Evaluation of the utility of probiotics for the prevention of infections in a model of the skin

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List of Abbreviations

ACTB	Beta-actin
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
B2M	Beta 2 microglobulin
BCA	Bicinchoninic Acid Assay
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
BV	Bacterial vaginosis
CAMP	Cathelicidin antimicrobial peptide
CD	Crohn's disease
CE	Cornified envelope
CFS	Cell-free supernatant
CFU	Colony forming units
CM	Conditioned medium
CnBP	Collagen binding proteins
CONS	Coagulase-negative staphylococci
CRAMP	Cathelicidin related antimicrobial peptide
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
EaP	Extracellular adherence protein
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay

EPS	Extracellular polysaccharide
FDA	Food and Drug Authority
FITC	Fluorescein isothiocyanate
FnBP	Fibronectin binding protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GMH	Glucomannan hydrolysates
GRAS	Generally recognised as safe
HA	Hyaluronic acid
Hbd	Human beta defensin
HMBS	Hydroxymethyl-bilane synthase
HBSS	Hanks buffered salt solution
HKLR	Heat-killed *L. reuteri*
HPLC	High performance liquid chromatography
HPRT1	Hypoxanthine phosphoribosyl transferase I
HSP	Heat shock protein
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFN	Interferon
IRF	Interferon regulatory factors
JAMS	Junction adhesion molecules
LAB	Lactic acid bacteria
LGG	*L. rhamnosus* GG
LPS	Lipopolysaccharide
LR	*L. reuteri*
LRH	*L. rhamnosus* AC 413

LS	*L. salivarius*
LTA	Lipoteichoic acid
MAMP	Microbial associated molecular pattern
MAPK	Mitogen activated protein kinase
mEASI	Modified eczema area sensitivity index
MIC	Minimum inhibitory concentration
MOI	Multiplicity of infection
MRS	Man-Rogosa Sharpe
MRSA	Methicillin resistant *S. aureus*
MSA	Mannitol salt agar
NF-κB	Nuclear factor-κ -B
NHEK	Normal human epidermal keratinocytes
NOD-2	Nucleotide-binding oligomerization domain containing protein-2
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PBS	Peptone buffered saline
PCR	Polymerase chain reaction
PGN	Peptidoglycan
PRR	Pattern recognition receptor
QRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RNA	Ribonucleic acid
RPL13A	Ribosomal protein L13a
RPL32	Ribosomal protein L32
SA	*S. aureus*
SB	*Stratum basale*

SC	*S. capitis*
SD-Ag	Silver sulphadiazine
SDHA	Succinate dehydrogenase complex, subunit A
SDS-PAGE	Sodium-dodecyl polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SG	*Stratum granulosum*
SSP	*Stratum spinosum*
TEER	Trans-epithelial electrical resistance
TEWL	Trans-epithelial water loss
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
TRAF-6	TNF receptor associated factor-6
TRIF	Toll–IL-1 receptor containing adaptor inducing IFN-β
TSST	Toxic shock syndrome toxin
TUNEL	Terminal deoxynucleotidyl Transferase dUTP nick end labeling
UC	Ulcerative colitis
VEC	Vaginal epithelial cells
WC	Whole cell
WCA	Wilkins-Chalgren Agar
WCB	Wilkins-Chalgren Broth
ZOI-1	Zona-Occludens-1

Abstract: Evaluation of the utility of probiotics for the prevention of infections in a model of the skin

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Probiotics have been defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. The beneficial effects of probiotics in the gut are well described and roles including immunomodulation and colonisation resistance have been documented. Recent reports suggest that topical use of probiotic bacteria may be an effective strategy to promote skin health or inhibit disease. Therefore, in this thesis the potential of probiotics to protect skin from pathogenic bacteria was assessed using primary keratinocytes as a model system, and the skin pathogen, *Staphylococcus aureus*. The ability of three probiotics, *L. reuteri* ATCC 55730, *L. rhamnosus* AC413 and *L. salivarius* UCC118 to inhibit the growth of *S. aureus* was tested using well-diffusion assays and spot on the lawn assays. All three probiotics inhibited the growth of *S. aureus* in well-diffusion assays, though this property was dependent on growth medium. Inhibition of *S. aureus* growth was principally via the production of organic acids rather than bacteriocin production. Next, to determine whether probiotics could protect keratinocytes, confluent normal human epidermal keratinocytes (NHEK) were infected with S*. aureus* (10^6 CFU/ml) in the presence or absence of the probiotic (10^8 CFU/ml). NHEK viability was measured using trypan blue exclusion assays. *L. reuteri* had a significant protective effect on NHEK when applied 1h prior to (P=0.0003), or simultaneously with *S. aureus* (P=0.002). *L. reuteri* did not however protect NHEK when applied 1h after *S. aureus* addition. There was no change in the number of viable *S. aureus* in cell culture assays. To determine whether the protective effect was due to the inhibition of adhesion, NHEK were either pre-exposed to the probiotic for 1h, simultaneously exposed to the probiotic and *S. aureus* for 1h, or exposed to the probiotic 30 minutes after *S. aureus* addition for 1h. Pre-exposure of NHEK to *L. reuteri* (exclusion) and simultaneous exposure to *L. reuteri* and *S. aureus* (competition) resulted in significantly less staphylococci adhering to NHEK (P=0.03 and P=0.008 respectively). However when *L. reuteri* was added after *S. aureus* (displacement), the number of adherent staphylococci was not reduced. The necessity of *S. aureus* adherence for the inactivation of NHEK was demonstrated using a α5β1 integrin blocking antibody. Finally, to compare the innate response of NHEK to probiotics with *S. capitis* and *S. aureus*, TLR-2, antimicrobial peptide (AMP) expression and IL-8 production were measured. TLR-2 protein (but not mRNA) expression was reduced in the presence of *S. aureus* (P=0.018). NHEK pre-exposed to *S. capitis* prior to *S. aureus* infection however, exhibited elevated TLR-2 protein and mRNA expression (P<0.0001 and P=0.009 respectively). NHEK pre-exposed to *L. reuteri* prior to *S. aureus* had no significant change in TLR-2 expression compared to untreated controls. ELISAs demonstrated that IL-8 production was significantly increased in NHEK pre-exposed to *L. reuteri* prior to *S. aureus* infection (P=0.0001). In conclusion, *L. reuteri* protected NHEK from the toxic effects of *S. aureus* at least partly through competitive exclusion of binding sites on NHEK. Finally, NHEK innate responses to probiotic bacteria were akin to those to the skin commensal, *S. capitis*. *L. reuteri* induced expression of a neutrophil chemoattractant, suggesting it could be of importance in priming the innate immune response against *S. aureus* infections. Taken together, these results suggest that probiotic bacteria could be used prophylactically within skin creams and soaps to prevent *S. aureus* colonisation and infection in skin.

