T., Kezic, S., Wolterbeek, R., Lavrijsen, A.P. and Bouwstra, J.A. 2012. Increase in short-chain ceramides correlates with an altered lipid organization and decreased barrier function in atopic eczema patients. *Journal of Lipid Research.* **53**(12), pp.2755-2766.

Jeng, L., Yamshchikov, A.V., Judd, S.E., Blumberg, H.M., Martin, G.S., Ziegler, T.R. and Tangpricha, V. 2009. Alterations in vitamin D status and anti-microbial peptide levels in patients in the intensive care unit with sepsis. *Journal of Translational Medicine.* **7**, pp.28-28.

Jenkins, A., Diep, B.A., Mai, T.T., Vo, N.H., Warrener, P., Suzich, J., Stover, C.K. and Sellman, B.R. 2015. Differential Expression and Roles of Staphylococcus aureus Virulence Determinants during Colonization and Disease. *mBio.* **6**(1), pp.e02272-02214.

Jeon, C., Yan, D., Nakamura, M., Sekhon, S., Bhutani, T., Berger, T. and Liao, W. 2017. Frequency and Management of Sleep Disturbance in Adults with Atopic Dermatitis: A Systematic Review. *Dermatology and Therapy.* **7**(3), pp.349-364.

Jiang, W.G., Sanders, A.J., Ruge, F. and Harding, K.G. 2012. Influence of interleukin-8 (IL-8) and IL-8 receptors on the migration of human keratinocytes, the role of PLC-γ and potential clinical implications. *Experimental and therapeutic medicine.* **3**(2), pp.231-236.

Jiao, D., Wong, C.-K., Qiu, H.-N., Dong, J., Cai, Z., Chu, M., Hon, K.-L., Tsang, M.S.-M. and Lam, C.W.-K. 2015. NOD2 and TLR2 ligands trigger the activation of basophils and eosinophils by interacting with dermal fibroblasts in atopic dermatitis-like skin inflammation. *Cellular and Molecular Immunology.* **13**(4), pp.535-550.

Jin, S., Park, C.O., Shin, J.U., Noh, J.Y., Lee, Y.S., Lee, N.R., Kim, H.R., Noh, S., Lee, Y., Lee, J.-H. and Lee, K.H. 2014. DAMP molecules S100A9 and S100A8 activated by IL-17A and house-dust mites are increased in atopic dermatitis. *Experimental Dermatology.* **23**(12), pp.938-941.

Johansson, E.K., Bergström, A., Kull, I., Lind, T., Söderhäll, C., van Hage, M., Wickman, M., Ballardini, N. and Wahlgren, C.-F. 2017. IgE sensitization in relation to preschool eczema and filaggrin mutation. *Journal of Allergy and Clinical Immunology.* **140**(6), pp.1572-1579.e1575.

Joly, S., Maze, C., McCray, P.B. and Guthmiller, J.M. 2004. Human β-Defensins 2 and 3 Demonstrate Strain-Selective Activity against Oral Microorganisms. *Journal of Clinical Microbiology.* **42**(3), pp.1024-1029.

Jones, P.H. and Watt, F.M. 1993. Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. *Cell.* **73**(4), pp.713-724.

Joo, H.S. and Otto, M. 2015. Mechanisms of resistance to antimicrobial peptides in staphylococci. *Biochim Biophys Acta.* **1848**(11 Pt B), pp.3055-3061.

Jordan, B. 2000. How to set up an ELISA. *Methods Mol Med.* **40**, pp.373-379.

Josefson, D. 2001. Bacteria killer found in sweat. *BMJ.* **323**(7323), pp.1206-1206.

Jung, M.Y., Chung, J.Y., Lee, H.Y., Park, J., Lee, D.Y. and Yang, J.M. 2015. Antibiotic Susceptibility of Staphylococcus aureus in Atopic Dermatitis: Current Prevalence of Methicillin-Resistant Staphylococcus aureus in Korea and Treatment Strategies. *Ann Dermatol.* **27**(4), pp.398-403.

Jung, S., Mysliwy, J., Spudy, B., Lorenzen, I., Reiss, K., Gelhaus, C., Podschun, R., Leippe, M. and Grotzinger, J. 2011. Human beta-defensin 2 and beta-defensin 3 chimeric peptides reveal the structural basis of the pathogen specificity of their parent molecules. *Antimicrob Agents Chemother.* **55**(3), pp.954-960.

Kagami, S., Saeki, H., Komine, M., Kakinuma, T., Tsunemi, Y., Nakamura, K., Sasaki, K., Asahina, A. and Tamaki, K. 2005. Interleukin-4 and interleukin-13 enhance CCL26 production in a human keratinocyte cell line, HaCaT cells. *Clinical and Experimental Immunology.* **141**(3), pp.459-466.

Kalinin, A.E., Kajava, A.V. and Steinert, P.M. 2002. Epithelial barrier function: assembly and structural features of the cornified cell envelope. *Bioessays.* **24**(9), pp.789-800.

Kaneko, K., Smetana-Just, U., Matsui, M., Young, A.R., John, S., Norval, M. and Walker, S.L. 2008. cis-urocanic acid initiates gene transcription in primary human keratinocytes. *Journal of Immunology.* **181**(1), pp.217-224.

Karlsson-Kanth, A., Tegmark-Wisell, K., Arvidson, S. and Oscarsson, J. 2006. Natural human isolates of Staphylococcus aureus selected for high production of proteases and alpha-hemolysin are sigmaB deficient. *Int J Med Microbiol.* **296**(4-5), pp.229-236.

Kato, Y., Pawankar, R., Kimura, Y. and Kawana, S. 2006. Increased expression of RANTES, CCR3 and CCR5 in the lesional skin of patients with atopic eczema. *Int Arch Allergy Immunol.* **139**(3), pp.245-257.

Kaufman, B.P., Guttman-Yassky, E. and Alexis, A.F. 2018. Atopic dermatitis in diverse racial and ethnic groups—Variations in epidemiology, genetics, clinical presentation and treatment. *Experimental Dermatology.* **27**(4), pp.340-357.

Kawai, T. and Akira, S. 2007a. Signaling to NF-kappaB by Toll-like receptors. *Trends Mol Med.* **13**(11), pp.460-469.

Kawai, T. and Akira, S. 2007b. TLR signaling. *Seminars in Immunology.* **19**(1), pp.24-32.

Kekki, O.M., Scheynius, A., Poikonen, S., Koskinen, A., Kautiainen, H. and Turjanmaa, K. 2013. Sensitization to Malassezia in children with atopic dermatitis combined with food allergy. *Pediatric Allergy and Immunology.* **24**(3), pp.244-249.

Kelleher, M., Dunn-Galvin, A., Hourihane, J.O.B., Murray, D., Campbell, L.E., McLean, W.H.I. and Irvine, A.D. 2015. Skin barrier dysfunction measured by transepidermal water loss at 2 days and 2 months predates and predicts atopic dermatitis at 1 year. *Journal of Allergy and Clinical Immunology.* **135**(4), pp.930-935.e931.

Kelly, S.E., Jones, D.B. and Fleming, S. 1989. Calgranulin expression in inflammatory dermatoses. *The Journal of Pathology.* **159**(1), pp.17-21.

Kelsey, J.A., Bayles, K.W., Shafii, B. and McGuire, M.A. 2006. Fatty acids and monoacylglycerols inhibit growth of Staphylococcus aureus. *Lipids.* **41**(10), pp.951-961.

Kennedy, E.A., Connolly, J., Hourihane, J.O., Fallon, P.G., McLean, W.H.I., Murray, D., Jo, J.H., Segre, J.A., Kong, H.H. and Irvine, A.D. 2017. 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. *J Allergy Clin Immunol.* **139**(1), pp.166-172.

Kezic, S., O'Regan, G.M., Yau, N., Sandilands, A., Chen, H., Campbell, L.E., Kroboth, K., Watson, R., Rowland, M., Irwin McLean, W.H. and Irvine, A.D. 2011. Levels of filaggrin degradation products are influenced by both filaggrin genotype and atopic dermatitis severity. *Allergy.* **66**(7), pp.934-940.

Kiatsurayanon, C., Niyonsaba, F., Smithrithee, R., Akiyama, T., Ushio, H., Hara, M., Okumura, K., Ikeda, S. and Ogawa, H. 2014. Host Defense (Antimicrobial) Peptide, Human β-Defensin-3, Improves the Function of the Epithelial Tight-Junction Barrier in Human Keratinocytes. *Journal of Investigative Dermatology.* **134**(8), pp.2163-2173.

Kielian, T., Cheung, A. and Hickey, W.F. 2001. Diminished virulence of an alpha-toxin mutant of Staphylococcus aureus in experimental brain abscesses. *Infection and immunity.* **69**(11), pp.6902-6911.

Kim, B.E., Bin, L., Ye, Y.-M., Ramamoorthy, P. and Leung, D.Y.M. 2013. IL-25 Enhances HSV-1 Replication by Inhibiting Filaggrin Expression, and Acts Synergistically with Th2 Cytokines to Enhance HSV-1 Replication. *Journal of Investigative Dermatology.* **133**(12), pp.2678-2685.

Kim, J.E., Kim, B.J., Jeong, M.S., Seo, S.J., Kim, M.N., Hong, C.K. and Ro, B.I. 2005. Expression and Modulation of LL-37 in Normal Human Keratinocytes, HaCaT cells, and Inflammatory Skin Diseases. *J Korean Med Sci.* **20**(4), pp.649-654.

Kim, S.-J., Lee, J.Y., Jun, D.Y., Song, J.-Y., Lee, W.-K., Cho, M.-J. and Kim, Y.H. 2009. Oral administration of Lactococcus lactis expressing Helicobacter pylori Cag7-ct383 protein induces systemic anti-Cag7 immune response in mice. *FEMS Immunol Med Microbiol.* **57**(3), pp.257-268.

Kime, L., Randall, C.P., Banda, F.I., Wright, J., Richardson, J., Empel, J., Parkhill, J. and O'Neill, A.J. 2019. Transient silencing of antibiotic resistance by mutation in Staphylococcus aureus represents a major potential source of unanticipated therapeutic failure. Manuscript in preparation.

Kimura, T., Sugaya, M., Suga, H., Morimura, S., Miyamoto, A., Kai, H., Kagami, S., Yanaba, K., Fujita, H., Asano, Y., Tada, Y., Kadono, T. and Sato, S. 2014. Variations in serum TARC and I-TAC levels reflect minor changes in disease activity and pruritus in atopic dermatitis. *Acta Derm Venereol.* **94**(3), pp.331-332.

Kirschner, N., Bohner, C., Rachow, S. and Brandner, J.M. 2010. Tight junctions: is there a role in dermatology? *Arch Dermatol Res.* **302**(7), pp.483-493.

Kisich, K.O., Carspecken, C.W., Fiéve, S., Boguniewicz, M. and Leung, D.Y.M. 2008. Defective killing of Staphylococcus aureus in atopic dermatitis is associated with reduced mobilization of human β-defensin-3. *Journal of Allergy and Clinical Immunology.* **122**(1), pp.62-68.

Knapp, H.R. and Melly, M.A. 1986. Bactericidal Effects of Polyunsaturated Fatty Acids. *Journal of Infectious Diseases.* **154**(1), pp.84-94.

Kobayashi, T., Glatz, M., Horiuchi, K., Kawasaki, H., Akiyama, H., Kaplan, Daniel H., Kong, Heidi H., Amagai, M. and Nagao, K. 2015. Dysbiosis and Staphylococcus aureus Colonization Drives Inflammation in Atopic Dermatitis. *Immunity.* **42**(4), pp.756-766.

Kocianova, S., Vuong, C., Yao, Y., Voyich, J.M., Fischer, E.R., DeLeo, F.R. and Otto, M. 2005. Key role of poly-gamma-DL-glutamic acid in immune evasion and virulence of Staphylococcus epidermidis. *The Journal of clinical investigation.* **115**(3), pp.688-694.

Kolar, S.L., Antonio Ibarra, J., Rivera, F.E., Mootz, J.M., Davenport, J.E., Stevens, S.M., Horswill, A.R. and Shaw, L.N. 2013. Extracellular proteases are key mediators of Staphylococcus aureus virulence via the global modulation of virulence-determinant stability. *MicrobiologyOpen.* **2**(1), pp.18-34.

Kolb, L. and Ferrer-Bruker, S.J. 2019. Atopic Dermatitis. *StatPearls.* Treasure Island (FL): StatPearls Publishing LLC.

Kong, C., Neoh, H.-M. and Nathan, S. 2016. Targeting Staphylococcus aureus Toxins: A Potential form of Anti-Virulence Therapy. *Toxins (Basel).* **8**(3), p72.

Kong, H.H., Oh, J., Deming, C., Conlan, S., Grice, E.A., Beatson, M.A., Nomicos, E., Polley, E.C., Komarow, H.D., Murray, P.R., Turner, M.L. and Segre, J.A. 2012. Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. *Genome Res.* **22**.

Koppenol-Raab, M., Sjoelund, V., Manes, N.P., Gottschalk, R.A., Dutta, B., Benet, Z.L., Fraser, I.D.C. and Nita-Lazar, A. 2017. Proteome and Secretome Analysis Reveals Differential Post-transcriptional Regulation of Toll-like Receptor Responses. *Molecular & cellular proteomics : MCP.* **16**(4 suppl 1), pp.S172-S186.

Kretschmer, D., Gleske, A.K., Rautenberg, M., Wang, R., Koberle, M., Bohn, E., Schoneberg, T., Rabiet, M.J., Boulay, F., Klebanoff, S.J., van Kessel, K.A., van Strijp, J.A., Otto, M. and Peschel, A. 2010. Human formyl peptide receptor 2 senses highly pathogenic Staphylococcus aureus. *Cell Host Microbe.* **7**(6), pp.463-473.

Krisanaprakornkit, S., Jotikasthira, D. and Dale, B.A. 2003. Intracellular calcium in signaling human beta-defensin-2 expression in oral epithelial cells. *J Dent Res.* **82**(11), pp.877-882.

Krisanaprakornkit, S., Kimball, J.R. and Dale, B.A. 2002. Regulation of Human -Defensin-2 in Gingival Epithelial Cells: The Involvement of Mitogen-Activated Protein

Kinase Pathways, But Not the NF- B Transcription Factor Family. *The Journal of Immunology.* **168**(1), pp.316-324.

Krogius-Kurikka, L., Lyra, A., Malinen, E., Aarnikunnas, J., Tuimala, J., Paulin, L., Makivuokko, H., Kajander, K. and Palva, A. 2009. Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers. *BMC Gastroenterol.* **9**, p95.

Kubo, T., Morita, H., Sugita, K. and Akdis, C.A. 2017. Chapter 1 - Introduction to Mechanisms of Allergic Diseases. In: O'Hehir, R.E., et al. eds. *Middleton's Allergy Essentials.* Elsevier, pp.1-27.

Kuo, I.H., Yoshida, T., De Benedetto, A. and Beck, L.A. 2013. The cutaneous innate immune response in patients with atopic dermatitis. *J Allergy Clin Immunol.* **131**.

Kusch, H. and Engelmann, S. 2014. Secrets of the secretome in Staphylococcus aureus. *Int J Med Microbiol.* **304**(2), pp.133-141.

Laborel-Préneron, E., Bianchi, P., Boralevi, F., Lehours, P., Fraysse, F., Morice-Picard, F., Sugai, M., Sato'O, Y., Badiou, C., Lina, G., Schmitt, A.-M., Redoulès, D., Casas, C. and Davrinche, C. 2015. Effects of the Staphylococcus aureus and Staphylococcus epidermidis Secretomes Isolated from the Skin Microbiota of Atopic Children on CD4+ T Cell Activation. *PLoS ONE.* **10**(10), pe0141067.

Lai, Y., Cogen, A.L., Radek, K.A., Park, H.J., Macleod, D.T., Leichtle, A., Ryan, A.F., Di Nardo, A. and Gallo, R.L. 2010. Activation of TLR2 by a small molecule produced by Staphylococcus epidermidis increases antimicrobial defense against bacterial skin infections. *J Invest Dermatol.* **130**(9), pp.2211-2221.

Lai, Y. and Gallo, R.L. 2008. Toll-like receptors in skin infections and inflammatory diseases. *Infectious disorders drug targets.* **8**(3), pp.144-155.

Lai, Y., Villaruz, A.E., Li, M., Cha, D.J., Sturdevant, D.E. and Otto, M. 2007. The human anionic antimicrobial peptide dermcidin induces proteolytic defence mechanisms in staphylococci. *Molecular Microbiology.* **63**(2), pp.497-506.

Lan, C.C.E., Wu, C.S., Huang, S.M., Wu, I.H. and Chen, G.S. 2013. High-Glucose Environment Enhanced Oxidative Stress and Increased Interleukin-8 Secretion From Keratinocytes: New Insights Into Impaired Diabetic Wound Healing. *Diabetes.* **62**(7), pp.2530-2538.

Landmann, L. 1988. The epidermal permeability barrier. *Anatomy and Embryology.* **178**(1), pp.1-13.

Langan, S.M. and Irvine, A.D. 2013. Childhood Eczema and the Importance of the Physical Environment. *J Invest Dermatol.* **133**(7), pp.1706-1709.

Langer, K., Breuer, K., Kapp, A. and Werfel, T. 2007. Staphylococcus aureus-derived enterotoxins enhance house dust mite-induced patch test reactions in atopic dermatitis. *Experimental Dermatology.* **16**(2), pp.124-129.

Larsen, N., Vogensen, F.K., van den Berg, F.W., Nielsen, D.S., Andreasen, A.S., Pedersen, B.K., Al-Soud, W.A., Sorensen, S.J., Hansen, L.H. and Jakobsen, M. 2010. Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. *PLoS One.* **5**(2), pe9085.