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Dedication

This thesis is lovingly dedicated to my dear sister Phoebe. "Go gcoinní Dia i mbos a láimhe thú" (May God keep you in the palm of his hand).

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Publications arising from this Thesis

Peer reviewed manuscripts

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Conference contributions

Prince, T., Mcbain, A. J., and O'Neill, C. A. (2011). *L. reuteri* can inhibit *S. aureus* adhesion to keratinocytes *in vitro*. European Society of Dermatological Research Annual Meeting, Barcelona, Spain.

Prince, T., Mcbain, A. J., and O'Neill, C. A. (2011). *L. reuteri* can inhibit the cytotoxic effects of *S.aureus* on keratinocytes *in vitro*. British Society of Investigative Dermatology Annual Meeting, Manchester, UK.

Prince, T., Mcbain, A. J., and O'Neill, C. A (2012). A comparison of the innate immune responses of keratinocytes to probiotics and skin commensals. European Society of Dermatological Research Annual Meeting, Venice, Italy.

Background

1.0 Introduction

Humans live in constant interaction with the external environment and therefore with constant exposure to microorganisms. The human foetus is essentially sterile, but following birth the epithelial surfaces of the body (e.g. skin, respiratory tract, gastrointestinal tract and urogenital tract) are colonised by microorganisms which form complex communities of transient and permanent bacterial species. The permanent bacterial residents are described as the commensal microflora, indicating a mutual beneficial relationship for both parties. The enormous bacterial population with which we co-exist daily can be demonstrated by the fact that approximately 10^{14} bacteria reside in the gut and 10^{12} bacteria reside on the skin while the human body possesses approximately 10^{13} somatic cells, considerably less than the number of bacteria residing within it (Luckey, 1972). Molecular biology techniques have allowed more comprehensive analysis of the diverse range of organisms that inhabit the body, as many are unculturable (Tlaskalová-Hogenová et al., 2004). Most work has concentrated on the gut microflora, and evidence suggests that certain enteric bacteria have a positive impact on health, e.g. regulating epithelial barrier function, modification of innate immune responses in the gut and inhibition of pathogen adherence to the gut epithelium. This has led to the development of probiotics, or the so called “friendly bacteria” which, when taken in sufficient quantities, may be used to promote health or combat disease (Section 1.2). Since enteric probiotics are generally considered non-pathogenic and safe, more recent studies have led to the concept that probiotics may have widespread applications beyond the gut. Potentially, probiotics could be used anywhere commensal microflora normally reside (Section 1.2).

In contrast to the gut, very little is known about the normal interactions between the skin microflora and the epidermis. Some interest has developed in the potentially beneficial effects of skin commensals, i.e. specific skin probiotics. However, the skin microflora can also be pathogenic under certain circumstances. Hence, the role of enteric probiotics in relation to skin health has begun to be examined (Krutmann, 2009). Application of probiotics could potentially aid in wound healing, or act as a preventative measure or adjunct therapy against infection. However, little work has been undertaken to date in this area. This project is one of the first of its kind to carry out preliminary investigations into the potential utility of probiotic bacteria as skin therapeutic agents. The primary aim was to determine whether a topical probiotic could be used to prevent or treat

common *Staphylococcus aureus* skin infections and what effect probiotics may have upon the innate immune responses of the skin. The results obtained may have therapeutic potential in terms of prevention of skin infections.