Lauderdale, K.J., Boles, B.R., Cheung, A.L. and Horswill, A.R. 2009. Interconnections between Sigma B, agr, and Proteolytic Activity in Staphylococcus aureus Biofilm Maturation. *Infection and Immunity.* **77**(4), pp.1623-1635.

Le, K.Y. and Otto, M. 2015. Quorum-sensing regulation in staphylococci-an overview. *Frontiers in microbiology.* **6**, pp.1174-1174.

Lebwohl, M.G., Del Rosso, J.Q., Abramovits, W., Berman, B., Cohen, D.E., Guttman-Yassky, E., Mancini, A.J. and Schachner, L.A. 2013. Pathways to Managing Atopic Dermatitis: Consensus From the Experts. *The Journal of Clinical and Aesthetic Dermatology.* **6**(7 Suppl), pp.S2-S18.

Lee, A.S., Cooper, B.S., Malhotra-Kumar, S., Chalfine, A., Daikos, G.L., Fankhauser, C., Carevic, B., Lemmen, S., Martínez, J.A., Masuet-Aumatell, C., Pan, A., Phillips, G., Rubinovitch, B., Goossens, H., Brun-Buisson, C. and Harbarth, S. 2013. Comparison of strategies to reduce meticillin-resistant Staphylococcus aureus rates in surgical patients: a controlled multicentre intervention trial. *BMJ Open.* **3**(9), pe003126.

Lee, C.-H. and Hwang, S.T.-Y. 2012. Pathophysiology of chemokines and chemokine receptors in dermatological science: A focus on psoriasis and cutaneous T-cell lymphoma. *Dermatologica Sinica.* **30**(4), pp.128-135.

Lee, N.R., Lee, H.-J., Yoon, N.Y., Kim, D., Jung, M. and Choi, E.H. 2016. Application of Topical Acids Improves Atopic Dermatitis in Murine Model by Enhancement of Skin Barrier Functions Regardless of the Origin of Acids. *Annals of dermatology.* **28**(6), pp.690-696.

Lee, S.-C. 2016. Various diagnostic criteria for atopic dermatitis (AD): A proposal of Reliable Estimation of Atopic Dermatitis in Childhood (REACH) criteria, a novel questionnaire-based diagnostic tool for AD. *The Journal of Dermatology.* **43**(4), pp.376-384.

Lee, S.-Y., Lee, E., Park, Y.M. and Hong, S.-J. 2018. Microbiome in the Gut-Skin Axis in Atopic Dermatitis. *Allergy, Asthma & Immunology Research.* **10**(4), p354.

Lee, W.J., Park, K.H., Cha, H.W., Sohn, M.Y., Park, K.D., Lee, S.-J. and Kim, D.W. 2014. The Expression of Involucrin, Loricrin, and Filaggrin in Cultured Sebocytes. *Annals of dermatology.* **26**(1), pp.134-137.

Leung, D.Y.M. 2003. Infection in atopic dermatitis. *Current Opinion in Pediatrics.* **15**(4), pp.399-404.

Leung, D.Y.M. 2013. New Insights into Atopic Dermatitis: Role of Skin Barrier and Immune Dysregulation. *Allergology International.* **62**(2), pp.151-161.

Leung, D.Y.M. and Bieber, T. 2003. Atopic dermatitis. *The Lancet.* **361**(9352), pp.151-160.

Leung, D.Y.M., Jain, N. and Leo, H.L. 2003. New concepts in the pathogenesis of atopic dermatitis. *Current Opinion in Immunology.* **15**(6), pp.634-638.

Leung, D.Y.M., Travers, J.B. and Norris, D.A. 1995. The role of superantigens in skin disease. *J Invest Dermatol.* **105**(1 Suppl), pp.37s-42s.

Ley, R.E., Turnbaugh, P.J., Klein, S. and Gordon, J.I. 2006. Microbial ecology: human gut microbes associated with obesity. *Nature.* **444**(7122), pp.1022-1023.

Leyden, J.J., Marples, R.R. and Kligman, A.M. 1974. Staphylococcus aureus in the lesions of atopic dermatitis. *British Journal of Dermatology.* **90**(5), pp.525-525.

Li, H., Xu, L., Wang, J., Wen, Y., Vuong, C., Otto, M. and Gao, Q. 2005. Conversion of Staphylococcus epidermidis Strains from Commensal to Invasive by Expression of the ica Locus Encoding Production of Biofilm Exopolysaccharide. *Infection and Immunity.* **73**(5), pp.3188-3191.

Liang, S.C., Tan, X.-Y., Luxenberg, D.P., Karim, R., Dunussi-Joannopoulos, K., Collins, M. and Fouser, L.A. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *The Journal of Experimental Medicine.* **203**(10), pp.2271-2279.

Lim, X., Tan, S.H., Koh, W.L.C., Chau, R.M.W., Yan, K.S., Kuo, C.J., van Amerongen, R., Klein, A.M. and Nusse, R. 2013. Interfollicular epidermal stem cells self-renew via autocrine Wnt signaling. *Science (New York, N.Y.).* **342**(6163), pp.1226-1230.

Lin, Y.-T., Wang, C.-T. and Chiang, B.-L. 2007. Role of Bacterial Pathogens in Atopic Dermatitis. *Clinical Reviews in Allergy & Immunology.* **33**(3), pp.167-177.

Lindsay, J.A. and Foster, S.J. 1999. Interactive regulatory pathways control virulence determinant production and stability in response to environmental conditions in Staphylococcus aureus. *Mol Gen Genet.* **262**(2), pp.323-331.

Liu, A.Y., Destoumieux, D., Wong, A.V., Park, C.H., Valore, E.V., Liu, L. and Ganz, T. 2002. Human β-Defensin-2 Production in Keratinocytes is Regulated by Interleukin-1, Bacteria, and the State of Differentiation. *Journal of Investigative Dermatology.* **118**(2), pp.275-281.

Liu, C., Ouyang, W., Xia, J., Sun, X., Zhao, L. and Xu, F. 2018. Tumor Necrosis Factor-alpha Is Required for Mast Cell-Mediated Host Immunity Against Cutaneous Staphylococcus aureus Infection. *J Infect Dis.* **218**(1), pp.64-74.

Liu, G., Saxena, D., Deng, H., Norman, R.G., Chen, Z., Abrams, W.R., Malamud, D. and Li, Y. 2010. Effect of protease inhibitors on the quantitative and qualitative assessment of oral microorganisms. *FEMS Microbiology Letters.* **312**(1), pp.63-70.

Liu, H., Lv, J., Qi, X., Ding, Y., Li, D., Hu, L., Wang, L. and Yu, F. 2015. The carriage of the serine-aspartate repeat protein-encoding sdr genes among Staphylococcus aureus lineages. *Braz J Infect Dis.* **19**(5), pp.498-502.

Liu, P.-F., Cheng, J.-S., Sy, C.-L., Huang, W.-C., Yang, H.-C., Gallo, R.L., Huang, C.-M. and Shu, C.-W. 2015. IsaB Inhibits Autophagic Flux to Promote Host Transmission of Methicillin-Resistant Staphylococcus aureus. *The Journal of investigative dermatology.* **135**(11), pp.2714-2722.

Liu, W., Dong, S.L., Xu, F., Wang, X.Q., Withers, T.R., Yu, H.D. and Wang, X. 2013. Effect of Intracellular Expression of Antimicrobial Peptide LL-37 on Growth of Escherichia coli Strain TOP10 under Aerobic and Anaerobic Conditions. *Antimicrobial Agents and Chemotherapy.* **57**(10), pp.4707-4716.

Llewelyn, M. and Cohen, J. 2002. Superantigens: microbial agents that corrupt immunity. *The Lancet. Infectious diseases.* **2**(3), pp.156-162.

Llor, C. and Bjerrum, L. 2014. Antimicrobial resistance: risk associated with antibiotic overuse and initiatives to reduce the problem. *Therapeutic Advances in Drug Safety.* **5**(6), pp.229-241.

Love, J.F., Tran-Winkler, H.J. and Wessels, M.R. 2012. Vitamin D and the Human Antimicrobial Peptide LL-37 Enhance Group A Streptococcus Resistance to Killing by Human Cells. *mBio.* **3**(5), pp.e00394-00312.

Lowy, F.D. 1998. Staphylococcus aureus Infections. *New England Journal of Medicine.* **339**(8), pp.520-532.

Lu, T., Jiao, X., Si, M., He, P., Zou, J., Zhang, S. and Zeng, K. 2016. The Correlation of Serums CCL11, CCL17, CCL26, and CCL27 and Disease Severity in Patients with Urticaria. *Disease markers.* **2016**, pp.1381760-1381760.

Lyon, G.J. and Novick, R.P. 2004. Peptide signaling in Staphylococcus aureus and other Gram-positive bacteria. *Peptides.* **25**(9), pp.1389-1403.

Mühl, H. and Pfeilschifter, J. 2003. Anti-inflammatory properties of pro-inflammatory interferon-γ. *International Immunopharmacology.* **3**(9), pp.1247-1255.

Macaluso, F., Nothnagel, M., Parwez, Q., Petrasch-Parwez, E., Bechara, F.G., Epplen, J.T. and Hoffjan, S. 2007. Polymorphisms in NACHT-LRR (NLR) genes in atopic dermatitis. *Experimental Dermatology.* **16**(8), pp.692-698.

Machura, E., Mazur, B., Golemiec, E., Pindel, M. and Halkiewicz, F. 2008. Staphylococcus aureus skin colonization in atopic dermatitis children is associated with decreased IFN-γ production by peripheral blood CD4+ and CD8+ T cells. *Pediatric Allergy and Immunology.* **19**(1), pp.37-45.

Mackey-Lawrence, N.M. and Jefferson, K.K. 2013. Regulation of Staphylococcus aureus immunodominant antigen B (IsaB). *Microbiological research.* **168**(2), pp.113-118.

Mackey-Lawrence, N.M., Potter, D.E., Cerca, N. and Jefferson, K.K. 2009. Staphylococcus aureus immunodominant surface antigen B is a cell-surface associated nucleic acid binding protein. *BMC Microbiol.* **9**, p61.

Mahlapuu, M., Håkansson, J., Ringstad, L. and Björn, C. 2016. Antimicrobial Peptides: An Emerging Category of Therapeutic Agents. *Frontiers in cellular and infection microbiology.* **6**, pp.194-194.

Majoie, I.M., Oldhoff, J.M., van Weelden, H., Laaper-Ertmann, M., Bousema, M.T., Sigurdsson, V., Knol, E.F., Bruijnzeel-Koomen, C.A. and de Bruin-Weller, M.S. 2009. Narrowband ultraviolet B and medium-dose ultraviolet A1 are equally effective in the treatment of moderate to severe atopic dermatitis. *Journal of the American Academy of Dermatology.* **60**(1), pp.77-84.

Malik, E., Dennison, S., Harris, F. and Phoenix, D. 2016. pH Dependent Antimicrobial Peptides and Proteins, Their Mechanisms of Action and Potential as Therapeutic Agents. *Pharmaceuticals (Basel).* **9**(4), p67.

Mallbris, L., Carlén, L., Wei, T., Heilborn, J., Nilsson, M.F., Granath, F. and Ståhle, M. 2010. Injury downregulates the expression of the human cathelicidin protein hCAP18/LL-37 in atopic dermatitis. *Experimental Dermatology.* **19**(5), pp.442-449.

Mandron, M., Ariès, M.-F., Brehm, R.D., Tranter, H.S., Acharya, K.R., Charveron, M. and Davrinche, C. 2006. Human dendritic cells conditioned with Staphylococcus aureus enterotoxin B promote TH2 cell polarization. *Journal of Allergy and Clinical Immunology.* **117**(5), pp.1141-1147.

Manku, M.S., Horrobin, D.F., Morse, N.L., Wright, S. and Burton, J.L. 1984. Essential fatty acids in the plasma phospholipids of patients with atopic eczema. *British Journal of Dermatology.* **110**(6), pp.643-648.

Marks, J.G. and Miller, J. 2006. *Lookingbill and Marks' Principles of Dermatology.* 4 ed. Elsevier Inc.

Matheson, M. 1999. Gram stain. *Community eye health.* **12**(30), pp.24-24.

Mathews, M., Jia, H.P., Guthmiller, J.M., Losh, G., Graham, S., Johnson, G.K., Tack, B.F. and McCray, P.B. 1999. Production of β-Defensin Antimicrobial Peptides by the Oral Mucosa and Salivary Glands. *Infection and Immunity.* **67**(6), pp.2740-2745.

Matsunaga, M.C. and Yamauchi, P.S. 2016. IL-4 and IL-13 Inhibition in Atopic Dermatitis. *J Drugs Dermatol.* **15**(8), pp.925-929.

Mauldin, E.A. and Peters-Kennedy, J. 2016. Chapter 6 - Integumentary System. In: Maxie, M.G. ed. *Jubb, Kennedy & Palmer's Pathology of Domestic Animals: Volume 1 (Sixth Edition).* W.B. Saunders, pp.509-736.e501.

Mazzoleni, G., Di Lorenzo, D. and Steimberg, N. 2009. Modelling tissues in 3D: the next future of pharmaco-toxicology and food research? *Genes Nutr.* **4**(1), pp.13-22.

McAdow, M., Missiakas, D.M. and Schneewind, O. 2012. Staphylococcus aureus Secretes Coagulase and von Willebrand Factor Binding Protein to Modify the Coagulation Cascade and Establish Host Infections. *Journal of Innate Immunity.* **4**(2), pp.141-148.

McAleer, M.A. and Irvine, A.D. 2013. The multifunctional role of filaggrin in allergic skin disease. *J Allergy Clin Immunol.* **131**(2), pp.280-291.

McCourt, J., O'Halloran, D.P., McCarthy, H., O'Gara, J.P. and Geoghegan, J.A. 2014. Fibronectin-binding proteins are required for biofilm formation by community-associated methicillin-resistant Staphylococcus aureus strain LAC. **353**(2), pp.157-164.

McKenney, D., Pouliot, K., Wang, Y., Murthy, V., Ulrich, M., Döring, G., Lee, J.C., Goldmann, D.A. and Pier, G.B. 2000. Vaccine potential of poly-1-6 β-d-N-succinylglucosamine, an immunoprotective surface polysaccharide of Staphylococcus aureus and Staphylococcus epidermidis. *Journal of Biotechnology.* **83**(1–2), pp.37-44.

McPherson, T. 2016. Current Understanding in Pathogenesis of Atopic Dermatitis. *Indian journal of dermatology.* **61**(6), pp.649-655.

Medzhitov, R. 2001. Toll-like receptors and innate immunity. *Nat Rev Immunol.* **1**(2), pp.135-145.

Medzhitov, R. and Janeway, C. 2000. Innate Immunity. *New England Journal of Medicine.* **343**(5), pp.338-344.

Megna, M., Napolitano, M., Patruno, C., Villani, A., Balato, A., Monfrecola, G., Ayala, F. and Balato, N. 2016. Systemic Treatment of Adult Atopic Dermatitis: A Review. *Dermatology and therapy.* **7**(1), pp.1-23.

Mehlin, C., Headley, C.M. and Klebanoff, S.J. 1999. An inflammatory polypeptide complex from Staphylococcus epidermidis: isolation and characterization. *J Exp Med.* **189**(6), pp.907-918.

Mekki, M.S., Mougel, A., Vinchent, A., Paquet, C., Copin, M.-C., Leroy, C., Kherrouche, Z., Bonte, J.-P., Melnyk, O., Vicogne, J. and Tulasne, D. 2018. Hypoxia leads to decreased autophosphorylation of the MET receptor but promotes its resistance to tyrosine kinase inhibitors. *Oncotarget.* **9**(43).

Méndez-Samperio, P. 2013. Recent advances in the field of antimicrobial peptides in inflammatory diseases. *Advanced biomedical research.* **2**, pp.50-50.

Meng, J., Moriyama, M., Feld, M., Buddenkotte, J., Buhl, T., Szollosi, A., Zhang, J., Miller, P., Ghetti, A., Fischer, M., Reeh, P.W., Shan, C., Wang, J. and Steinhoff, M. 2018. New mechanism underlying IL-31-induced atopic dermatitis. *J Allergy Clin Immunol.* **141**(5), pp.1677-1689.e1678.

Menzies, B.E. and Kenoyer, A. 2005. Staphylococcus aureus Infection of Epidermal Keratinocytes Promotes Expression of Innate Antimicrobial Peptides. *Infection and Immunity.* **73**(8), pp.5241-5244.

Menzies, B.E. and Kenoyer, A. 2006. Signal Transduction and Nuclear Responses in Staphylococcus aureus- Induced Expression of Human -Defensin 3 in Skin Keratinocytes. *Infection and Immunity.* **74**(12), pp.6847-6854.

Méric, G., Miragaia, M., de Been, M., Yahara, K., Pascoe, B., Mageiros, L., Mikhail, J., Harris, L.G., Wilkinson, T.S., Rolo, J., Lamble, S., Bray, J.E., Jolley, K.A., Hanage, W.P., Bowden, R., Maiden, M.C.J., Mack, D., de Lencastre, H., Feil, E.J., Corander, J. and Sheppard, S.K. 2015. Ecological Overlap and Horizontal Gene Transfer in Staphylococcus aureus and Staphylococcus epidermidis. *Genome Biology and Evolution.* **7**(5), pp.1313-1328.

Miajlovic, H., Fallon, P.G., Irvine, A.D. and Foster, T.J. 2010. Effect of filaggrin breakdown products on growth of and protein expression by Staphylococcus aureus. *The Journal of Allergy and Clinical Immunology.* **126**(6), pp.1184-1190.e1183.

Michalek, M., Gelhaus, C., Hecht, O., Podschun, R., Schröder, J.M., Leippe, M. and Grötzinger, J. 2009. The human antimicrobial protein psoriasin acts by permeabilization of bacterial membranes. *Developmental & Comparative Immunology.* **33**(6), pp.740-746.

Midorikawa, K., Ouhara, K., Komatsuzawa, H., Kawai, T., Yamada, S., Fujiwara, T., Yamazaki, K., Sayama, K., Taubman, M.A., Kurihara, H., Hashimoto, K. and Sugai, M. 2003. Staphylococcus aureus Susceptibility to Innate Antimicrobial Peptides, β-Defensins and CAP18, Expressed by Human Keratinocytes. *Infection and Immunity.* **71**(7), pp.3730-3739.

Milani, M. 2016. The epidermal barrier function and antimicrobial peptides in atopic dermatitis: The role of topical modulation. *OA Dermatology.* **1**(1), p2.

Mildner, M., Ballaun, C., Stichenwirth, M., Bauer, R., Gmeiner, R., Buchberger, M., Mlitz, V. and Tschachler, E. 2006. Gene silencing in a human organotypic skin model. *Biochemical and Biophysical Research Communications.* **348**(1), pp.76-82.

Miller, L.S. 2008. Toll-Like Receptors in Skin. *Advances in Dermatology.* **24**, pp.71-87.

Miller, L.S. and Modlin, R.L. 2007. Toll-like receptors in the skin. *Semin Immunopathol.* **29**(1), pp.15-26.

Miquel, S., Martin, R., Rossi, O., Bermudez-Humaran, L.G., Chatel, J.M., Sokol, H., Thomas, M., Wells, J.M. and Langella, P. 2013. Faecalibacterium prausnitzii and human intestinal health. *Curr Opin Microbiol.* **16**(3), pp.255-261.

Mittermann, I., Wikberg, G., Johansson, C., Lupinek, C., Lundeberg, L., Crameri, R., Valenta, R. and Scheynius, A. 2016. IgE Sensitization Profiles Differ between Adult Patients with Severe and Moderate Atopic Dermatitis. *PLoS ONE.* **11**(5), pe0156077.

Mohamadzadeh, M., Muller, M., Hultsch, T., Enk, A., Saloga, J. and Knop, J. 1994. Enhanced expression of IL-8 in normal human keratinocytes and human keratinocyte cell line HaCaT in vitro after stimulation with contact sensitizers, tolerogens and irritants. *Exp Dermatol.* **3**(6), pp.298-303.

Monecke, S., Müller, E., Büchler, J., Stieber, B. and Ehricht, R. 2014. Staphylococcus aureus In Vitro Secretion of Alpha Toxin (hla) Correlates with the Affiliation to Clonal Complexes. *PLOS ONE.* **9**(6), pe100427.

Moran, J.C., Alorabi, J.A. and Horsburgh, M.J. 2017. Comparative Transcriptomics Reveals Discrete Survival Responses of S. aureus and S. epidermidis to Sapienic Acid. *Frontiers in Microbiology.* **8**, p33.

Morandi, S., Brasca, M., Andrighetto, C., Lombardi, A. and Lodi, R. 2009. Phenotypic and Genotypic Characterization of Staphylococcus aureus Strains from Italian Dairy Products. *International journal of microbiology.* **2009**, pp.501362-501362.

Moreillon, P., Entenza, J.M., Francioli, P., McDevitt, D., Foster, T.J., François, P. and Vaudaux, P. 1995. Role of Staphylococcus aureus coagulase and clumping factor in pathogenesis of experimental endocarditis. *Infection and Immunity.* **63**(12), pp.4738-4743.

Moriwaki, M., Iwamoto, K., Niitsu, Y., Matsushima, A., Yanase, Y., Hisatsune, J., Sugai, M. and Hide, M. 2019. Staphylococcus aureus from atopic dermatitis skin accumulates in the lysosomes of keratinocytes with induction of IL-1alpha secretion via TLR9. *Allergy.* **74**(3), pp.560-571.

Mørk, G., Schjerven, H., Mangschau, L., Søyland, E. and Brandtzaeg, P. 2003. Proinflammatory cytokines upregulate expression of calprotectin (L1 protein, MRP-8/MRP-14) in cultured human keratinocytes. *British Journal of Dermatology.* **149**(3), pp.484-491.

Mu, Q., Kirby, J., Reilly, C.M. and Luo, X.M. 2017. Leaky Gut As a Danger Signal for Autoimmune Diseases. *Frontiers in immunology.* **8**, pp.598-598.

Mukaida, N., Okamoto, S., Ishikawa, Y. and Matsushima, K. 1994. Molecular mechanism of interleukin-8 gene expression. *J Leukoc Biol.* **56**(5), pp.554-558.

Mukherjee, P. and Mani, S. 2013. Methodologies to decipher the cell secretome. *Biochimica et biophysica acta.* **1834**(11), pp.2226-2232.

Murakami, M., Ohtake, T., Dorschner, R.A., Gallo, R.L., Schittek, B. and Garbe, C. 2002. Cathelicidin Anti-Microbial Peptide Expression in Sweat, an Innate Defense System for the Skin. *Journal of Investigative Dermatology.* **119**(5), pp.1090-1095.

Nakamura, M.T. and Nara, T.Y. 2004. Structure, function, and dietary regulation of delta6, delta5, and delta9 desaturases. *Annual review of nutrition.* **24**, pp.345-376.

Nakashige, T.G., Zhang, B., Krebs, C. and Nolan, E.M. 2015. Human calprotectin is an iron-sequestering host-defense protein. *Nat Chem Biol.* **11**(10), pp.765-771.

Nakatsuji, T., Chen, T.H., Narala, S., Chun, K.A., Two, A.M., Yun, T., Shafiq, F., Kotol, P.F., Bouslimani, A., Melnik, A.V., Latif, H., Kim, J.N., Lockhart, A., Artis, K., David, G., Taylor, P., Streib, J., Dorrestein, P.C., Grier, A., Gill, S.R., Zengler, K., Hata, T.R., Leung, D.Y. and Gallo, R.L. 2017. Antimicrobials from human skin

commensal bacteria protect against Staphylococcus aureus and are deficient in atopic dermatitis. *Science translational medicine.* **9**(378).

Nakatsuji, T., Chen, T.H., Two, A.M., Chun, K.A., Narala, S., Geha, R.S., Hata, T.R. and Gallo, R.L. 2016. Staphylococcus aureus Exploits Epidermal Barrier Defects in Atopic Dermatitis to Trigger Cytokine Expression. *J Invest Dermatol.* **136**(11), pp.2192-2200.

Nakazato, J., Kishida, M., Kuroiwa, R., Fujiwara, J., Shimoda, M. and Shinomiya, N. 2008. Serum levels of Th2 chemokines, CCL17, CCL22, and CCL27, were the important markers of severity in infantile atopic dermatitis. *Pediatr Allergy Immunol.* **19**(7), pp.605-613.

Namkung, J.-H., Lee, J.-E., Kim, E., Kim, H.-J., Seo, E.-Y., Jang, H.-Y., Shin, E.-S., Cho, E.-Y. and Yang, J.-M. 2011. Association of polymorphisms in genes encoding IL-4, IL-13 and their receptors with atopic dermatitis in a Korean population. *Experimental Dermatology.* **20**(11), pp.915-919.

Namvar, A.E., Bastarahang, S., Abbasi, N., Ghehi, G.S., Farhadbakhtiarian, S., Arezi, P., Hosseini, M., Baravati, S.Z., Jokar, Z. and Chermahin, S.G. 2014. Clinical characteristics of Staphylococcus epidermidis: a systematic review. *GMS hygiene and infection control.* **9**(3), pp.Doc23-Doc23.

National Collaborating Centre for Women's and Children's Health. 2007. National Institute for Health and Clinical Excellence: Guidance. *Atopic Eczema in Children: Management of Atopic Eczema in Children from Birth up to the Age of 12 Years.* London: RCOG Press.

Nedoszytko, B., Sokołowska-Wojdyło, M., Ruckemann-Dziurdzińska, K., Roszkiewicz, J. and Nowicki, R.J. 2014. Chemokines and cytokines network in the pathogenesis of the inflammatory skin diseases: atopic dermatitis, psoriasis and skin mastocytosis. *Postepy dermatologii i alergologii.* **31**(2), pp.84-91.

Nell, M.J., Tjabringa, G.S., Vonk, M.J., Hiemstra, P.S. and Grote, J.J. 2004. Bacterial products increase expression of the human cathelicidin hCAP-18/LL-37 in cultured human sinus epithelial cells. *FEMS Immunol Med Microbiol.* **42**(2), pp.225-231.

Neufeld, K.M., Kang, N., Bienenstock, J. and Foster, J.A. 2011. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterol Motil.* **23**(3), pp.255-264, e119.

New England Biolabs. c2019. *NEB Tm Calculator.* [Online]. Available from: https://tmcalculator.neb.com/#!/main

Nguyen, M.T., Hanzelmann, D., Härtner, T., Peschel, A. and Götz, F. 2016. Skin-Specific Unsaturated Fatty Acids Boost the Staphylococcus aureus Innate Immune Response. *Infection and Immunity.* **84**(1), pp.205-215.

Nickel, R.G., Casolaro, V., Wahn, U., Beyer, K., Barnes, K.C., Plunkett, B.S., Freidhoff, L.R., Sengler, C., Plitt, J.R., Schleimer, R.P., Caraballo, L., Naidu, R.P., Levett, P.N., Beaty, T.H. and Huang, S.K. 2000. Atopic Dermatitis Is Associated with a Functional Mutation in the Promoter of the C-C Chemokine RANTES. *Journal of immunology (Baltimore, Md. : 1950).* **164**(3), pp.1612-1616.

Niebuhr, M., Gathmann, M., Scharonow, H., Mamerow, D., Mommert, S., Balaji, H. and Werfel, T. 2011a. Staphylococcal alpha-toxin is a strong inducer of interleukin-17 in humans. *Infect Immun.* **79**(4), pp.1615-1622.

Niebuhr, M., Mamerow, D., Heratizadeh, A., Satzger, I. and Werfel, T. 2011b. Staphylococcal α-Toxin Induces a Higher T Cell Proliferation and Interleukin-31 in Atopic Dermatitis. *International Archives of Allergy and Immunology.* **156**(4), pp.412-415.

Niebuhr, M., Scharonow, H., Gathmann, M., Mamerow, D. and Werfel, T. 2010. Staphylococcal exotoxins are strong inducers of IL-22: A potential role in atopic dermatitis. *J Allergy Clin Immunol.* **126**(6), pp.1176-1183.e1174.

Niessen, C.M. 2007. Tight junctions/adherens junctions: basic structure and function. *J Invest Dermatol.* **127**(11), pp.2525-2532.

Nishijima, S., Namura, S., Higashida, T. and Kawai, S. 1997. Staphylococcus Aureus in the Anterior Nares and Subungual Spaces of the Hands in Atopic Dermatitis. *Journal of International Medical Research.* **25**(3), pp.155-158.

Niyonsaba, F., Iwabuchi, K., Someya, A., Hirata, M., Matsuda, H., Ogawa, H. and Nagaoka, I. 2002. A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis. *Immunology.* **106**(1), pp.20-26.

Niyonsaba, F., Suzuki, A., Ushio, H., Nagaoka, I., Ogawa, H. and Okumura, K. 2009. The human antimicrobial peptide dermcidin activates normal human keratinocytes. *British Journal of Dermatology.* **160**(2), pp.243-249.

Niyonsaba, F., Ushio, H., Nakano, N., Ng, W., Sayama, K., Hashimoto, K., Nagaoka, I., Okumura, K. and Ogawa, H. 2006. Antimicrobial Peptides Human [beta]-Defensins Stimulate Epidermal Keratinocyte Migration, Proliferation and Production of Proinflammatory Cytokines and Chemokines. *J Invest Dermatol.* **127**(3), pp.594-604.

Nocadello, S., Minasov, G., Shuvalova, L., Dubrovska, I., Sabini, E., Bagnoli, F., Grandi, G. and Anderson, W.F. 2016. Crystal structures of the components of the Staphylococcus aureus leukotoxin ED. *Acta Crystallogr D Struct Biol.* **72**(1), pp.113-120.

Nodake, Y., Matsumoto, S., Miura, R., Honda, H., Ishibashi, G., Matsumoto, S., Dekio, I. and Sakakibara, R. 2015. Pilot study on novel skin care method by augmentation with Staphylococcus epidermidis, an autologous skin microbe – A blinded randomized clinical trial. *Journal of Dermatological Science.* **79**(2), pp.119-126.

Nomura, I., Gao, B., Boguniewicz, M., Darst, M.A., Travers, J.B. and Leung, D.Y.M. 2003a. Distinct patterns of gene expression in the skin lesions of atopic dermatitis and psoriasis. *Journal of Allergy and Clinical Immunology.* **112**(6), pp.1195-1202.

Nomura, I., Goleva, E., Howell, M.D., Hamid, Q.A., Ong, P.Y., Hall, C.F., Darst, M.A., Gao, B., Boguniewicz, M., Travers, J.B. and Leung, D.Y.M. 2003b. Cytokine Milieu of Atopic Dermatitis, as Compared to Psoriasis, Skin Prevents Induction of Innate Immune Response Genes. *The Journal of Immunology.* **171**(6), pp.3262-3269.

Noonan, F.P. and De Fabo, E.C. 1992. Immunosuppression by ultraviolet B radiation: initiation by urocanic acid. *Immunology Today.* **13**(7), pp.250-254.

Norris, D.A. 2005. Mechanisms of action of topical therapies and the rationale for combination therapy. *J Am Acad Dermatol.* **53**(1 Suppl 1), pp.S17-25.

Novak, N. 2012. An update on the role of human dendritic cells in patients with atopic dermatitis. *Journal of Allergy and Clinical Immunology.* **129**(4), pp.879-886.

Novick, R.P. 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Molecular Microbiology.* **48**(6), pp.1429-1449.

Nowicka, D. and Grywalska, E. 2018. The Role of Immune Defects and Colonization of Staphylococcus aureus in the Pathogenesis of Atopic Dermatitis. *Analytical cellular pathology (Amsterdam).* **2018**, pp.1956403-1956403.

Nowicki, R., Trzeciak, M., Wilkowska, A., Sokołowska-Wojdyło, M., Ługowska-Umer, H., Barańska-Rybak, W., Kaczmarski, M., Kowalewski, C., Kruszewski, J., Maj, J., Silny, W., Śpiewak, R. and Petranyuk, A. 2015. Atopic dermatitis: current treatment guidelines. Statement of the experts of the Dermatological Section, Polish Society of Allergology, and the Allergology Section, Polish Society of Dermatology. *Postepy dermatologii i alergologii.* **32**(4), pp.239-249.

Nutten, S. 2015. Atopic Dermatitis: Global Epidemiology and Risk Factors. *Annals of Nutrition and Metabolism.* **66**(Suppl. 1), pp.8-16.

Nygaard, T.K., Pallister, K.B., DuMont, A.L., DeWald, M., Watkins, R.L., Pallister, E.Q., Malone, C., Griffith, S., Horswill, A.R., Torres, V.J. and Voyich, J.M. 2012. Alpha-toxin induces programmed cell death of human T cells, B cells, and monocytes during USA300 infection. *PLoS One.* **7**(5), pe36532.

O'Neill, A.J., Larsen, A.R., Henriksen, A.S. and Chopra, I. 2004. A Fusidic Acid-Resistant Epidemic Strain of Staphylococcus aureus Carries the fusB Determinant, whereas fusA Mutations Are Prevalent in Other Resistant Isolates. *Antimicrob Agents Chemother.* **48**(9), pp.3594-3597.

O'Neill, A.J., Larsen, A.R., Skov, R., Henriksen, A.S. and Chopra, I. 2007a. Characterization of the Epidemic European Fusidic Acid-Resistant Impetigo Clone of Staphylococcus aureus. *Journal of Clinical Microbiology.* **45**(5), pp.1505-1510.

O'Neill, A.J., McLaws, F., Kahlmeter, G., Henriksen, A.S. and Chopra, I. 2007b. Genetic Basis of Resistance to Fusidic Acid in Staphylococci. *Antimicrob Agents Chemother.* **51**(5), pp.1737-1740.

O'Regan, G.M., Sandilands, A., McLean, W.H.I. and Irvine, A.D. 2008. Filaggrin in atopic dermatitis. *J Allergy Clin Immunol.* **122**(4), pp.689-693.

O'Neill, A.J. 2010. Staphylococcus aureus SH1000 and 8325-4: comparative genome sequences of key laboratory strains in staphylococcal research. *Letters in Applied Microbiology.* **51**(3), pp.358-361.

Oh, J., Byrd, A.L., Deming, C., Conlan, S., Kong, H.H. and Segre, J.A. 2014. Biogeography and individuality shape function in the human skin metagenome. *Nature.* **514**(7520), pp.59-64.

Oh, J., Freeman, A.F., Park, M., Sokolic, R., Candotti, F., Holland, S.M., Segre, J.A. and Kong, H.H. 2013. The altered landscape of the human skin microbiome in patients with primary immunodeficiencies. *Genome Res.* **23**.

Oh, S.-H., Choi, Y.-B., Kim, J.-H., Weihl, C.C. and Ju, J.-S. 2017. Comparisons of ELISA and Western blot assays for detection of autophagy flux. *Data in brief.* **13**, pp.696-699.

Olivares, M., Neef, A., Castillejo, G., Palma, G.D., Varea, V., Capilla, A., Palau, F., Nova, E., Marcos, A., Polanco, I., Ribes-Koninckx, C., Ortigosa, L., Izquierdo, L. and Sanz, Y. 2015. The HLA-DQ2 genotype selects for early intestinal microbiota composition in infants at high risk of developing coeliac disease. *Gut.* **64**(3), pp.406-417.