1.1 The skin and its commensal microbiota

1.1.1 Structure of the skin

The skin is the largest sensory organ of the body. Its most important role is as a barrier against external influences and water loss. The significance of skin as a barrier can be observed in those exhibiting defective barrier function, such as burns victims and certain skin conditions such as Pemphigus, where epidermal blistering and resultant fluid imbalance occurs (MacKie, 1997a). The skin is composed of three main layers, the epidermis, the dermis and the hypodermis (Figure 1.1). The epidermis is the main outer layer of the skin and composed of the main cell type, the keratinocyte. The epidermis is stratified into different layers. Beneath the epidermis lies the dermis, a connective tissue layer providing the vascular supply to the skin (Moran and Rowley, 1988, MacKie, 1997b). Below the dermis lies the hypodermis, a layer composed mostly of adipose tissue and collagen, ranging in thickness according to the person (Figure 1.1) (Thibodeau and Patton, 1999). It is the epidermis that forms the main physical barrier of the skin.

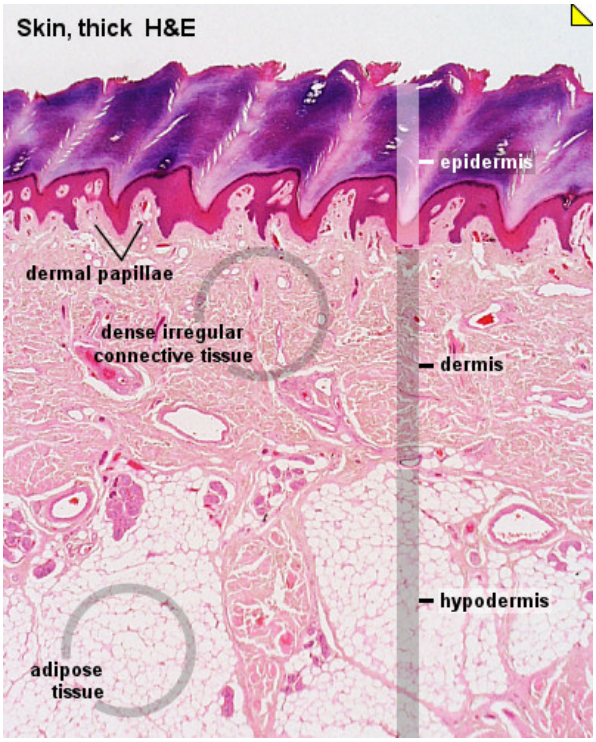


Figure 1.1. Structure of the skin (Slomianka, 2009).
Diagram demonstrating the three main regions of the skin, the epidermis, the dermis, and the hypodermis.

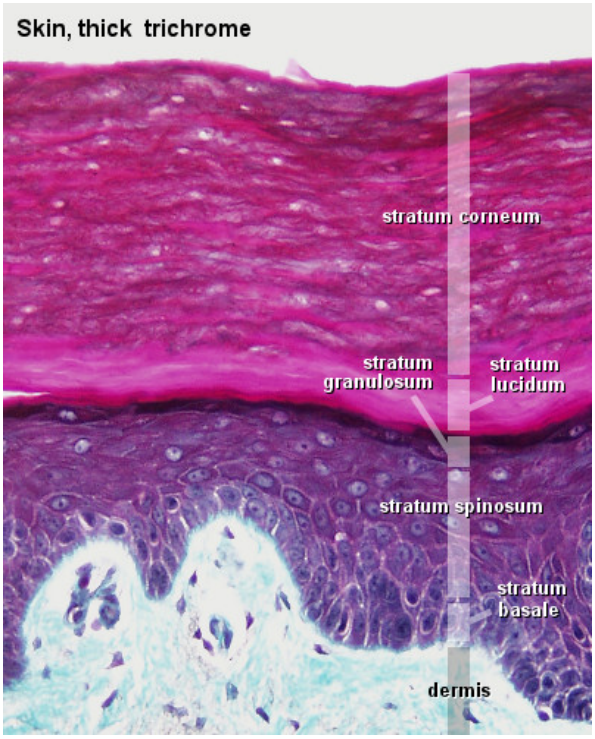


Figure 1.2. Structure of the epidermis (Slomianka, 2009).
Photograph of thick skin detailing the different layers of the epidermis, the *stratum corneum*, *stratum lucidum*, *stratum granulosum*, *stratum spinosum* and *stratum basale*.

The epidermis consists of four main layers (Figure 1.2). The innermost layer is the *stratum basale*, composed mostly of dividing keratinocytes (McEwan Jenkinson, 1993). When a keratinocyte divides in the *stratum basale*, one cell remains in the basal layer, while the other cell moves upwards through the epidermis (MacKie, 1997b). These dividing cells become steadily more keratinised until they reach the outermost layer where they are fully keratinised, flattened and inert. These cells or “squames” may then be shed into the atmosphere, a process called desquamation (McEwan Jenkinson, 1993). Above the *stratum basale* is the *stratum spinosum* where the majority of living keratinocytes reside, attached to one another by desmosomes (Moran and Rowley, 1988). Above this is the *stratum granulosum*, a granular layer where cells of the epidermis begin to lose their nuclei. This layer is so called because of the presence of numerous granules of keratohyalin within the cells (Moran and Rowley, 1988). The *stratum granulosum* may be composed of two or three layers of cells (Hunter et al., 1989). Lamellar bodies found in the cells of the *stratum granulosum* are organelles which are responsible for the release of the lipids which form the lipid layers of the overlying *stratum corneum* and play a role in the prevention of water loss (Proksch et al., 2008).