Olson, M.E., Todd, D.A., Schaeffer, C.R., Paharik, A.E., Van Dyke, M.J., Büttner, H., Dunman, P.M., Rohde, H., Cech, N.B., Fey, P.D. and Horswill, A.R. 2014. Staphylococcus epidermidis agr quorum-sensing system: signal identification, cross talk, and importance in colonization. *Journal of bacteriology.* **196**(19), pp.3482-3493.

Olsson, T., Gulliksson, H., Palmeborn, M., Bergstrom, K. and Thore, A. 1983. Leakage of adenylate kinase from stored blood cells. *J Appl Biochem.* **5**(6), pp.437-445.

Ommori, R., Ouji, N., Mizuno, F., Kita, E., Ikada, Y. and Asada, H. 2013. Selective induction of antimicrobial peptides from keratinocytes by staphylococcal bacteria. *Microbial Pathogenesis.* **56**, pp.35-39.

Omoe, K., Ishikawa, M., Shimoda, Y., Hu, D.-L., Ueda, S. and Shinagawa, K. 2002. Detection of seg, seh, and sei genes in Staphylococcus aureus isolates and determination of the enterotoxin productivities of S. aureus isolates Harboring seg, seh, or sei genes. *Journal of clinical microbiology.* **40**(3), pp.857-862.

Ong, P.Y. 2012. Editorial update on emerging treatments of atopic dermatitis. *Expert Opin Emerg Drugs.* **17**(2), pp.129-133.

Ong, P.Y., Ohtake, T., Brandt, C., Strickland, I., Boguniewicz, M., Ganz, T., Gallo, R.L. and Leung, D.Y.M. 2002. Endogenous Antimicrobial Peptides and Skin Infections in Atopic Dermatitis. *New England Journal of Medicine.* **347**(15), pp.1151-1160.

Oppenheim, J.J., Biragyn, A., Kwak, L.W. and Yang, D. 2003. Roles of antimicrobial peptides such as defensins in innate and adaptive immunity. *Ann Rheum Dis.* **62**(suppl 2), pp.ii17-ii21.

Otto, M. 2001. Staphylococcus aureus and Staphylococcus epidermidis peptide pheromones produced by the accessory gene regulator agr system. *Peptides.* **22**(10), pp.1603-1608.

Otto, M. 2009. Staphylococcus epidermidis – the “accidental” pathogen. *Nature reviews. Microbiology.* **7**(8), pp.555-567.

Otto, M. 2010. Staphylococcus colonization of the skin and antimicrobial peptides. *Expert review of dermatology.* **5**(2), pp.183-195.

Otto, M. 2012. Molecular basis of Staphylococcus epidermidis infections. *Seminars in Immunopathology.* **34**(2), pp.201-214.

Otto, M. 2013. Community-associated MRSA: What makes them special? *Int J Med Microbiol.* **303**(6-7), pp.324-330.

Otto, M. 2014. Staphylococcus aureus toxins. *Current Opinion in Microbiology.* **17**, pp.32-37.

Otto, M., Echner, H., Voelter, W. and Götz, F. 2001. Pheromone Cross-Inhibition between Staphylococcus aureus and Staphylococcus epidermidis. *Infection and Immunity.* **69**(3), pp.1957-1960.

Otto, M., Süßmuth, R., Vuong, C., Jung, G. and Götz, F. 1999. Inhibition of virulence factor expression in Staphylococcus aureus by the Staphylococcus epidermidis agr pheromone and derivatives. *FEBS Letters.* **450**(3), pp.257-262.

Ouhara, K., Komatsuzawa, H., Kawai, T., Nishi, H., Fujiwara, T., Fujiue, Y., Kuwabara, M., Sayama, K., Hashimoto, K. and Sugai, M. 2008. Increased resistance

to cationic antimicrobial peptide LL-37 in methicillin-resistant strains of Staphylococcus aureus. *J Antimicrob Chemother.* **61**(6), pp.1266-1269.

Paller, A.S., Kabashima, K. and Bieber, T. 2017. Therapeutic pipeline for atopic dermatitis: End of the drought? *J Allergy Clin Immunol.* **140**(3), pp.633-643.

Paller, A.S., Kong, H.H., Seed, P., Naik, S., Scharschmidt, T.C., Gallo, R.L., Luger, T. and Irvine, A.D. 2019. The microbiome in patients with atopic dermatitis. *Journal of Allergy and Clinical Immunology.* **143**(1), pp.26-35.

Palmer, C.N.A., Irvine, A.D., Terron-Kwiatkowski, A., Zhao, Y., Liao, H., Lee, S.P., Goudie, D.R., Sandilands, A., Campbell, L.E., Smith, F.J.D., O'Regan, G.M., Watson, R.M., Cecil, J.E., Bale, S.J., Compton, J.G., DiGiovanna, J.J., Fleckman, P., Lewis-Jones, S., Arseculeratne, G., Sergeant, A., Munro, C.S., El Houate, B., McElreavey, K., Halkjaer, L.B., Bisgaard, H., Mukhopadhyay, S. and McLean, W.H.I. 2006. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet.* **38**(4), pp.441-446.

Pane-Farre, J., Jonas, B., Hardwick, S.W., Gronau, K., Lewis, R.J., Hecker, M. and Engelmann, S. 2009. Role of RsbU in Controlling SigB Activity in Staphylococcus aureus following Alkaline Stress. **191**(8), pp.2561-2573.

Panther, D.J. and Jacob, S.E. 2015. The Importance of Acidification in Atopic Eczema: An Underexplored Avenue for Treatment. *Journal of clinical medicine.* **4**(5), pp.970-978.

Park, J.-M., Jo, J.-H., Jin, H., Ko, H.-C., Kim, M.-B., Kim, J.-M., Kim, D.-W., Jang, H.-S. and Kim, B.-S. 2016. Change in Antimicrobial Susceptibility of Skin-Colonizing Staphylococcus aureus in Korean Patients with Atopic Dermatitis during Ten-Year Period. *Annals of dermatology.* **28**(4), pp.470-478.

Park, K.Y., Park, M.K., Kim, E.J., Lee, M.-K. and Seo, S.J. 2011. FCεRI Gene Promoter Polymorphisms and Total IgE Levels in Susceptibility to Atopic Dermatitis in Korea. *J Korean Med Sci.* **26**(7), p870.

Park, Y.H., Jang, W.H., Seo, J.A., Park, M., Lee, T.R., Park, Y.H., Kim, D.K. and Lim, K.M. 2012. Decrease of ceramides with very long-chain fatty acids and downregulation of elongases in a murine atopic dermatitis model. *J Invest Dermatol.* **132**(2), pp.476-479.

Parsons, J.B., Yao, J., Frank, M.W., Jackson, P. and Rock, C.O. 2012. Membrane Disruption by Antimicrobial Fatty Acids Releases Low-Molecular-Weight Proteins from Staphylococcus aureus. *Journal of Bacteriology.* **194**(19), pp.5294-5304.

Paulino, L.C., Tseng, C.-H. and Blaser, M.J. 2008. Analysis of Malassezia microbiota in healthy superficial human skin and in psoriatic lesions by multiplex real-time PCR. *FEMS Yeast Research.* **8**(3), pp.460-471.

Pellerin, L., Henry, J., Hsu, C.-Y., Balica, S., Jean-Decoster, C., Méchin, M.-C., Hansmann, B., Rodriguez, E., Weindinger, S., Schmitt, A.-M., Serre, G., Paul, C. and Simon, M. 2013. Defects of filaggrin-like proteins in both lesional and nonlesional atopic skin. *Journal of Allergy and Clinical Immunology.* **131**(4), pp.1094-1102.

Peltonen, J.M., PylkkÄNen, L., JansÉN, C.T., Volanen, I., Lehtinen, T., Laihia, J.K. and Leino, L. 2014. Three Randomised Phase I/IIa Trials of 5% Cis-urocanic Acid Emulsion Cream in Healthy Adult Subjects and in Patients with Atopic Dermatitis. *Acta Dermato-Venereologica.* **94**(4), pp.415-420.

Pempkowiak, M.R.J.B.G.K.J.W.R.G.N.J. 2001. Western blotting versus ELISA detection of stress proteins induced in the blue mussel Mytilus edulis exposed to cadmium and tributyltin. *Biomarkers.* **6**(6), pp.400-405.

Peng Jia, H., Schutte, B.C., Schudy, A., Linzmeier, R., Guthmiller, J.M., Johnson, G.K., Tack, B.F., Mitros, J.P., Rosenthal, A., Ganz, T. and McCray, P.B. 2001. Discovery of new human β-defensins using a genomics-based approach. *Gene.* **263**(1-2), pp.211-218.

Peng, W. and Novak, N. 2015. Pathogenesis of atopic dermatitis. *Clin Exp Allergy.* **45**(3), pp.566-574.

Percoco, G., Merle, C., Jaouen, T., Ramdani, Y., Bénard, M., Hillion, M., Mijouin, L., Lati, E., Feuilloley, M., Lefeuvre, L., Driouich, A. and Follet-Gueye, M.-L. 2013. Antimicrobial peptides and pro-inflammatory cytokines are differentially regulated across epidermal layers following bacterial stimuli. *Experimental Dermatology.* **22**(12), pp.800-806.

Pereira, L.B. 2014. Impetigo - review. *Anais Brasileiros de Dermatologia.* **89**(2), pp.293-299.

Periasamy, S., Joo, H.S., Duong, A.C., Bach, T.H., Tan, V.Y., Chatterjee, S.S., Cheung, G.Y. and Otto, M. 2012. How Staphylococcus aureus biofilms develop their characteristic structure. *Proc Natl Acad Sci U S A.* **109**(4), pp.1281-1286.

Peschel, A. and Götz, F. 1996. Analysis of the Staphylococcus epidermidis genes epiF, -E, and -G involved in epidermin immunity. *Journal of Bacteriology.* **178**(2), pp.531-536.

Pfalzgraff, A., Brandenburg, K. and Weindl, G. 2018. Antimicrobial Peptides and Their Therapeutic Potential for Bacterial Skin Infections and Wounds. *Frontiers in Pharmacology.* **9**, p281.

Pförtner, H., Burian, M.S., Michalik, S., Depke, M., Hildebrandt, P., Dhople, V.M., Pané-Farré, J., Hecker, M., Schmidt, F. and Völker, U. 2014. Activation of the alternative sigma factor SigB of Staphylococcus aureus following internalization by epithelial cells – An in vivo proteomics perspective. *Int J Med Microbiol.* **304**(2), pp.177-187.

Pincelli, C. and Marconi, A. 2010. Keratinocyte stem cells: friends and foes. *J Cell Physiol.* **225**(2), pp.310-315.

Pinto, M., Morange, M. and Bensaude, O. 1991. Denaturation of proteins during heat shock. In vivo recovery of solubility and activity of reporter enzymes. *Journal of Biological Chemistry.* **266**(21), pp.13941-13946.

Pitt, J.J. 2009. Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. *The Clinical biochemist. Reviews.* **30**(1), pp.19-34.

Pivarcsi, A. and Homey, B. 2005. Chemokine networks in atopic dermatitis: traffic signals of disease. *Current allergy and asthma reports.* **5**(4), pp.284-290.

Pollitt, E.J.G., West, S.A., Crusz, S.A., Burton-Chellew, M.N. and Diggle, S.P. 2014. Cooperation, quorum sensing, and evolution of virulence in Staphylococcus aureus. *Infection and immunity.* **82**(3), pp.1045-1051.

Popov, L., Kovalski, J., Grandi, G., Bagnoli, F. and Amieva, M.R. 2014. Three-Dimensional Human Skin Models to Understand Staphylococcus aureus Skin Colonization and Infection. *Front Immunol.* **5**, p41.

Prachi, P. 2013. Characterization of the Staphylococcus aureus bone sialoprotein-binding protein SdrE and the serine protease EpiP, Ph.D. thesis, University of Bologna.

Prescott, S.L., Larcombe, D.-L., Logan, A.C., West, C., Burks, W., Caraballo, L., Levin, M., Etten, E.V., Horwitz, P., Kozyrskyj, A. and Campbell, D.E. 2017. The skin microbiome: impact of modern environments on skin ecology, barrier integrity, and systemic immune programming. *The World Allergy Organization journal.* **10**(1), pp.29-29.

Price, L.B., Hungate, B.A., Koch, B.J., Davis, G.S. and Liu, C.M. 2017. Colonizing opportunistic pathogens (COPs): The beasts in all of us. *PLOS Pathogens.* **13**(8), pe1006369.

Proksch, E. 2018. pH in nature, humans and skin. *J Dermatol.* **45**(9), pp.1044-1052.

Proksch, E., Brandner, J.M. and Jensen, J.M. 2008. The skin: an indispensable barrier. *Experimental Dermatology.* **17**(12), pp.1063-1072.

Proksch, E., Fölster-Holst, R. and Jensen, J.-M. 2006. Skin barrier function, epidermal proliferation and differentiation in eczema. *Journal of Dermatological Science.* **43**(3), pp.159-169.

Proksch, E., Jensen, J.M. and Elias, P.M. 2003. Skin lipids and epidermal differentiation in atopic dermatitis. *Clin Dermatol.* **21**(2), pp.134-144.

Pruett, S.T., Bushnev, A., Hagedorn, K., Adiga, M., Haynes, C.A., Sullards, M.C., Liotta, D.C. and Merrill, A.H. 2008. Thematic Review Series: Sphingolipids. Biodiversity of sphingoid bases (“sphingosines”) and related amino alcohols. *Journal of Lipid Research.* **49**(8), pp.1621-1639.

Qazi, S.N., Counil, E., Morrissey, J., Rees, C.E., Cockayne, A., Winzer, K., Chan, W.C., Williams, P. and Hill, P.J. 2001. agr expression precedes escape of internalized Staphylococcus aureus from the host endosome. *Infection and immunity.* **69**(11), pp.7074-7082.

Queck, S.Y., Jameson-Lee, M., Villaruz, A.E., Bach, T.-H.L., Khan, B.A., Sturdevant, D.E., Ricklefs, S.M., Li, M. and Otto, M. 2008. RNAIII-Independent Target Gene Control by the agr Quorum-Sensing System: Insight into the Evolution of Virulence Regulation in Staphylococcus aureus. *Molecular Cell.* **32**(1), pp.150-158.

Rabeony, H., Petit-Paris, I., Garnier, J., Barrault, C., Pedretti, N., Guilloteau, K., Jegou, J.-F., Guillet, G., Huguier, V., Lecron, J.-C., Bernard, F.-X. and Morel, F. 2014. Inhibition of Keratinocyte Differentiation by the Synergistic Effect of IL-17A, IL-22, IL-1α, TNFα and Oncostatin M. *PLoS ONE.* **9**(7), pe101937.

Rademacher, F., Simanski, M., Gläser, R. and Harder, J. 2018. Skin microbiota and human 3D skin models. *Experimental Dermatology.* **27**(5), pp.489-494.

Rademacher, F., Simanski, M. and Harder, J. 2016. RNase 7 in Cutaneous Defense. *International Journal of Molecular Sciences.* **17**(4), p560.

Rakesh, K. and Yung-Sheng, H. 2006. Gamma Linolenic Acid: An Antiinflammatory Omega-6 Fatty Acid. *Current Pharmaceutical Biotechnology.* **7**(6), pp.531-534.

Ravipaty, S. and Reilly, J.P. 2010. Comprehensive characterization of methicillin-resistant Staphylococcus aureus subsp. aureus COL secretome by two-dimensional liquid chromatography and mass spectrometry. *Molecular & cellular proteomics : MCP.* **9**(9), pp.1898-1919.

Rawlings, A.V. 2006. Ethnic skin types: are there differences in skin structure and function? *Int J Cosmet Sci.* **28**(2), pp.79-93.

Rawlings, A.V. and Harding, C.R. 2004. Moisturization and skin barrier function. *Dermatologic Therapy.* **17**, pp.43-48.

Rayner, C. and Munckhof, W.J. 2005. Antibiotics currently used in the treatment of infections caused by Staphylococcus aureus. *Internal Medicine Journal.* **35**, pp.S3-S16.

Reginald, K., Westritschnig, K., Linhart, B., Focke-Tejkl, M., Jahn-Schmid, B., Eckl-Dorna, J., Heratizadeh, A., Stöcklinger, A., Balic, N., Spitzauer, S., Niederberger, V., Werfel, T., Thalhamer, J., Weidinger, S., Novak, N., Ollert, M., Hirschl, A.M. and Valenta, R. 2011. Staphylococcus aureus fibronectin-binding protein specifically binds IgE from patients with atopic dermatitis and requires antigen presentation for cellular immune responses. *Journal of Allergy and Clinical Immunology.* **128**(1), pp.82-91.e88.

Reinholz, M., Ruzicka, T. and Schauber, J. 2012. Cathelicidin LL-37: An Antimicrobial Peptide with a Role in Inflammatory Skin Disease. *Annals of Dermatology.* **24**(2), pp.126-135.

Richards, G.M., Oresajo, C.O. and Halder, R.M. 2003. Structure and function of ethnic skin and hair. *Dermatol Clin.* **21**(4), pp.595-600.

Rieg, S., Garbe, C., Sauer, B., Kalbacher, H. and Schittek, B. 2004. Dermcidin is constitutively produced by eccrine sweat glands and is not induced in epidermal cells under inflammatory skin conditions. *British Journal of Dermatology.* **151**(3), pp.534-539.

Rieg, S., Steffen, H., Seeber, S., Humeny, A., Kalbacher, H., Dietz, K., Garbe, C. and Schittek, B. 2005. Deficiency of dermcidin-derived antimicrobial peptides in sweat of patients with atopic dermatitis correlates with an impaired innate defense of human skin in vivo. *J Immunol.* **174**(12), pp.8003-8010.