Superficial to the *stratum granulosum* may be a thin layer named the *stratum lucidum*, composed of clear cells containing a precursor to keratin, eleidin, which acts to prevent water movement. This layer is absent in thin skin, but clearly present in thick skin (Figure 1.2), such as on the soles of the feet (Thibodeau and Patton, 1999). Finally, the outermost layer, the *stratum corneum*, or "horny layer" is composed of a stratified coat of non-nucleated cells, of varying thickness, surrounded by cornified envelopes (CE) composed of both protein and lipid components (Figure 1.3) (Proksch et al., 2008, Thibodeau and Patton, 1999). Corneocytes of the *stratum corneum* are embedded in lipid layers giving the *stratum corneum* a brick and mortar appearance (Figure 1.3) (Elias, 2007). The predominant lipids found in the *stratum corneum* include ceramide, free fatty acids and cholesterol, and act to prevent water movement (Proksch et al., 2008) in addition to preventing bacterial translocation, in a manner similar to the effect of mucins in the intestinal epithelial barrier (Mack et al., 1999).

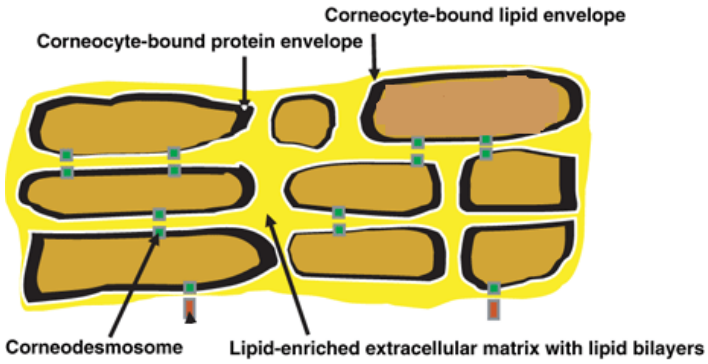


Figure 1.3. Structure of the *stratum corneum* (Proksch et al., 2008).
Diagram demonstrating the "brick and mortar" appearance of the *stratum corneum* and the protein and lipid envelopes of corneocytes.

The flattened shape and mechanical resistance of corneocytes is created by the formation of keratin filaments, through the aggregation of the proteins filaggrin and profilaggrin with keratin (Proksch et al., 2008). Filaggrin is instrumental in barrier function, as demonstrated in skin disorders such as ichthyosis vulgaris, where it is lost or expression is reduced (Smith et al., 2006). The protein envelope of the corneocyte is formed through the aggregation of proteins such as involucrin and loricrin through the action of transglutamases (Stephen and Steinert, 1994, Proksch et al., 2008, Nemes and Steinert, 1999). The barrier function of the skin is often attributed solely to the presence of the toughened cornified envelope of the corneocytes, though evidence suggests that epidermal cell junctions play an additional role (Section 1.1.2).

The epidermis also contains other cell types, including melanocytes (the pigment-producing cells), Merkel cells (cells which play a role in sensation), and Langerhans cells which are the antigen presenting cells of the epidermis (Kamel, 1994).

In total, the physical barrier of the epidermis can be described as having several different components, including the *stratum corneum* possessing its corneocytes with tough corneal envelopes, and keratinocytes of the *stratum granulosum* and spinosum connected by tight junctions, adherens junctions, desmosomes and gap junctions (Proksch et al., 2008).

1.1.2 Barrier function in skin

Tight junctions are cell-cell junctions that play a role in barrier function and control the paracellular movement of molecules through the skin (Brandner, 2009). The presence of tight junctions was not recognised in skin until relatively recently (Pummi et al., 2001, Furuse et al., 2002). In human skin, a variety of tight junction proteins have been identified including occludin, claudins, JAMs, Cingulin, ZO-1, and symplekin (Brandner, 2009). Tight junction proteins are expressed differentially throughout the epidermis. For example, occludin is found only at the level of the *stratum granulosum* while claudin-1 can be found in all living layers of the epidermis (Figure 1.4) (Brandner, 2009). However, the tight junction structures themselves are found in the *stratum granulosum* (Brandner, 2009). It has been hypothesised that tight junctions may act as an additional barrier in cases where the *stratum corneum* is compromised (Proksch et al., 2008). Tight junctions are also found in hair follicles (Brandner et al., 2003).

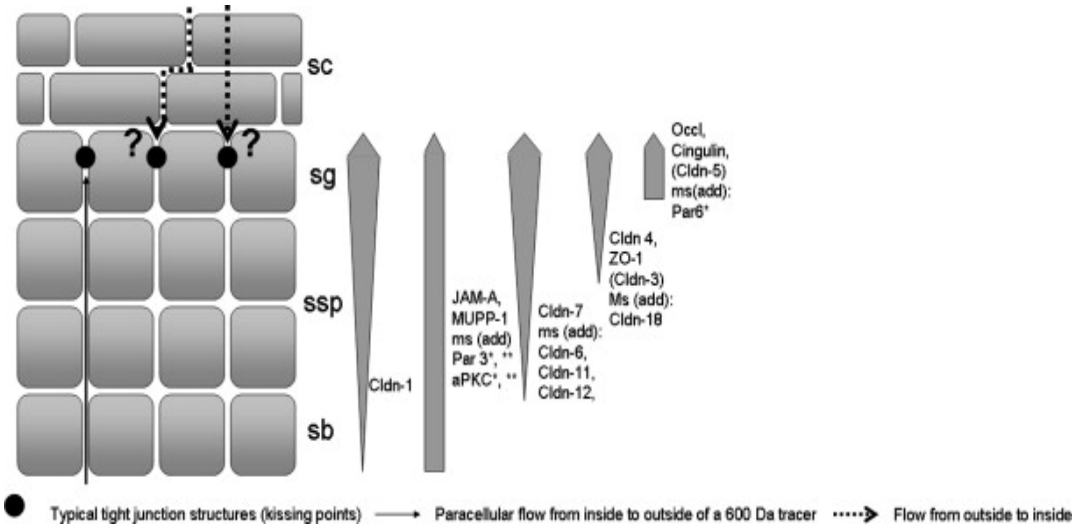


Figure 1.4. Tight junctions in the epidermis (Brandner, 2009).
Tight junction proteins present in the different layers of the epidermis, and the area where tight junction structures are commonly formed in the *stratum granulosum*. sc = *stratum corneum*, sg= *stratum granulosum*, ssp= *stratum spinosum*, sb = *stratum basale*.

Aside from the *stratum corneum* and cell-cell junctions that form the physical barrier of the skin, the skin also possesses a “chemical” barrier exemplified by the movement of ions through the skin. Efflux pumps on keratinocytes in the *stratum basale* prevent the build up of toxins from the blood into the skin. Expression of these multidrug resistant efflux pumps has been shown to be regulated by cytokines, and their expression may be modified in skin diseases such as psoriasis (Dreuw et al., 2004). Finally, the other main component of the skin barrier is the innate immune system of the skin, through the production of cytokines, chemokines and antimicrobial peptides. It is against each of these components which exogenous bacteria must compete to colonise the skin and/or cause infection.

1.1.3 Microenvironment of the skin

The microenvironment at the skin surface undoubtedly has an effect on the type and number of commensal and pathogenic organisms that may colonise it. Both skin temperature and surface humidity vary from site to site and in response to both environmental and internal stimuli, potentially affecting colonisation by bacteria (McEwan Jenkinson, 1993). The pH of the skin may vary but in general tends to be acidic in adults (Aly and Bibel, 1993). The importance of the acid mantle is not only important in limiting the growth of certain bacteria on the skin such as *S. aureus* (Noble, 1993, Lambers et al., 2006), but also relevant to the production of ceramides, because many of the enzymes required to produce ceramides may work only at an acidic pH. Pockets of carbon dioxide and oxygen trapped in the outer layer of the epidermis provide a diverse environment for exploitation by microbes (McEwan Jenkinson, 1993). The “emulsion” of both sweat and sebum in the *stratum corneum*, in addition to the lipid-composed cement between corneocytes may act as a medium on which bacterial microflora may survive because keratin is not suitable for bacterial growth. This may explain why bacteria are more commonly found in the upper layers of the *stratum corneum* and within hair follicles (McEwan Jenkinson, 1993). Skin biopsies have demonstrated staphylococcal passage through the *stratum corneum* lipid layers (Miller et al., 1988). Furthermore, the *stratum corneum* lipids may possess antimicrobial activity against some skin bacteria, e.g. staphylococci. This suggests that the lamellar layers of the *stratum corneum* play a particularly important role in antimicrobial defence (Miller et al., 1988).

1.1.4 Commensal microbiota of the skin

The skin provides a diverse ecological niche for the growth of both commensal and pathogenic bacteria. Traditional culturing techniques have revealed that the commensal bacteria residing on the skin are most often Gram positive species though some Gram negative bacteria are known to colonise the skin of individuals. Table 1.1 lists the commonly accepted dominant species found on the skin using culture techniques.

Table 1.1 Common microorganisms on the skin.

Gram positive	Coagulase-negative staphylococci (e.g. *S. epidermidis and S. capitis*)
	Staphylococcus aureus
	Corynebacterium jeikeium
	Micrococcus spp.
	Propionibacteria acnes
Gram negative	*Acinetobacter* spp.
	Pseudomonas spp.

Molecular studies of the skin microbiota have revealed that this view of the skin microflora is grossly oversimplified (Grice et al., 2008, Grice et al., 2009, Grice et al., 2010, Gao et al., 2007). Grice *et al* (2008) performed a 16S ribosomal subunit gene sequencing analysis of the skin microbiota of the inner elbow, and found that microbial diversity on the epidermis was much larger and more varied than previously thought. Approximately 113 different phylotypes were found, of which the proteobacteria appeared the most prevalent. Interestingly, the commonly held belief that the predominant organisms found on the skin are *Staphylococcus epidermidis* and *Propionibacterium acnes* proved false, with these two organisms representing fewer than 5% of the entire bacterial community. Three different methods were utilised for sampling the microbiota; (i) using a swabbing technique, (ii) skin scrapings, and (iii) a punch biopsy technique, to include the different layers of the skin in the analysis. All three methods were capable of capturing the most dominant phylotypes of bacteria present and organisms were found in several layers of the epidermis, indicating bacterial penetration of the *stratum corneum*. However, a drawback of the use of molecular techniques is that 16S sequencing does not differentiate between viable and dead organisms.