Rinaldi, M., Moroni, P., Leino, L., Laihia, J., Paape, M.J. and Bannerman, D.D. 2006. Effect of Cis-Urocanic Acid on Bovine Neutrophil Generation of Reactive Oxygen Species. *Journal of Dairy Science.* **89**(11), pp.4188-4201.

Rippke, F., Schreiner, V., Doering, T. and Maibach, H.I. 2004. Stratum corneum pH in atopic dermatitis: impact on skin barrier function and colonization with Staphylococcus Aureus. *Am J Clin Dermatol.* **5**(4), pp.217-223.

Rippke, F., Schreiner, V. and Schwanitz, H.J. 2002. The acidic milieu of the horny layer: new findings on the physiology and pathophysiology of skin pH. *Am J Clin Dermatol.* **3**(4), pp.261-272.

Rodrigues-Hoffmann, A. 2017. The cutaneous ecosystem: the roles of the skin microbiome in health and its association with inflammatory skin conditions in humans and animals. *Vet Dermatol.* **28**(1), pp.60-e15.

Roebuck, K.A. 1999. Regulation of interleukin-8 gene expression. *J Interferon Cytokine Res.* **19**(5), pp.429-438.

Ross, K.F. and Herzberg, M.C. 2001. Calprotectin expression by gingival epithelial cells. *Infection and immunity.* **69**(5), pp.3248-3254.

Rudack, C., Sachse, F., Albert, N., Becker, K. and Von Eiff, C. 2009. Immunomodulation of Nasal Epithelial Cells by Staphylococcus aureus-Derived Serine Proteases. *Journal of immunology (Baltimore, Md. : 1950).* **183**(11), pp.7592-7601.

Ryan, C., Shaw, R.E., Cockerell, C.J., Hand, S. and Ghali, F.E. 2013. Novel Sodium Hypochlorite Cleanser Shows Clinical Response and Excellent Acceptability in the Treatment of Atopic Dermatitis. *Pediatric Dermatology.* **30**(3), pp.308-315.

Sabaté-Brescó, M., Harris, L.G., Thompson, K., Stanic, B., Morgenstern, M., O'Mahony, L., Richards, R.G. and Moriarty, T.F. 2017. Pathogenic Mechanisms and Host Interactions in Staphylococcus epidermidis Device-Related Infection. *Frontiers in Microbiology.* **8**, p1401.

Saenz, S.A., Noti, M. and Artis, D. 2010. Innate immune cell populations function as initiators and effectors in Th2 cytokine responses. *Trends Immunol.* **31**(11), pp.407-413.

Sakr, A., Brégeon, F., Mège, J.-L., Rolain, J.-M. and Blin, O. 2018. Staphylococcus aureus Nasal Colonization: An Update on Mechanisms, Epidemiology, Risk Factors, and Subsequent Infections. *Frontiers in Microbiology.* **9**, p2419.

Salava, A. and Lauerma, A. 2014. Role of the skin microbiome in atopic dermatitis. *Clinical and Translational Allergy.* **4**, p33.

Sambrook, J. and Russell, D.W. 2001. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press.

Sandilands, A., Sutherland, C., Irvine, A.D. and McLean, W.H.I. 2009. Filaggrin in the frontline: Role in skin barrier function and disease. *Journal of Cell Science.* **122**(9), pp.1285-1294.

SanMiguel, A.J., Meisel, J.S., Horwinski, J., Zheng, Q. and Grice, E.A. 2017. Topical Antimicrobial Treatments Can Elicit Shifts to Resident Skin Bacterial Communities and Reduce Colonization by Staphylococcus aureus Competitors. *Antimicrobial agents and chemotherapy.* **61**(9), pp.e00774-00717.

Sanyal, D. and Greenwood, D. 1993. An electronmicroscope study of glycopeptide antibiotic-resistant strains of Staphylococcus epidermidis. *J Med Microbiol.* **39**(3), pp.204-210.

Sasaki, T., Kano, R., Sato, H., Nakamura, Y., Watanabe, S. and Hasegawa, A. 2003. Effects of staphylococci on cytokine production from human keratinocytes. *Br J Dermatol.* **148**(1), pp.46-50.

Sass, V., Schneider, T., Wilmes, M., Korner, C., Tossi, A., Novikova, N., Shamova, O. and Sahl, H.G. 2010. Human beta-defensin 3 inhibits cell wall biosynthesis in Staphylococci. *Infection and Immunity.* **78**(6), pp.2793-2800.

Sassi, M., Sharma, D., Brinsmade, S.R., Felden, B. and Augagneur, Y. 2015. Genome Sequence of the Clinical Isolate Staphylococcus aureus subsp. aureus Strain UAMS-1. *Genome Announc.* **3**(1).

Savage, V.J., Chopra, I. and O'Neill, A.J. 2013a. Staphylococcus aureus Biofilms Promote Horizontal Transfer of Antibiotic Resistance. *Antimicrobial Agents and Chemotherapy.* **57**(4), pp.1968-1970.

Savage, V.J., Chopra, I. and O'Neill, A.J. 2013b. Population Diversification in Staphylococcus aureus Biofilms May Promote Dissemination and Persistence. *PLOS ONE.* **8**(4), pe62513.

Scharschmidt, T.C. and Fischbach, M.A. 2013. What lives on our skin: ecology, genomics and therapeutic opportunities of the skin microbiome. *Drug Discovery Today: Disease Mechanisms.* **10**(3-4), pp.e83-e89.

Schauber, J., Dorschner, R.A., Coda, A.B., Büchau, A.S., Liu, P.T., Kiken, D., Helfrich, Y.R., Kang, S., Elalieh, H.Z., Steinmeyer, A., Zügel, U., Bikle, D.D., Modlin, R.L. and Gallo, R.L. 2007. Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D–dependent mechanism. *Journal of Clinical Investigation.* **117**(3), pp.803-811.

Schauber, J., Dorschner, R.A., Yamasaki, K., Brouha, B. and Gallo, R.L. 2006. Control of the innate epithelial antimicrobial response is cell-type specific and dependent on relevant microenvironmental stimuli. *Immunology.* **118**(4), pp.509-519.

Schauber, J. and Gallo, R.L. 2008. Antimicrobial peptides and the skin immune defense system. *Journal of Allergy and Clinical Immunology.* **122**(2), pp.261-266.

Schibli, D.J., Hunter, H.N., Aseyev, V., Starner, T.D., Wiencek, J.M., McCray, P.B., Tack, B.F. and Vogel, H.J. 2002. The Solution Structures of the Human β-Defensins Lead to a Better Understanding of the Potent Bactericidal Activity of HBD3 against Staphylococcus aureus. *Journal of Biological Chemistry.* **277**(10), pp.8279-8289.

Schittek, B. 2012. The Multiple Facets of Dermcidin in Cell Survival and Host Defense. *Journal of Innate Immunity.* **4**(4), pp.349-360.

Schittek, B., Hipfel, R., Sauer, B., Bauer, J., Kalbacher, H., Stevanovic, S., Schirle, M., Schroeder, K., Blin, N., Meier, F., Rassner, G. and Garbe, C. 2001. Dermcidin: a novel human antibiotic peptide secreted by sweat glands. *Nat Immunol.* **2**(12), pp.1133-1137.

Schneider, L., Tilles, S., Lio, P., Boguniewicz, M., Beck, L., Lebovidge, J., Novak, N., Bernstein, D., Blessing-Moore, J., Khan, D., Lang, D., Nicklas, R., Oppenheimer, J., Portnoy, J., Randolph, C., Schuller, D., Spector, S. and Wallace, D. 2013. Atopic dermatitis: A practice parameter update 2012. *Journal of Allergy and Clinical Immunology.* **131**(2), pp.295-e227.

Schonthaler, Helia B., Guinea-Viniegra, J., Wculek, Stefanie K., Ruppen, I., Ximénez-Embún, P., Guío-Carrión, A., Navarro, R., Hogg, N., Ashman, K. and Wagner, Erwin F. 2013. S100A8-S100A9 Protein Complex Mediates Psoriasis by Regulating the Expression of Complement Factor C3. *Immunity.* **39**(6), pp.1171-1181.

Schram, M.E., Leeflang, M.M., JP, D.E.N.O., Spuls, P.I. and Bos, J.D. 2011. Validation and refinement of the Millennium Criteria for atopic dermatitis. *J Dermatol.* **38**(9), pp.850-858.

Schröder, J.M. 2010. The role of keratinocytes in defense against infection. *Curr Opin Infect Dis.* **23**(2), pp.106-110.

Schröder, J.M. 2011. Antimicrobial Peptides in Healthy Skin and Atopic Dermatitis. *Allergology International.* **60**(1), pp.17-24.

Schröder, J.M. and Harder, J. 1999. Human beta-defensin-2. *The International Journal of Biochemistry & Cell Biology.* **31**(6), pp.645-651.

Schröder, J.M., Harder, J.J.C. and CMLS, M.L.S. 2006. Antimicrobial skin peptides and proteins. *Cell Mol Life Sci.* **63**(4), pp.469-486.

Schroder, K., Hertzog, P.J., Ravasi, T. and Hume, D.A. 2004. Interferon-γ: an overview of signals, mechanisms and functions. *Journal of Leukocyte Biology.* **75**(2), pp.163-189.

Schwandner, R., Dziarski, R., Wesche, H., Rothe, M. and Kirschning, C.J. 1999. Peptidoglycan- and Lipoteichoic Acid-induced Cell Activation Is Mediated by Toll-like Receptor 2. *Journal of Biological Chemistry.* **274**(25), pp.17406-17409.

Sechet, E., Telford, E., Bonamy, C., Sansonetti, P.J. and Sperandio, B. 2018. Natural molecules induce and synergize to boost expression of the human antimicrobial peptide β-defensin-3. *Proc Natl Acad Sci U S A.* **115**(42), pp.E9869-E9878.

Sehra, S., Yao, Y., Howell, M.D., Nguyen, E.T., Kansas, G.S., Leung, D.Y.M., Travers, J.B. and Kaplan, M.H. 2010. IL-4 regulates skin homeostasis and the predisposition towards allergic skin inflammation. *Journal of immunology (Baltimore, Md. : 1950).* **184**(6), pp.3186-3190.

Seidenari, S. and Giusti, G. 1995. Objective assessment of the skin of children affected by atopic dermatitis: a study of pH, capacitance and TEWL in eczematous and clinically uninvolved skin. *Acta Derm Venereol.* **75**(6), pp.429-433.

Sela, U., Euler, C.W., Correa Da Rosa, J. and Fischetti, V.A. 2018. Strains of bacterial species induce a greatly varied acute adaptive immune response: The contribution of the accessory genome. *PLOS Pathogens.* **14**(1), pe1006726.

Seltmann, J., Roesner, L.M., von Hesler, F.-W., Wittmann, M. and Werfel, T. 2015. IL-33 impacts on the skin barrier by downregulating the expression of filaggrin. *Journal of Allergy and Clinical Immunology.* **135**(6), pp.1659-1661.e1654.

Semprini, S., Capon, F., Tacconelli, A., Giardina, E., Orecchia, A., Mingarelli, R., Gobello, T., Zambruno, G., Botta, A., Fabrizi, G. and Novelli, G. 2002. Evidence for differential S100 gene over-expression in psoriatic patients from genetically heterogeneous pedigrees. *Human Genetics.* **111**(4), pp.310-313.

Senyürek, I., Paulmann, M., Sinnberg, T., Kalbacher, H., Deeg, M., Gutsmann, T., Hermes, M., Kohler, T., Götz, F., Wolz, C., Peschel, A. and Schittek, B. 2009. Dermcidin-Derived Peptides Show a Different Mode of Action than the Cathelicidin LL-37 against Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy.* **53**(6), pp.2499-2509.

Shallcross, L.J. and Davies, D.S.C. 2014. Antibiotic overuse: a key driver of antimicrobial resistance. *The British Journal of General Practice.* **64**(629), pp.604-605.

Shaw, T., Currie, G.P., Koudelka, C.W. and Simpson, E. 2011. Eczema Prevalence in the United States: Data from the 2003 National Survey of Children's Health. *Journal of Investigative Dermatology.* **131**(1), pp.67-73.

Sheffield, P., Weinberger, K. and Kinney, P. 2011. Climate Change, Aeroallergens, and Pediatric Allergic Disease. *Mt. Sinai J. Med.* **78**(1), pp.78-84.

Shinji, H., Yosizawa, Y., Tajima, A., Iwase, T., Sugimoto, S., Seki, K. and Mizunoe, Y. 2011. Role of Fibronectin-Binding Proteins A and B in In Vitro Cellular Infections and In Vivo Septic Infections by Staphylococcus aureus. *Infection and Immunity.* **79**(6), pp.2215-2223.

Shiohara, T. 2011. *Pathogenesis and Management of Atopic Dermatitis.* Karger.

Shirakawa, T., Deichmann, K.A., Izuhara, K., Mao, X.-Q., Adra, C.N. and Hopkin, J.M. 2000. Atopy and asthma: genetic variants of IL-4 and IL-13 signalling. *Immunology Today.* **21**(2), pp.60-64.

Shire, S.J. 2015. 2 - Analytical tools used in the formulation and assessment of stability of monoclonal antibodies (mAbs). In: Shire, S.J. ed. *Monoclonal Antibodies.* Woodhead Publishing, pp.17-44.

Silva, J.S., Vespa, G.N., Cardoso, M.A., Aliberti, J.C. and Cunha, F.Q. 1995. Tumor necrosis factor alpha mediates resistance to Trypanosoma cruzi infection in mice by inducing nitric oxide production in infected gamma interferon-activated macrophages. *Infection and Immunity.* **63**(12), pp.4862-4867.

Silverberg, J., Hanifin, J. and Simpson, E. 2013. Climatic Factors Are Associated with Childhood Eczema Prevalence in the United States. *J. Invest. Dermatol.* **133**(7), pp.1752-1759.

Simanski, M., Dressel, S., Gläser, R. and Harder, J. 2010. RNase 7 Protects Healthy Skin from Staphylococcus aureus Colonization. *Journal of Investigative Dermatology.* **130**(12), pp.2836-2838.

Simanski, M., Gläser, R. and Harder, J. 2014. Human skin engages different epidermal layers to provide distinct innate defense mechanisms. *Experimental Dermatology.* **23**(4), pp.230-231.

Simanski, M., Rademacher, F., Schroder, L., Glaser, R. and Harder, J. 2016. The Inflammasome and the Epidermal Growth Factor Receptor (EGFR) Are Involved in the Staphylococcus aureus-Mediated Induction of IL-1alpha and IL-1beta in Human Keratinocytes. *PLoS One.* **11**(1), pe0147118.

Simanski, M., Rademacher, F., Schröder, L., Schumacher, H.M., Gläser, R. and Harder, J. 2013. IL-17A and IFN-γ Synergistically Induce RNase 7 Expression via STAT3 in Primary Keratinocytes. *PLoS ONE.* **8**(3), pe59531.

Simon, D. and Bieber, T. 2014. Systemic therapy for atopic dermatitis. *Allergy.* **69**(1), pp.46-55.

Simpson, C.L., Patel, D.M. and Green, K.J. 2011. Deconstructing the skin: cytoarchitectural determinants of epidermal morphogenesis. *Nature reviews. Molecular cell biology.* **12**(9), pp.565-580.

Simpson, E.L., Villarreal, M., Jepson, B., Rafaels, N., David, G., Hanifin, J., Taylor, P., Boguniewicz, M., Yoshida, T., De Benedetto, A., Barnes, K.C., Leung, D.Y.M. and Beck, L.A. 2018. Patients with Atopic Dermatitis Colonized with Staphylococcus aureus Have a Distinct Phenotype and Endotype. *J Invest Dermatol.* **138**(10), pp.2224-2233.

Smalley, K.S.M., Lioni, M. and Herlyn, M. 2006. Life ins't flat: Taking cancer biology to the next dimension. *In Vitro Cellular & Developmental Biology - Animal.* **42**(8), pp.242-247.

Smeden, J., Janssens, M., Kaye, E.C.J., Caspers, P.J., Lavrijsen, A.P., Vreeken, R.J. and Bouwstra, J.A. 2014. The importance of free fatty acid chain length for the skin barrier function in atopic eczema patients. *Experimental Dermatology.* **23**(1), pp.45-52.

Soltis, R.D., Hasz, D., Morris, M.J. and Wilson, I.D. 1979. The effect of heat inactivation of serum on aggregation of immunoglobulins. *Immunology.* **36**(1), pp.37-45.

Son, E.D., Kim, H.-J., Park, T., Shin, K., Bae, I.-H., Lim, K.-M., Cho, E.-G. and Lee, T.R. 2014. Staphylococcus aureus inhibits terminal differentiation of normal human keratinocytes by stimulating interleukin-6 secretion. *Journal of Dermatological Science.* **74**(1), pp.64-71.

Sorensen, O.E., Follin, P., Johnsen, A.H., Calafat, J., Tjabringa, G.S., Hiemstra, P.S. and Borregaard, N. 2001. Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. *Blood.* **97**(12), pp.3951-3959.

Sorensen, O.E., Thapa, D.R., Rosenthal, A., Liu, L., Roberts, A.A. and Ganz, T. 2005. Differential Regulation of β-Defensin Expression in Human Skin by Microbial Stimuli. *Journal of immunology (Baltimore, Md. : 1950).* **174**(8), pp.4870-4879.

Speziali, C.D., Dale, S.E., Henderson, J.A., Vines, E.D. and Heinrichs, D.E. 2006. Requirement of Staphylococcus aureus ATP-Binding Cassette-ATPase FhuC for Iron-Restricted Growth and Evidence that It Functions with More than One Iron Transporter. *Journal of Bacteriology.* **188**(6), pp.2048-2055.