Bacteria on the skin may also work to modify their own environment and influence the microbiome. For example, *S. pyogenes* growth is inhibited by free fatty acids liberated from sebaceous triglycerides by commensal bacteria such as propionibacteria (Hentges, 1993). Many bacteria, such as *S. epidermidis,* are also known to produce antimicrobial factors (e.g. phenol soluble modulins) which undoubtedly affect the composition of the microflora (Cogen et al., 2009, Cogen et al., 2010).

1.1.4.1 Interpersonal variation

Whether the skin microbiota changes significantly from person to person is unclear. Grice *et al* (2008) concluded that the microbiome of the inner elbow did not appear to change significantly from person to person. However, Gao *et al* (2010) demonstrated that there were significant differences in the microbiota of forearm skin between individuals sampled. However, these two studies analysed the microbiota of different skin sites and used different methods, suggesting that interpersonal variation differs depending upon the site sampled.

1.1.4.2 Site-specific variation

In 2009, Grice *et al* performed another study incorporating skin from different sites and found that the dominant microbiota was influenced by location. For example, staphylococci and propionibacteria dominated in sebaceous sites while corynebacteria dominated in moist sites (Grice et al., 2009). Gao *et al* (2010) demonstrated that the primary phyla found on the skin of the forearm were *Actinobacteria, Firmicutes* and *Proteobacteria*. Other studies have confirmed that anatomically distinct skin sites such as the ear (Frank et al., 2003), hands (Fierer et al., 2008) and forehead (Dekio et al., 2005) possess different bacterial microbiota.

1.1.4.3 Temporal variation

Studies suggest that the host microbiota appears to be dynamic, changing over time, though this appears to be dependent on the site sampled. Gao *et al* (2010) found that proportions of streptococci and staphylococci on the skin varied over 1 month, while the number of total bacteria did not (Gao et al., 2010). A study of the microbiota of the external ear canal contrasted with this finding suggesting the bacterial microbiota did not change significantly over 14 months (Frank et al., 2003). Finally, Grice *et al* (2009) found that stability of the microbiota over 4-6 months varied according to the site analysed. However, further work must be performed to confirm this (Rosenthal et al., 2011, Costello et al., 2009).

1.1.4.4 Ageing and the bacterial microflora

The skin microflora is known to change with age. At birth, infants are essentially sterile, and colonisation of the skin is dependent on the method of delivery of the neonate. Vaginally born infants possess microflora synonymous with the mothers vaginal microflora (e.g. lactobacilli and prevotella spp.) while infants born by caesarean section possess microflora found commonly on adult skin (e.g. staphylococci, corynebacteria). It has been hypothesised that initial colonisation with the vaginal microflora protects against pathogenic infection because vaginally delivered infants are known to develop less methicillin resistant *S. aureus* (MRSA) infections (Dominguez-Bello et al., 2010). Additionally, unlike adult skin, the skin microflora of infants does not vary with site regardless of the method of delivery (Dominguez-Bello et al., 2010). Culture based studies have shown that the quantity of bacteria on the skin of the face is at its maximum during adulthood and then begins to decline with increasing age (Leyden et al., 1975). Aged skin appears to possess more diptheroids and streptococci compared to normal adult skin (Somerville, 1969).

1.1.4.5 Quantities of bacteria on the skin

Culture-dependent methods utilising colony counts have revealed that aerobic bacterial numbers on the skin may range from 10^2 bacteria/cm^2 on dry forearm skin to 10^7 bacteria/cm^2 on skin that is moist (such as the axilla) while anaerobic numbers may be up to 10^6 bacteria/cm^2 (Leyden et al., 1987). Grice *et al* (2008) utilised a quantitative PCR strategy to estimate the number of bacteria found in different skin samples. Swabs found approximately 1 x 10^4 bacteria/cm^2, while scrapings found 5 x 10^4 bacteria/cm^2 and punch biopsies collected 1 x 10^6 bacteria/cm^2 (Grice et al., 2008). A study of the microbial flora on the hands of homemakers revealed that the average number of bacteria found on the hands of individuals was approximately 5 x 10^5 CFU (5.72 $\log_{10}$ CFU) (Larson et al., 2003). Colony counts of less than 10^6 *S. aureus* per cm^2 of skin are thought to represent colonisation while an amount equalling or more than 10^6 suggests an infection (Leyden et al., 1987).

1.1.4.6 The skin microbiota in disease

The notion that the skin microbiota may change with disease is not a new one. Gao *et al* (2008) compared the microbiota of psoriatic skin to that of uninvolved skin and normal healthy skin. The phylum Firmicutes dominated in psoriatic skin compared to uninvolved skin and skin from healthy controls.

Additionally, the genus streptococcus was found more frequently in psoriatic lesions compared to psoriatic uninvolved skin (Gao et al., 2008). In contrast, significantly more Propionibacteria were found in normal skin and uninvolved skin compared to psoriatic lesions. Interestingly, while staphylococcal and streptococcal species were favoured in psoriatic lesions, *S. aureus* and *S. pyogenes* were present only in small numbers (Gao et al., 2008). Another study in 2009 utilising a mouse model of icthyotic skin disorders found a shift in the microbiome of the skin, where higher levels of corynebacteria and streptococci were found in diseased skin (Scharschmidt et al., 2009).