Steinbakk, M., Naess-Andresen, C.F., Fagerhol, M.K., Lingaas, E., Dale, I. and Brandtzaeg, P. 1990. Antimicrobial actions of calcium binding leucocyte L1 protein, calprotectin. *The Lancet.* **336**(8718), pp.763-765.

Steubesand, N., Kiehne, K., Brunke, G., Pahl, R., Reiss, K., Herzig, K.-H., Schubert, S., Schreiber, S., Folsch, U.R., Rosenstiel, P. and Arlt, A. 2009. The expression of the beta-defensins hBD-2 and hBD-3 is differentially regulated by NF-kappaB and MAPK/AP-1 pathways in an in vitro model of Candida esophagitis. *BMC Immunology.* **10**(1), p36.

Stillwell, W. and Wassall, S.R. 2003. Docosahexaenoic acid: membrane properties of a unique fatty acid. *Chemistry and Physics of Lipids.* **126**(1), pp.1-27.

Stobberingh, M.I.A.R., Wolffs, P.F.G., Hopstaken, R.M., Heyer, M.D., Bruggeman, C.A. and E, E. 2012. Spread of the epidemic European fusidic acid-resistant impetigo clone (EEFIC) in general practice patients in the south of The Netherlands. *Journal of Antimicrobial Chemotherapy.* **67**(5).

Stoitzner, P., Zanella, M., Ortner, U., Lukas, M., Tagwerker, A., Janke, K., Lutz, M.B., Schuler, G., Echtenacher, B., Ryffel, B., Koch, F. and Romani, N. 1999. Migration of langerhans cells and dermal dendritic cells in skin organ cultures: augmentation by TNF-alpha and IL-1beta. *Journal of Leukocyte Biology.* **66**(3), pp.462-470.

Stuetz, A., Baumann, K., Grassberger, M., Wolff, K. and Meingassner, J.G. 2006. Discovery of topical calcineurin inhibitors and pharmacological profile of pimecrolimus. *Int Arch Allergy Immunol.* **141**(3), pp.199-212.

Su, C., Yang, T., Wu, Z., Zhong, J., Huang, Y., Huang, T. and Zheng, E. 2017. Differentiation of T-helper cells in distinct phases of atopic dermatitis involves Th1/Th2 and Th17/Treg. *European Journal of Inflammation.* **15**(1), pp.46-52.

Su, Y.-C. and Wong, A.C.L. 1996. Detection of Staphylococcal Enterotoxin H by an Enzyme-Linked Immunosorbent Assay. *Journal of Food Protection.* **59**(3), pp.327-330.

Suarato, G., Bertorelli, R. and Athanassiou, A. 2018. Borrowing From Nature: Biopolymers and Biocomposites as Smart Wound Care Materials. *Frontiers in bioengineering and biotechnology.* **6**, pp.137-137.

Suárez, A.L., Feramisco, J.D., Koo, J. and Steinhoff, M. 2012. Psychoneuroimmunology of psychological stress and atopic dermatitis: pathophysiologic and therapeutic updates. *Acta dermato-venereologica.* **92**(1), pp.7-15.

Sudhakara, P., Gupta, A., Bhardwaj, A. and Wilson, A. 2018. Oral Dysbiotic Communities and Their Implications in Systemic Diseases. *Dentistry journal.* **6**(2), p10.

Sugimoto, S., Morizane, S., Sugihara, S., Kobashi, M. and Iwatsuki, K. 2017. TLR signaling regulates the expression of serine protease inhibitors in epidermal keratinocytes. *Journal of Dermatological Science.* **86**(2), pp.e71-e72.

Sugino, K., Imokawa, G. and Maibach, H.I. 1993. Ethnic difference of varied stratum corneum function in relation to stratum corneum lipids. *Journal of Dermatological Science.* **6**(1), p108.

Suter, M.M., Schulze, K., Bergman, W., Welle, M., Roosje, P. and Müller, E. 2009. The keratinocyte in epidermal renewal and defence. *Veterinary Dermatology.* **20**(5-6), pp.515-532.

Sybilski, A.J., Raciborski, F., Lipiec, A., Tomaszewska, A., Lusawa, A., Samel-Kowalik, P., Walkiewicz, A., Krzych-Fałta, E. and Samoliński, B. 2015. Epidemiology of atopic dermatitis in Poland according to the Epidemiology of Allergic Disorders in Poland (ECAP) study. *Journal of Dermatology.* **42**(2), pp.140-147.

Syed, A.K., Reed, T.J., Clark, K.L., Boles, B.R. and Kahlenberg, J.M. 2015. Staphlyococcus aureus Phenol-Soluble Modulins Stimulate the Release of Proinflammatory Cytokines from Keratinocytes and Are Required for Induction of Skin Inflammation. **83**(9), pp.3428-3437.

Takeda, K., Tanaka, T., Shi, W., Matsumoto, M., Minami, M., Kashiwamura, S., Nakanishi, K., Yoshida, N., Kishimoto, T. and Akira, S. 1996. Essential role of Stat6 in IL-4 signalling. *Nature.* **380**(6575), pp.627-630.

Takematsu, H. and Tagami, H. 1993. Mode of release of interleukin-8 from proliferating human epidermal keratinocytes in vitro. *Experimental Dermatology.* **2**(3), pp.121-124.

Takeuchi, O. and Akira, S. 2010. Pattern Recognition Receptors and Inflammation. *Cell.* **140**(6), pp.805-820.

Takigawa, H., Nakagawa, H., Kuzukawa, M., Mori, H. and Imokawa, G. 2005. Deficient production of hexadecenoic acid in the skin is associated in part with the vulnerability of atopic dermatitis patients to colonization by Staphylococcus aureus. *Dermatology.* **211**(3), pp.240-248.

Tanaka, M. 1994. IgE-Mediated Hypersensitivity and Contact Sensitivity to Multiple Environmental Allergens in Atopic Dermatitis. *Archives of Dermatology.* **130**(11), p1393.

Tang, A., Caballero, A.R., Bierdeman, M.A., Marquart, M.E., Foster, T.J., Monk, I.R. and O'Callaghan, R.J. 2019. Staphylococcus aureus Superantigen-Like Protein SSL1: A Toxic Protease. *Pathogens.* **8**(1).

Tartaglia-Polcini, A., Bonnart, C., Micheloni, A., Cianfarani, F., Andrè, A., Zambruno, G., Hovnanian, A. and D'Alessio, M. 2006. SPINK5, the Defective Gene in Netherton Syndrome, Encodes Multiple LEKTI Isoforms Derived from Alternative Pre-mRNA Processing. *Journal of Investigative Dermatology.* **126**(2), pp.315-324.

Tauber, M., Balica, S., Hsu, C.-Y., Jean-Decoster, C., Lauze, C., Redoules, D., Viodé, C., Schmitt, A.-M., Serre, G., Simon, M. and Paul, C.F. 2016. Staphylococcus aureus density on lesional and nonlesional skin is strongly associated with disease severity in atopic dermatitis. *Journal of Allergy and Clinical Immunology.* **137**(4), pp.1272-1274.e1273.

Taylor, K., Clarke, D.J., McCullough, B., Chin, W., Seo, E., Yang, D., Oppenheim, J., Uhrin, D., Govan, J.R.W., Campopiano, D.J., Macmillan, D., Barran, P. and Dorin, J.R. 2008. Analysis and Separation of Residues Important for the Chemoattractant and Antimicrobial Activities of β-Defensin 3. *Journal of Biological Chemistry.* **283**(11), pp.6631-6639.

Thimmappaiah Jagadeesh, A., Prakash, P.Y., Karthik Rao, N. and Ramya, V. 2017. Culture characterization of the skin microbiome in Type 2 diabetes mellitus: A focus on the role of innate immunity. *Diabetes Res Clin Pract.* **134**, pp.1-7.

Thoendel, M., Kavanaugh, J.S., Flack, C.E. and Horswill, A.R. 2011. Peptide Signaling in the Staphylococci. *Chemical Reviews.* **111**(1), pp.117-151.

Thompson, M.R., Kaminski, J.J., Kurt-Jones, E.A. and Fitzgerald, K.A. 2011. Pattern Recognition Receptors and the Innate Immune Response to Viral Infection. *Viruses.* **3**(6), pp.920-940.

Thomsen, S.F. 2014. Atopic Dermatitis: Natural History, Diagnosis, and Treatment. *ISRN Allergy.* **2014**, p7.

Tirumurugaan, K.G., Balachandran, C. and Raj, G.D. 2015. Chapter Twelve - Demonstration of Rabies Viral Nucleic Acids by In-Situ Polymerase Chain Reaction. In: Rupprecht, C. and Nagarajan, T. eds. *Current Laboratory Techniques in Rabies Diagnosis, Research and Prevention, Volume 2.* Academic Press, pp.129-141.

Tjabringa, G.S., Ninaber, D.K., Drijfhout, J.W., Rabe, K.F. and Hiemstra, P.S. 2006. Human cathelicidin LL-37 is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors. *Int Arch Allergy Immunol.* **140**(2), pp.103-112.

Toda, M., Leung, D.Y., Molet, S., Boguniewicz, M., Taha, R., Christodoulopoulos, P., Fukuda, T., Elias, J.A. and Hamid, Q.A. 2003. Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. *J Allergy Clin Immunol.* **111**(4), pp.875-881.

Tomczak, H., Wróbel, J., Jenerowicz, D., Sadowska-Przytocka, A., Wachal, M., Adamski, Z. and Czarnecka-Operacz, M.M. 2019. The Role of Staphylococcus aureus in Atopic Dermatitis - Microbiological and Immunological Implications. *Advances in Dermatology and Allergology.* **36**(4), pp.485-491.

Tomi, N.S., Kranke, B. and Aberer, E. 2005. Staphylococcal toxins in patients with psoriasis, atopic dermatitis, and erythroderma, and in healthy control subjects. *J Am Acad Dermatol.* **53**(1), pp.67-72.

Tong, S.Y.C., Davis, J.S., Eichenberger, E., Holland, T.L. and Fowler, V.G. 2015. Staphylococcus aureus Infections: Epidemiology, Pathophysiology, Clinical Manifestations, and Management. *Clinical Microbiology Reviews.* **28**(3), pp.603-661.

Torrent, M., Badia, M., Moussaoui, M., Sanchez, D., Nogués, M.V. and Boix, E. 2010. Comparison of human RNase 3 and RNase 7 bactericidal action at the Gram-negative and Gram-positive bacterial cell wall. *FEBS Journal.* **277**(7), pp.1713-1725.

Totte, J.E., van der Feltz, W.T., Hennekam, M., van Belkum, A., van Zuuren, E.J. and Pasmans, S.G. 2016. Prevalence and odds of Staphylococcus aureus carriage in atopic dermatitis: a systematic review and meta-analysis. *Br J Dermatol.* **175**(4), pp.687-695.

Traber, K.E., Lee, E., Benson, S., Corrigan, R., Cantera, M., Shopsin, B. and Novick, R.P. 2008. agr function in clinical Staphylococcus aureus isolates. *Microbiology.* **154**(8), pp.2265-2274.

Trumbo, P., Schlicker, S., Yates, A.A. and Poos, M. 2002. Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein and amino acids. *J Am Diet Assoc.* **102**(11), pp.1621-1630.

Tuchscherr, L., Bischoff, M., Lattar, S.M., Noto Llana, M., Pförtner, H., Niemann, S., Geraci, J., Van de Vyver, H., Fraunholz, M.J., Cheung, A.L., Herrmann, M., Völker, U., Sordelli, D.O., Peters, G. and Löffler, B. 2015. Sigma Factor SigB Is Crucial to Mediate Staphylococcus aureus Adaptation during Chronic Infections. *PLoS Pathogens.* **11**(4), pe1004870.

Turner, J., Cho, Y., Dinh, N.-N., Waring, A.J. and Lehrer, R.I. 1998. Activities of LL-37, a Cathelin-Associated Antimicrobial Peptide of Human Neutrophils. *Antimicrobial Agents and Chemotherapy.* **42**(9), pp.2206-2214.

Turner, M.D., Nedjai, B., Hurst, T. and Pennington, D.J. 2014. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research.* **1843**(11), pp.2563-2582.

Van Crombruggen, K., Vogl, T., Pérez-Novo, C., Holtappels, G. and Bachert, C. 2016. Differential release and deposition of S100A8/A9 proteins in inflamed upper airway tissue. *European Respiratory Journal.* **47**(1), pp.264-274.

Van de Guchte, M., Van der Vossen, J.M., Kok, J. and Venema, G. 1989. Construction of a lactococcal expression vector: expression of hen egg white lysozyme in Lactococcus lactis subsp. lactis. *Applied and Environmental Microbiology.* **55**(1), p224.

Van Der Does, A.M., Bergman, P., Agerberth, B. and Lindbom, L. 2012. Induction of the human cathelicidin LL-37 as a novel treatment against bacterial infections. *J Leukoc Biol.* **92**(4), pp.735-742.

Van Der Meulen, T., Harmsen, H., Bootsma, H., Spijkervet, F., Kroese, F. and Vissink, A. 2016. The microbiome-systemic diseases connection. *Oral Diseases.* **22**(8), pp.719-734.

Van Drongelen, V., Haisma, E.M., Out-Luiting, J.J., Nibbering, P.H. and El Ghalbzouri, A. 2014. Reduced filaggrin expression is accompanied by increased Staphylococcus aureus colonization of epidermal skin models. *Clinical & Experimental Allergy.* **44**(12), pp.1515-1524.

Van Smeden, J. and Bouwstra, J.A. 2016. Stratum Corneum Lipids: Their Role for the Skin Barrier Function in Healthy Subjects and Atopic Dermatitis Patients. *Curr Probl Dermatol.* **49**, pp.8-26.

Van Zuuren, E.J., Fedorowicz, Z., Christensen, R., Lavrijsen, A. and Arents, B.W.M. 2017. Emollients and moisturisers for eczema. *Cochrane Database Syst Rev.* **2**, pCd012119.

Vandamme, D., Landuyt, B., Luyten, W. and Schoofs, L. 2012. A comprehensive summary of LL-37, the factotum human cathelicidin peptide. *Cellular Immunology.* **280**(1), pp.22-35.

Vandecandelaere, I., Depuydt, P., Nelis, H.J. and Coenye, T. 2014. Protease production by Staphylococcus epidermidis and its effect on Staphylococcus aureus biofilms. *Pathogens and Disease.* **70**(3), pp.321-331.

Vandenesch, F., Lina, G. and Henry, T. 2012. Staphylococcus aureus Hemolysins, bi-component Leukocidins, and Cytolytic Peptides: A Redundant Arsenal of Membrane-Damaging Virulence Factors? *Frontiers in Cellular and Infection Microbiology.* **2**, p12.

Varothai, S., Nitayavardhana, S. and Kulthanan, K. 2013. Moisturizers for patients with atopic dermatitis. *Asian Pac J Allergy Immunol.* **31**(2), pp.91-98.

Vashi, N.A., de Castro Maymone, M.B. and Kundu, R.V. 2016. Aging Differences in Ethnic Skin. *The Journal of clinical and aesthetic dermatology.* **9**(1), pp.31-38.

Ventola, C.L. 2015. The antibiotic resistance crisis: part 1: causes and threats. *P & T : a peer-reviewed journal for formulary management.* **40**(4), pp.277-283.

Vlahopoulos, S., Boldogh, I., Casola, A. and Brasier, A.R. 1999. Nuclear factor-kappaB-dependent induction of interleukin-8 gene expression by tumor necrosis factor alpha: evidence for an antioxidant sensitive activating pathway distinct from nuclear translocation. *Blood.* **94**(6), pp.1878-1889.

Vogel, C. and Marcotte, E.M. 2012. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nature Reviews Genetics.* **13**(4), pp.227-232.

Von Eiff, C., Peters, G. and Heilmann, C. 2002. Pathogenesis of infections due to coagulase-negative staphylococci. *Lancet Infect Dis.* **2**(11), pp.677-685.

Vrieze, A., Van Nood, E., Holleman, F., Salojarvi, J., Kootte, R.S., Bartelsman, J.F., Dallinga-Thie, G.M., Ackermans, M.T., Serlie, M.J., Oozeer, R., Derrien, M., Druesne, A., Van Hylckama Vlieg, J.E., Bloks, V.W., Groen, A.K., Heilig, H.G., Zoetendal, E.G., Stroes, E.S., de Vos, W.M., Hoekstra, J.B. and Nieuwdorp, M. 2012. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology.* **143**(4), pp.913-916.e917.

Vu, A.T., Baba, T., Chen, X., Le, T.A., Kinoshita, H., Xie, Y., Kamijo, S., Hiramatsu, K., Ikeda, S., Ogawa, H., Okumura, K. and Takai, T. 2010. Staphylococcus aureus membrane and diacylated lipopeptide induce thymic stromal lymphopoietin in keratinocytes through the Toll-like receptor 2-Toll-like receptor 6 pathway. *J Allergy Clin Immunol.* **126**(5), pp.985-993, 993.e981-983.

Vuong, C., Durr, M., Carmody, A.B., Peschel, A., Klebanoff, S.J. and Otto, M. 2004a. Regulated expression of pathogen-associated molecular pattern molecules in Staphylococcus epidermidis: quorum-sensing determines pro-inflammatory capacity and production of phenol-soluble modulins. *Cell Microbiol.* **6**(8), pp.753-759.

Vuong, C., Gerke, C., Greg, Elizabeth and Otto, M. 2003. Quorum-Sensing Control of Biofilm Factors in Staphylococcus epidermidis. *Journal of Infectious Diseases.* **188**(5), pp.706-718.

Vuong, C., Kocianova, S., Yao, Y., Carmody, A.B. and Otto, M. 2004b. Increased colonization of indwelling medical devices by quorum-sensing mutants of Staphylococcus epidermidis in vivo. *Journal of Infectious Diseases.* **190**(8), pp.1498-1505.