Acne vulgaris has long been associated with *Propionibacterium acnes.* However, a recent study revealed that healthy sebaceous follicles were colonised by *P. acnes* alone, while acne lesions were found to have multiple organisms including *P. acnes, S. epidermidis* and *Corynebacterium* spp, suggesting that a re-evaluation of the role of *S. epidermidis* may be necessary (Bek-Thomsen et al., 2008).

Atopic dermatitis has long been associated with *S. aureus* skin colonisation*,* using culture based techniques (Williams et al., 1990). A recent molecular analysis of the skin microbiota of atopic individuals during flares with and without treatment, compared to normal skin, revealed that atopic skin during flares had a greater proportion of staphylococci, particularly *S. aureus,* compared to treated and normal skin. In addition, atopic flares were also associated with increased *S. epidermidis* colonisation of atopic skin. In contrast, normal skin and treated atopic skin had increased bacterial diversity in the colonising microbiota (Kong et al., 2012).

If skin disease is associated with disruptions to the normal microbiota of the skin, then it follows that it could be possible to enable a return to health by restoring the natural balance of bacteria upon the skin. Additionally, if skin disease due to disruptions in the chemical barrier could be restored by the commensal microflora, it stands to reason that a bacteriotherapy approach may be of use.

1.1.5 Innate immune system of the skin

Although the epidermis possesses a tough physical barrier (the *stratum corneum*) that helps to combat infection, this barrier can occasionally be breached e.g. due to wounds. The keratinocytes of the epidermis also act as a barrier against invasion through innate immune responses. This is through interaction of bacteria with Toll-like receptors (TLRs) and subsequent cytokine and antimicrobial peptide (AMP) production.

1.1.5.1 Toll-like receptors in the skin and keratinocytes

Toll-like receptors are a class of pattern recognition receptor found on many types of cells throughout the body which are known to recognise microbial associated molecular patterns (MAMPs) or specific ligands associated with bacteria, viruses and fungi (Miller and Modlin, 2007). The TLRs present on keratinocytes provide a first response to microbial assaults. Low molecular weight hyaluronic acid and RNA released from damaged cells during injury or wounding can activate TLRs on epidermal keratinocytes triggering an immune defence response against potential invaders (Gariboldi et al., 2008, Lai et al., 2009). Many studies have related the role of TLR activation in skin diseases such as atopic dermatitis, *acne vulgaris*, *Staphylococcus aureus* infections, leprosy, candiasis and psoriasis (Miller and Modlin, 2007). Because many of these involve bacterial infection, TLR agonists to activate the innate immune response may be of use in the treatment of skin disease (Miller and Modlin, 2007, Baker et al., 2003).

In humans, there are 10 TLRs, each recognising a different ligand (Table 1.2). Keratinocytes of the epidermis express TLR-1, -2, -3, -4, -5, -6, -9, and -10 mRNA (Lebre et al., 2006). Other cells of the skin also possess TLRs, notably the Langerhans cells. In the dermis and hypodermis, dendritic cells, macrophages, T and B cells, fibroblasts and adipocytes along with vascular endothelial cells may express TLRs (Miller and Modlin, 2007). Triggering of each TLR leads to a signalling cascade culminating in the production of a variety of cytokines and chemokines, and the production of antimicrobial peptides by keratinocytes (Sumikawa et al., 2006).

Table 1.2. Toll-like receptors and their ligands (Adapted from Takeda *et al*., 2003).

TLR-1	Tri-acyl lipopeptides (bacteria, mycobacteria) Soluble factors (*Neisseria meningitides*)
TLR-2	Lipoprotein/lipopeptides (a variety of pathogens) Peptidoglycan (Gram positive bacteria) Lipoteichoic acid (Gram positive bacteria) Lipoarabinomannan (mycobacteria) A phenol-soluble modulin (*Staphylococcus epidermidis*) Glycoinositolphospholipids (*Trypanosoma Cruzi*) Glycolipids (*Treponema maltophilum*) Porins (*Neisseria*) Zymosan (fungi) Atypical LPS (*Leptospira interrogans*) Atypical LPS (*Porphyromonas gingivalis*) HSP70 (host)
TLR-3	Double-stranded RNA
TLR-4	LPS (Gram negative bacteria) Taxol (plant) Fusion protein (viral) Envelope proteins (viral) HSP60 (*Chlamydia pneumoniae*) HSP60 (host) HSP70 (host) Type III repeat extra domain A of fibronectin (host) Oligosaccharides of hyaluronic acid (host) Polysaccharide fragments of heparan sulfate (host) Fibrinogen (host)
TLR-5	Flagellin (bacteria)
TLR-6	Di-acyl lipopeptides (mycoplasma)
TLR-7	Imidazoquinoline (synthetic compounds) Loxoribine (synthetic compounds) Bropirimine (synthetic compounds)
TLR-8	Single stranded RNA (viral)
TLR-9	CpG DNA (bacteria)
TLR-10	Unknown

The presence of TLRs on keratinocytes raises the question of why the commensal skin microflora does not cause inflammation on a constant basis. It may be that TLR stimulation is negatively regulated by the presence of inhibitory TLR-like molecules (Hornef and Bogdan, 2005). Another theory is that certain cells are less responsive than others. For example, Abreu *et al* (2001) found that intestinal epithelial cells (IECs) express little TLR-4 and its co-receptor MD-2, suggesting this is a mechanism by which IECs prevent dysregulation of the inflammatory response in the presence of commensal bacteria (Abreu et al., 2001). Epidermal keratinocytes express TLR-2 predominantly cytoplasmically rather than expressed on the cell surface ready to interact with its associated MAMP. This may be a mechanism by which cells avoid over-stimulation by the commensal bacteria residing on the skin, while retaining the ability to respond quickly if needed (Begon et al., 2007). Furthermore, TLR-2 expression in the epidermis is concentrated predominantly in the basal layers, suggesting that keratinocytes closer to the surface avoid over-stimulation by the commensal microbiota (Begon et al., 2007).