Vuong, C. and Otto, M. 2002. Staphylococcus epidermidis infections. *Microbes and Infection.* **4**(4), pp.481-489.

Vuong, C., Saenz, H.L., Götz, F. and Otto, M. 2000. Impact of the agr Quorum-Sensing System on Adherence to Polystyrene in Staphylococcus aureus. *Journal of Infectious Diseases.* **182**(6), pp.1688-1693.

Walker, A.W., Ince, J., Duncan, S.H., Webster, L.M., Holtrop, G., Ze, X., Brown, D., Stares, M.D., Scott, P., Bergerat, A., Louis, P., McIntosh, F., Johnstone, A.M., Lobley, G.E., Parkhill, J. and Flint, H.J. 2011. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *Isme j.* **5**(2), pp.220-230.

Walter, B.A., Alexander, J., Julian, L., Martin, R., Keith, R. and Peter. 2002. *Molecular Biology of the Cell. 4th edition. Introduction to Pathogens.* New York: Garland Science.

Wan, D.C., Wong, V.W., Longaker, M.T., Yang, G.P. and Wei, F.-C. 2014. Moisturizing different racial skin types. *The Journal of clinical and aesthetic dermatology.* **7**(6), pp.25-32.

Wang, B. and Tom. 2016. Regulation of Virulence in Staphylococcus aureus : Molecular Mechanisms and Remaining Puzzles. *Cell Chemical Biology.* **23**(2), pp.214-224.

Wang, B., Yao, M., Lv, L., Ling, Z. and Li, L. 2017. The Human Microbiota in Health and Disease. *Engineering.* **3**(1), pp.71-82.

Wang, R., Braughton, K.R., Kretschmer, D., Bach, T.H., Queck, S.Y., Li, M., Kennedy, A.D., Dorward, D.W., Klebanoff, S.J., Peschel, A., DeLeo, F.R. and Otto, M. 2007. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med.* **13**(12), pp.1510-1514.

Wang, R., Khan, B.A., Cheung, G.Y., Bach, T.H., Jameson-Lee, M., Kong, K.F., Queck, S.Y. and Otto, M. 2011. Staphylococcus epidermidis surfactant peptides promote biofilm maturation and dissemination of biofilm-associated infection in mice. *J Clin Invest.* **121**(1), pp.238-248.

Wang, T.T., Nestel, F.P., Bourdeau, V., Nagai, Y., Wang, Q., Liao, J., Tavera-Mendoza, L., Lin, R., Hanrahan, J.W., Mader, S. and White, J.H. 2004. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J Immunol.* **173**(5), pp.2909-2912.

Wang, Y.H. and Liu, Y.J. 2009. Thymic stromal lymphopoietin, OX40-ligand, and interleukin-25 in allergic responses. *Clin Exp Allergy.* **39**(6), pp.798-806.

Wanke, I., Steffen, H., Christ, C., Krismer, B., Götz, F., Peschel, A., Schaller, M. and Schittek, B. 2011. Skin Commensals Amplify the Innate Immune Response to Pathogens by Activation of Distinct Signaling Pathways. *Journal of Investigative Dermatology.* **131**(2), pp.382-390.

Warters, R.L., Packard, A.T., Kramer, G.F., Gaffney, D.K. and Moos, P.J. 2009. Differential Gene Expression in Primary Human Skin Keratinocytes and Fibroblasts in Response to Ionizing Radiation. *Radiation Research.* **172**(1), pp.82-95.

Wedi, B., Wieczorek, D., Stünkel, T., Breuer, K. and Kapp, A. 2002. Staphylococcal exotoxins exert proinflammatory effects through inhibition of eosinophil apoptosis, increased surface antigen expression (CD11b, CD45, CD54, and CD69), and enhanced cytokine-activated oxidative burst, thereby triggering allergic inflammatory reactions. *Journal of Allergy and Clinical Immunology.* **109**(3), pp.477-484.

Wehkamp, J., Harder, J., Wehkamp, K., Wehkamp-von Meissner, B., Schlee, M., Enders, C., Sonnenborn, U., Nuding, S., Bengmark, S., Fellermann, K., Schröder, J.M. and Stange, E.F. 2004. NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by Escherichia coli Nissle 1917: a novel effect of a probiotic bacterium. *Infect Immun.* **72**(10), pp.5750-5758.

Wehkamp, K., Schwichtenberg, L., Schröder, J.-M.M. and Harder, J. 2006. Pseudomonas Aeruginosa- and IL-1β-Mediated Induction of Human β-Defensin-2 in Keratinocytes Is Controlled by NF-κB and AP-1. *Journal of Investigative Dermatology.* **126**(1), pp.121-127.

Weinberg, A., Jin, G., Sieg, S. and McCormick, T.S. 2012. The Yin and Yang of Human Beta-Defensins in Health and Disease. *Frontiers in immunology.* **3**, p294.

Whiting, R.C., Sackitey, S., Calderone, S., Morely, K. and Phillips, J.G. 1996. Model for the survival of Staphylococcus aureus in nongrowth environments. *Int J Food Microbiol.* **31**(1-3), pp.231-243.

Wichmann, K., Uter, W., Weiss, J., Breuer, K., Heratizadeh, A., Mai, U. and Werfel, T. 2009. Isolation of α-toxin-producing Staphylococcus aureus from the skin of highly sensitized adult patients with severe atopic dermatitis. *British Journal of Dermatology.* **161**(2), pp.300-305.

Wikramanayake, T.C., Stojadinovic, O. and Tomic-Canic, M. 2014. Epidermal Differentiation in Barrier Maintenance and Wound Healing. *Advances in wound care.* **3**(3), pp.272-280.

Williams, H.C., Strachan, D.P. and Hay, R.J. 1994. Childhood eczema: disease of the advantaged? *BMJ.* **308**(6937), pp.1132-1135.

Williams, J.V., Vowels, B.R., Honig, P.J. and Leyden, J.J. 1998. S. aureus isolation from the lesions, the hands, and the anterior nares of patients with atopic dermatitis. *Pediatr Dermatol.* **15**(3), pp.194-198.

Williams, R.E.A., Gibson, A.G., Aitchison, T.C., Lever, R. and Mackie, R.M. 1990. Assessment of a contact-plate sampling technique and subsequent quantitative bacterial studies in atopic dermatitis. *British Journal of Dermatology.* **123**(4), pp.493-501.

Wirtanen, G. and Salo, S. 2016. Chapter 5 - Biofilm Risks. In: Lelieveld, H., et al. eds. *Handbook of Hygiene Control in the Food Industry (Second Edition).* San Diego: Woodhead Publishing, pp.55-79.

Wittmann, M., McGonagle, D. and Werfel, T. 2014. Cytokines as therapeutic targets in skin inflammation. *Cytokine & Growth Factor Reviews.* **25**(4), pp.443-451.

Wohl, D.L., Curry, W.J., Mauger, D., Miller, J. and Tyrie, K. 2015. Intrapartum antibiotics and childhood atopic dermatitis. *Journal of the American Board of Family Medicine : JABFM.* **28**(1), pp.82-89.

Wollina, U. 2017. Microbiome in atopic dermatitis. *Clinical, Cosmetic and Investigational Dermatology.* **10**, pp.51-56.

Wong, C.-K., Chu, I., Hon, K.-L., Tsang, M. and Lam, C. 2016. Aberrant Expression of Bacterial Pattern Recognition Receptor NOD2 of Basophils and Microbicidal Peptides in Atopic Dermatitis. *Molecules.* **21**(4), p471.

Wright, J.A. and Nair, S.P. 2012. The lipoprotein components of the Isd and Hts transport systems are dispensable for acquisition of heme by Staphylococcus aureus. *FEMS Microbiol Lett.* **329**(2), pp.177-185.

Xhindoli, D., Pacor, S., Benincasa, M., Scocchi, M., Gennaro, R. and Tossi, A. 2016. The human cathelicidin LL-37 — A pore-forming antibacterial peptide and host-cell modulator. *Biochimica et Biophysica Acta (BBA) - Biomembranes.* **1858**(3), pp.546-566.

Yamanaka, K. and Mizutani, H. 2011. The role of cytokines/chemokines in the pathogenesis of atopic dermatitis. *Curr Probl Dermatol.* **41**, pp.80-92.

Yamasaki, K., Schauber, J., Coda, A., Lin, H., Dorschner, R.A., Schechter, N.M., Bonnart, C., Descargues, P., Hovnanian, A. and Gallo, R.L. 2006. Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin. *The FASEB Journal.* **20**(12), pp.2068-2080.

Yamazaki, Y., Nakamura, Y. and Núñez, G. 2017. Role of the microbiota in skin immunity and atopic dermatitis. *Allergology International.* **66**(4), pp.539-544.

Yang, D., Chertov, O., Bykovskaia, S.N., Chen, Q., Buffo, M.J., Shogan, J., Anderson, M., Schröder, J.M., Wang, J.M., Howard, O.M.Z. and Oppenheim, J.J. 1999. β-Defensins: Linking innate and adaptive immunity through dendritic and T cell CCR6. *Science.* **286**(5439), pp.525-528.

Yang, D., Chertov, O. and Oppenheim, J.J. 2001. Participation of mammalian defensins and cathelicidins in anti-microbial immunity: receptors and activities of human defensins and cathelicidin (LL-37). *J Leukoc Biol.* **69**(5), pp.691-697.

Yang, H., Wang, M., Yu, J. and Wei, H. 2015. Aspartate inhibits Staphylococcus aureus biofilm formation. *FEMS Microbiol Lett.* **362**(7), pp.fnv025-fnv025.

Yang, L., Mao-Qiang, M., Taljebini, M., Elias, P.M. and Feingold, K.R. 1995. Topical stratum corneum lipids accelerate barrier repair after tape stripping, solvent treatment and some but not all types of detergent treatment. *Br J Dermatol.* **133**(5), pp.679-685.

Yao, Y., Sturdevant, D.E. and Otto, M. 2005. Genomewide analysis of gene expression in Staphylococcus epidermidis biofilms: insights into the pathophysiology of S. epidermidis biofilms and the role of phenol-soluble modulins in formation of biofilms. *J Infect Dis.* **191**(2), pp.289-298.

Yarbrough, K.B., Neuhaus, K.J. and Simpson, E.L. 2013. The effects of treatment on itch in atopic dermatitis. *Dermatologic therapy.* **26**(2), pp.110-119.

Ye, Y.-M., Kim, B.E., Shin, Y.S., Park, H.-S. and Leung, D.Y.M. 2014. Increased epidermal filaggrin in chronic idiopathic urticaria is associated with severity of urticaria. *Annals of Allergy, Asthma & Immunology.* **112**(6), pp.533-538.

Young Shin, S., Bajpai, V., T Hou, C., Choi, U.-K., Kim, H.-R. and Chul, S. 2005. Antibacterial activity of bioconverted linoleic acid produced by Pseudomonas aeruginosa PR3. *Journal of Applied Biological Chemistry.* **48**.

Yousef, H., Alhajj, M. and Sharma, S. 2019. Anatomy, Skin (Integument), Epidermis. *StatPearls.* Treasure Island (FL): StatPearls Publishing LLC.

Yui, S., Nakatani, Y. and Mikami, M. 2003. Calprotectin (S100A8/S100A9), an inflammatory protein complex from neutrophils with a broad apoptosis-inducing activity. *Biol Pharm Bull.* **26**(6), pp.753-760.

Zanger, P., Holzer, J., Schleucher, R., Scherbaum, H., Schittek, B. and Gabrysch, S. 2010. Severity of Staphylococcus aureus infection of the skin is associated with inducibility of human beta-defensin 3 but not human beta-defensin 2. *Infect Immun.* **78**(7), pp.3112-3117.

Zanger, P., Nurjadi, D., Vath, B. and Kremsner, P.G. 2011. Persistent Nasal Carriage of Staphylococcus aureus Is Associated with Deficient Induction of Human β-Defensin 3 after Sterile Wounding of Healthy Skin In Vivo. *Infection and Immunity.* **79**(7), pp.2658-2662.

Zaniboni, M.C., Samorano, L.P., Orfali, R.L. and Aoki, V. 2016. Skin barrier in atopic dermatitis: beyond filaggrin. *Anais brasileiros de dermatologia.* **91**(4), pp.472-478.

Zanoni, T.B., Tiago, M., Faião-Flores, F., De Moraes Barros, S.B., Bast, A., Hageman, G., De Oliveira, D.P. and Maria-Engler, S.S. 2014. Basic Red 51, a permitted semi-permanent hair dye, is cytotoxic to human skin cells: Studies in monolayer and 3D skin model using human keratinocytes (HaCaT). *Toxicology Letters.* **227**(2), pp.139-149.

Zhang, L.-J. and Gallo, R.L. 2016. Antimicrobial peptides. *Current Biology.* **26**(1), pp.R14-R19.

Ziboh, V.A., Miller, C.C. and Cho, Y. 2000. Metabolism of polyunsaturated fatty acids by skin epidermal enzymes: generation of antiinflammatory and antiproliferative metabolites. *The American Journal of Clinical Nutrition.* **71**(1), pp.361s-366s.

Zipperer, A., Konnerth, M.C., Laux, C., Berscheid, A., Janek, D., Weidenmaier, C., Burian, M., Schilling, N.A., Slavetinsky, C., Marschal, M., Willmann, M., Kalbacher, H., Schittek, B., Brötz-Oesterhelt, H., Grond, S., Peschel, A. and Krismer, B. 2016. Human commensals producing a novel antibiotic impair pathogen colonization. *Nature.* **535**, p511.

Zollner, Wichelhaus, Hartung, Von, M., Wagner, Brade and Kaufmann. 2000. Colonization with superantigen-producing Staphylococcus aureus is associated with increased severity of atopic dermatitis. *Clinical & Experimental Allergy.* **30**(7), pp.994-1000.

8. Appendices and supplemental material

8.1. Suppliers of the products used in the study

Supplier	Location
Appleton Woods	Birmingham B38 8SE, UK
Beckman-Coulter Genomics	Bishop's Stortford CM22 6TA, UK
BEI Resources	Manassas 20110-2209 Virginia, USA
Berthold Technologies	Harpenden AL5 4UT, UK
Bibby Scientific	Stone ST15 0SA, UK
Biolegend	London NW5 1LB, UK
Biolog, Inc	Hayward 94545 California, USA
Bio-rad	Watford WD17 1ET, UK
Camlab	Cambridge CB24 5WE, UK
Delta-Lab	Barcelona 08191, Spain
EdgeBio	San Jose 95131 California, USA
Eppendorf	Stevenage SG1 2FP, UK
Eurofins	Ebersberg 85560, Germany
Expedeon	Cambridge CB24 5QE, UK
GE Healthcare	Amersham HP7 9NA, UK
Hycult Biotech	Cambridge CB23 8SQ, UK
Implen	Munich 81829, Germany
Lonza	Castleford WF10 2JT, UK
Merck	Watford WD18 8YH, UK
MyBiosource	San Diego 92195-3308 California, USA
New England Biolabs	Hitchin SG4 0TY, UK
Oxoid	Basingstoke RG24 8PW, UK
Peprotech	London W6 8LL, UK
Pro Lab Diagnostics	Birkenhead CH62 3QL, UK
Promega	Fitchburg 53711 Wisconsin, USA
PromoCell	Heidelberg 69126, Germany
Public Health England	Salisbury SP4 0JG, UK
Qiagen	Manchester M15 6SH, UK
Sapphire	Manchester M15 6SH, UK
Sarstedt	Leicester LE4 1AW, UK
Sigma-Aldrich	Gillingham SP8 4XT, UK
Terumo	Prescot L34 9GT, UK
Thermo-Fisher Scientific	Paisley PA4 9RF, UK
Woundchek	Gargrave BD23 3RX, UK

8.2. Gram staining, pastorex test and MIC determination

8.2.1. Gram staining

During Gram staining (Pro Lab Diagnostics), a drop of sterile water was placed on a slide with a sterile loop. A single staphylococcal colony from a TSA plate was picked up with the loop and stirred in the drop of water. The slide was air-dried, passed several times through a flame (heat fixation) and then placed on a staining tray. Initially, the fixed smear was flooded with crystal violet solution for 1 min. The slide was rinsed with tap water and was then flooded with iodine solution. After 1 min, it was rinsed again with water. Acetone (Sigma-Aldrich) was poured on the slide and after 2-3 s it was rinsed with water. Then, the slide was flooded with carbol fuchsin solution for 30 s, rinsed with water and dried on absorbent paper. Finally, the slide was examined under a microscope using oil-immersion (Sigma-Aldrich). Gram-positive bacteria retain the crystal violet dye, whereas Gram-negative bacteria are stained red (Matheson, 1999).

8.2.2. Pastorex test

The manufacturer’s protocol was followed (Bio-rad). Briefly, one drop of latex test and one of negative control latex reagent were applied onto an agglutination card. A single colony of the test culture on a loop was mixed into each of the drops. Catalase-positive colonies can be discriminated by this method as they form aggregates in the latex test (Bio-rad).

8.2.3. Broth microdilutions and MIC determination

The MIC protocol was followed according to the CLSI guidelines (Cockerill et al., 2012). Briefly, a doubling dilution series of the compound to be tested was prepared. The most concentrated stock was at 10X the final required concentration. Then, 10 µL was added to wells of a flat-bottomed 96-well plate in duplicates. The cell density of MHB-II staphylococcal overnight cultures was

determined using $OD_{625\ nm}$. Cells were diluted in MHB-II to achieve a cell number of ~ 5 x 10^5 CFU/mL. Then, 90 µL of the diluted culture was added to each compound-containing well. The edge of the plate was sealed with parafilm and the plate was incubated at 37°C with agitation on a Heidolph Inkubator 1000 for 16-18 h. MICs were determined by evaluation of turbidity of the wells with constantly increasing concentration of the tested compound.

8.3. Oligonucleotide primers used in this study

Primers for verification of *S. epidermidis* skin-isolated strains		
Primer	**Sequence (5' → 3')**	**Comments**
S. epidermidis rpoB 2491 forward	GGCGAACCACCAACAGTAGA (20)	~ 600 bp amplicon from *S. epidermidis* (Eurofins)
S. epidermidis rpoB 3241 reverse	TGAACGTCACGCACTTCCAT (20)	

Primers for verification of staphylococcal strains before phenotypic characterisation		
Primer	**Sequence (5' → 3')**	**Comments**
F3, *S. aureus rpoB* forward	AGTCTATCACACCTCAACAA	~ 600 bp amplicon from *S. aureus* (Aubry-Damon et al., 1998)
F4, *S. aureus rpoB* reverse	TAATAGCCGCACCAGAATCA	
S. epidermidis rpoB forward	AATTTGGTTTTATTGAAACGCCA	~ 250 bp amplicon from *S. epidermidis* (Work conducted by Dr Liam Sharkey)
S. epidermidis rpoB reverse	GGTTAGAGTCGTCATTTTCTAAG	

8.4. Liquid chromatography-mass spectrometry

8.4.1. Initial LC-MS – Protein Identification (PTM mapping)

In-solution digestion

Dithiothreitol was added to a final concentration of 10 mM and heated to 56°C for 30 min with shaking. The Eppendorf was left to cool before adding

iodoacetic acid to a final concentration of 55 mM and incubated at room temperature in the dark for 30 min with shaking. Trypsin solution (20 ng/µL in 25 mM ammonium bicarbonate) was added to a 1:50 ratio (protease:total protein content). The samples were incubated at 37°C with shaking for 18 h. The digest reaction was stopped by adding 5 µL of 1% formic acid.

Sep-Pak C18 Purification

The Sep-Pak column was equilibrated with 1 mL acetonitrile followed by 1 mL 0.1% trifluoroacetic acid. Then, 500 µL 0.1% trifluoroacetic acid was added to the peptide digest, this mix was passed through the column. The column was washed with 1 mL 0.1% trifluoroacetic acid. Peptides were then eluted from the column with 500 µL 50% acetonitrol, 50% water, 0.1% formic acid. The eluant was dried by vacuum centrifugation. The peptides were reconstituted in 20 µL 0.1% aqueous trifluoroacetic acid.

Liquid chromatography-mass spectrometry

Liquid chromatograph separation of the peptide mixtures was performed on an ACQUITY M-Class UPLC (Waters UK, Manchester). Firstly, 1 µL of each sample was loaded onto a Symmetry C18 trap column (180 µM i.d. * 20 mm) and washed with 1% acetonitrile/0.1% formic acid for 5 min at 5 µL/min. After valve switching, the peptides were then separated on a HSS T3 C18, 75 µm i.d. x 150 mm analytical column (Waters UK, Manchester) by gradient elution of 1-60% solvent B in A over 30 min. at 0.3 µL/min. Solvent A was 0.1% formic acid in water, solvent B was 0.1% formic acid in acetonitrile.

The column eluant was directly interfaced to a Xevo G2-XS Q-TOF mass spectrometer via a Z-spray nanoflow electrospray source. The MS was operated in positive TOF mode using a capillary voltage of 3.0 kV, cone voltage of 40 V, source offset of 80 V, backing pressure of 3.58 mbar and a trap bias of 2 V. The source temperature was 80°C. Argon was used as the buffer gas at a pressure of 8.6×10^{-3} mbar in the trap and transfer regions. Mass calibration was performed using [Glu]-fibrinopeptide (GFP) at a concentration of 100 fmol/µL. GFP was also used as a lock mass calibrant

with a 1 s lock spray scan taken every 30 s during acquisition. Ten scans were averaged to determine the lock mass correction factor. Data acquisition was using data dependent analysis with a 0.2 s scan MS over m/z 350-2000 being followed by five 0.5 s MS/MS taken of the five most intense ions in the MS spectrum. CE applied was dependent upon charge state and mass of the ion selected. Dynamic exclusion of 60 s was used. Data processing was performed using the MassLynx v4.1 suite of software supplied with the mass spectrometer. Peptide MS/MS data were processed with PEAKS Studio (Bioinformatic Solutions Inc, Waterloo, Ontario, Canada) and searched against the Uniprot database for *Staphylococcus aureus*. Carbamiodomethylation was selected as a fixed modification, variable modifiacations were set for oxidation of methionine and deamidation of glutamine and asparagine. MS mass tolerance was 15 ppm, and fragment ion mass tolerance was 0.05 Da. The false discovery rate was set to 1% and only proteins with 3 or more unique peptides were reported.

8.4.2. Improved sensitivity LC-MS

Protein Digest Method: S-Trap micro ultra-high recovery protocol

First, 12.5 µL SDS solubilisation buffer (10% SDS, 100 mM triethylammonium bicarbonate TEAB, pH 7.55) was added to 12.5 µL lysate sample. To reduce the proteins DTT was added to a final concentration of 20 mM then incubated for 10 min at 95°C. Samples were allowed to cool to room temperature before adding iodoacetamide to a final concentration of 40 mM and incubated for 30 min at 20°C to alkylate the proteins. 4 µL of 12% phosphoric acid were then added to aid protein trap binding. 165 µL S-trap protein binding buffer (90% aqueous methanol containing a final concentration of 100 mM TEAB, pH 7.1) was added to the S-Trap micro column. 1 µg trypsin was added to the acidified sample and immediately mixed by pipetting up and down. The acidified lysate plus trypsin was immediately transferred to S-Trap binding buffer within the spin again by mixing up and down. The micro column was placed in a 2 mL tube for flowthrough and centrifuged at 4,000 x *g* until the sample had passed through the S-Trap column. At this stage the protein became trapped within

the protein-trapping matrix of the spin column. The captured protein was washed by adding 150 µL S-Trap protein binding buffer, before being centrifuged at 4,000 x *g* for 1 min. This was step was repeated three times whilst rotating the S-Trap micro column 180°C between centrifugations. 0.5 µg of trypsin in 25 µL of 50 mM TEAB, pH 8 to the top of the protein trap. The column was capped loosely and incubated in a clean 1.5 mL tube for 1 h at 47°C without shaking. Peptides were eluted with 40 µL each of 50 mM TEAB added to the trypsin solution and then centrifuged at 4,000 x *g*. Further elution rounds added 40 µL 0.2% aqueous formic acid, then 35 µL 50% acetonitrile, 0.2% formic acid. Peptides were dried down and resuspended in 0.1% trifluoroacetic acid for MS analysis.

Liquid chromatography-mass spectrometry

Liquid chromatography separation of the peptide mixtures was performed on an ACQUITY M-Class UPLC (Waters UK, Manchester). Initially, 1 µL of each sample was loaded onto a Symmetry C18 trap column (180 µM i.d. * 20mm) and washed with 1% acetonitrile/0.1% formic acid for 5 min at 5 µL/min. After valve switching, the peptides were then separated on a HSS T3 C18, 75 µm i.d. x 150 mm analytical column (Waters UK, Manchester) by gradient elution of 1-60% solvent B in A over 30 min. at 0.3 µL/min. Solvent A was 0.1% formic acid in water, solvent B was 0.1% formic acid in acetonitrile. The column eluant was directly interfaced to a quadrupole orthogonal time of flight mass spectrometer (Xevo QTOF G2-XS, Waters UK, Manchester) via a Z-spray nanoflow electrospray source. The MS was operated in positive TOF mode using a capillary voltage of 3.0 kV, cone voltage of 40 V, source offset of 80 V. The source temperature was 80°C. Mass calibration was performed using [Glu]-fibrinopeptide (GFP) at a concentration of 250 fmolµL. GFP was also used as a lock mass calibrant with a 1 s lock spray scan taken every 30 s during acquisition. Ten scans were averaged to determine the lock mass correction factor. Data acquisition was using data dependent analysis with a 0.2 s scan MS over m/z 350-2000 being followed by five 0.5 s MS/MS (m/z 50-2000) taken of the four most intense ions in the MS spectrum. CE applied was dependent upon charge state and mass of the ion selected. Dynamic

exclusion of 60 s was used. Data processing was performed using the MassLynx v4.1 suite of software supplied with the mass spectrometer. Peptide MS/MS data were processed with PEAKS Studio (Bioinformatic Solutions Inc, Waterloo, Ontario, Canada) and searched against the Uniprot database, with entries confined to *Staphylococcus aureus*. Carbamiodomethylation was selected as a fixed modification, variable modifiacations were set for oxidation of methionine and deamidation of glutamine and asparagine. MS mass tolerance was 20 ppm, and fragment ion mass tolerance was 0.05 Da. The false discovery rate was set to 1% and only proteins with 3 or more unique peptides were reported.

8.5. Phenotypic characterisation

8.5.1. Susceptibility determination of *S. aureus* and *S. epidermidis*

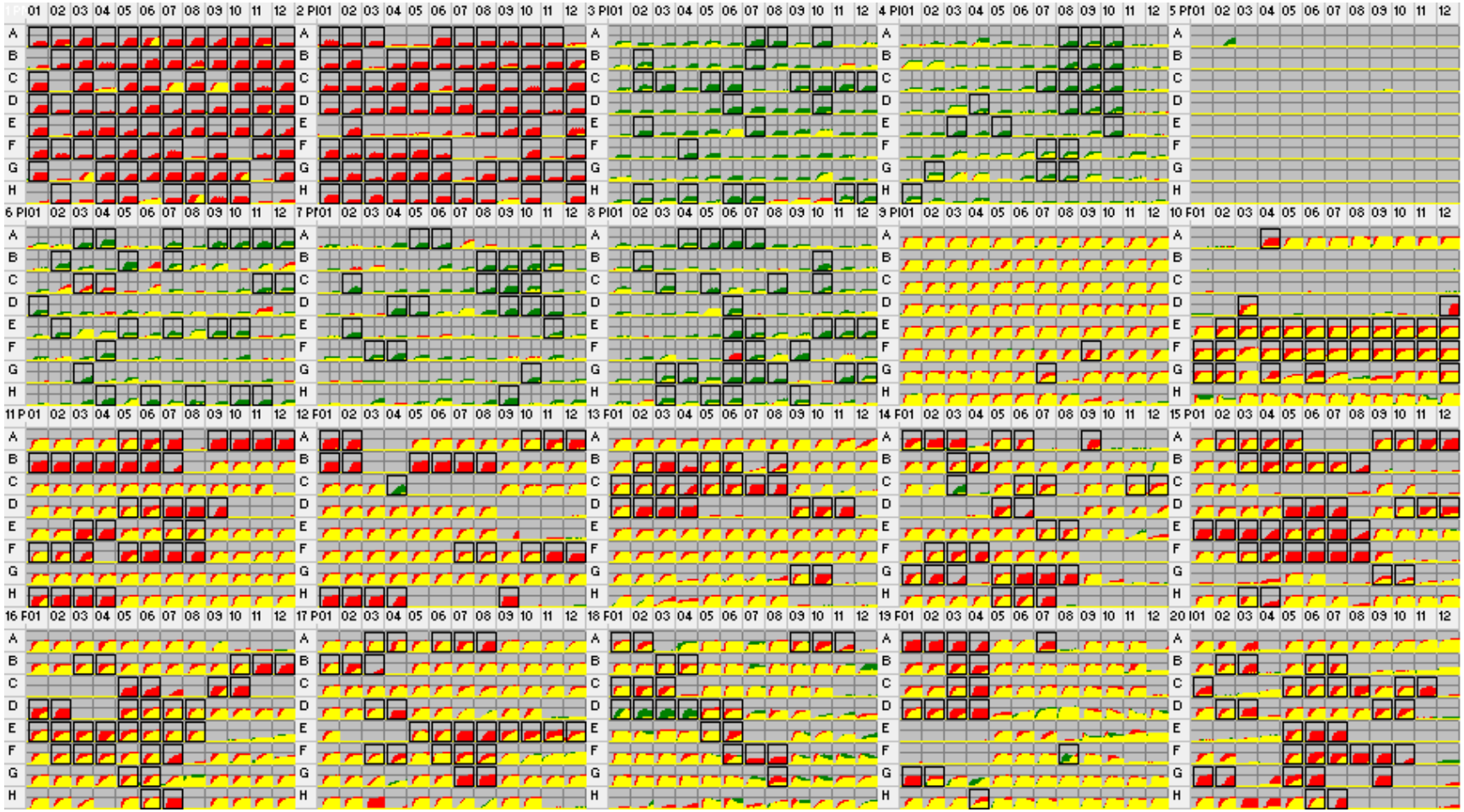
The susceptibility of the four staphylococcal strains (*S. aureus* USA300 and CS6-EEFIC and *S. epidermidis* NCTC11047 and NCTC6513) for the compounds identified by Biolog was examined using broth microdilutions and MIC determination. These compounds were tested in a 2-fold series of dilutions, starting from the highest concentration in which each compound is soluble, as shown below.

Compound	Final concentration range
D-alanine	50 mg/mL - 1.5625 mg/mL
L-alanine	50 mg/mL - 1.5625 mg/mL
L-citrulline	50 mg/mL - 1.5625 mg/mL
L-homoserine	50 mg/mL - 25 mg/mL
Phe-Ala	50 mg/mL - 25 mg/mL
Leu-Ala	50 mg/mL - 6.25 mg/mL
Ala-Phe	5 mg/mL - 0.0049 mg/mL
D-aspartic acid	1.25 mg/mL - 0.3125 mg/mL

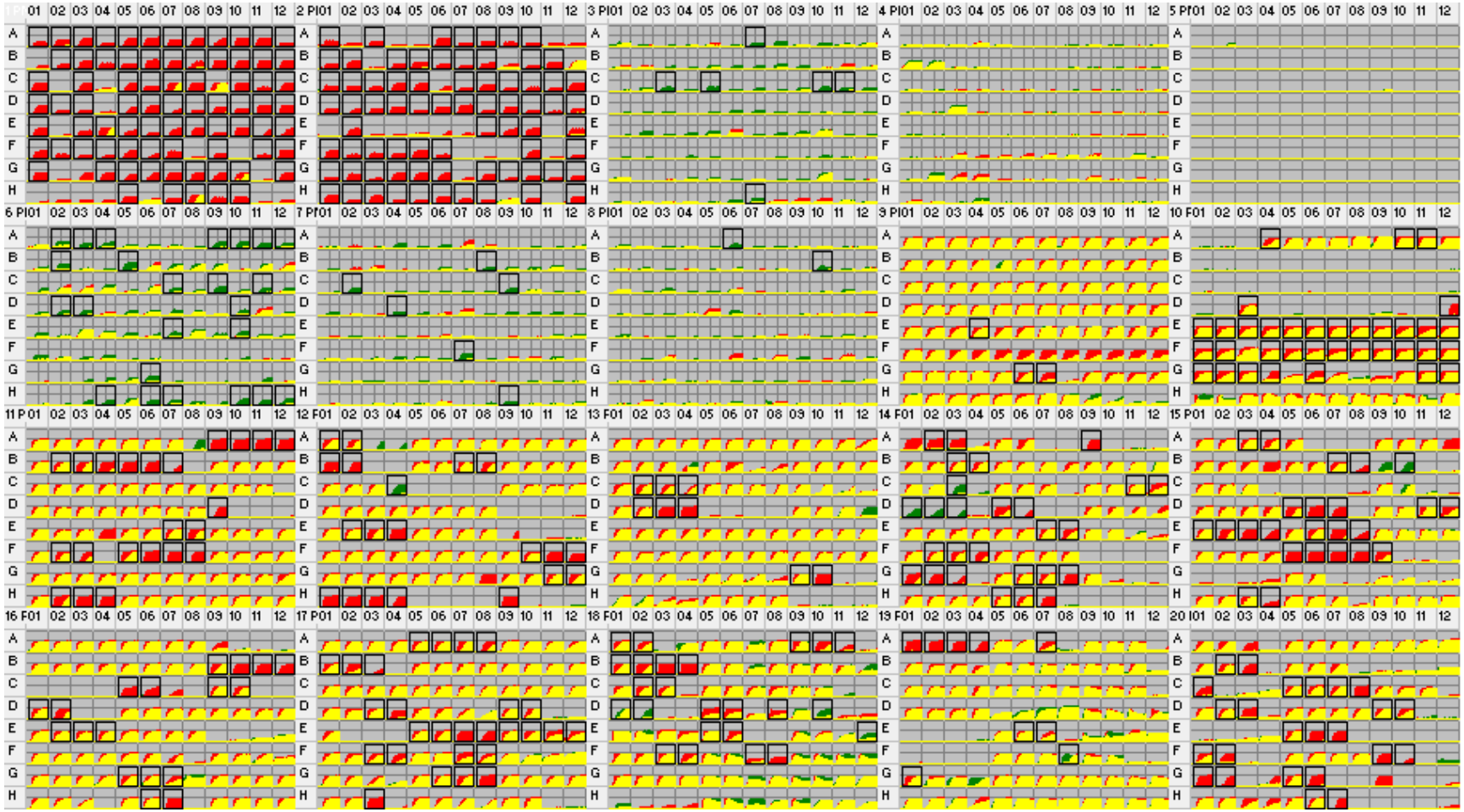
8.5.2. Phenotypic characterisation: preliminary results

The data presented were produced during the phenotypic characterisation of *S. aureus* USA300, *S. aureus* CS6-EEFIC, *S. epidermidis* NCTC11047 and *S. epidermidis* NCTC6513, performed by Biolog.

A) *S. epidermidis* NCTC6513 (green) versus *S. aureus* USA300 (red)



B) *S. epidermidis* NCTC11047 (green) versus *S. aureus* USA300 (red)



C) *S. aureus* CS6-EEFIC (green) versus *S. aureus* USA300 (red)