TLRs also act as a link between the adaptive and innate immune systems. For example, TLRs on cells activated by their respective MAMPs not only alert innate immune cells but also induce the production of IL-12, which instigates the T_H-1 adaptive immune response (Miller and Modlin, 2007, Hsieh et al., 1993).

1.1.5.2 Toll-like receptor signalling

Toll-like receptor signalling can be broadly split into two categories: 1) Myd88-dependent signalling and 2) Myd88-independent signalling (Figure 1.5). They can be summarised as follows:

1) TLRs, once activated by their corresponding MAMPs interact with an adapter molecule called Myd88. Activation results in a signalling cascade leading to activation of nuclear factor-κB (NF-κB) and other kinases (e.g. c-JUN-terminal kinase, ERK, p38, and mitogen activated protein kinases (MAPKs)). The activated NF-κB translocates to the nucleus and acts as a transcription factor to induce activation of proinflammatory genes involved in the innate immune response. MAPKs can lead to the activation of alternate transcription factors that can also induce expression of proinflammatory genes in the nucleus (Valins et al., 2010) (Miller and Modlin, 2007).

2) Myd88-independent signalling also leads to activation of either NF-κB or MAPKs but without the use of the Myd88 complex. Instead, Toll–IL-1 receptor containing adaptor inducing IFN-β (TRIF) activates TNF receptor associated factor (TRAF-6). Alternatively, Type-I interferon's (IFN-α and IFN-β) may activate IFN regulatory factors (IRF-3 and IRF-7) through TRIF (Valins et al., 2010) (Miller and Modlin, 2007).

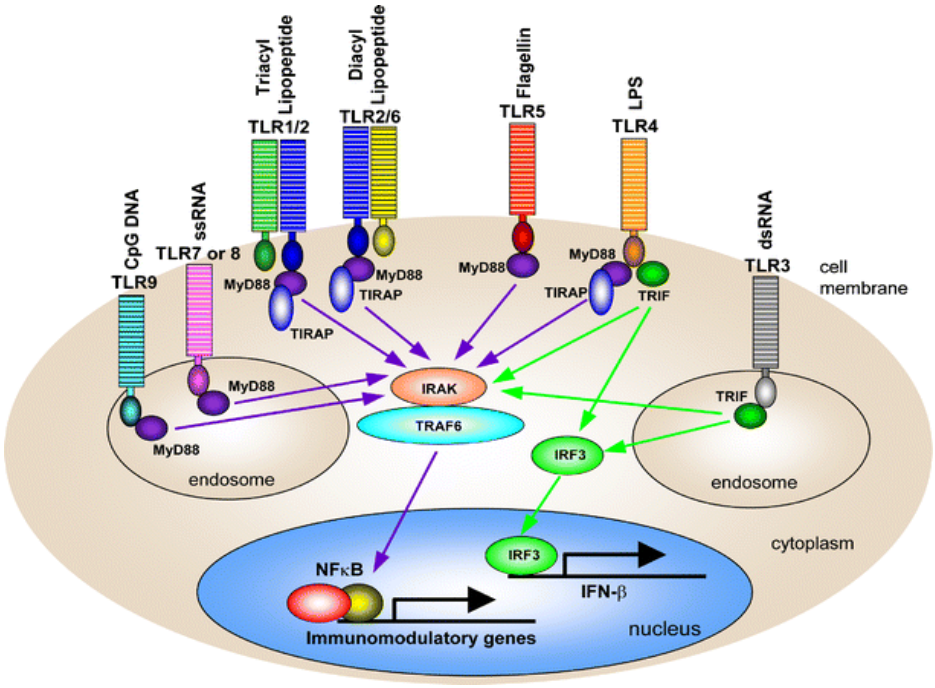


Figure 1.5. Toll-like Receptors (TLRs) (Miller and Modlin, 2007).
TLRs 1,2,4,5,& 6 are expressed on the cell membrane while TLRs 3, 7 and 9 are found on the membranes of intracellular endosomes. TLRs interact with their adapter molecule (MyD88/TRIF) which recruit signalling molecules IRAK and TRAF6. These may then act on transcription factor NF-κB which in turn causes the transcription of proinflammatory genes and the release of cytokines.

1.1.5.3 Cytokine signalling in skin/keratinocytes

The keratinocytes of the epidermis are the first living cells to come into contact with external insults such as bacteria and viruses. As such, they must be able to respond to attack promptly. Keratinocytes regulate inflammation in the skin through the production and response to cytokines and chemokines (Gröne, 2002) and antimicrobial peptides (AMPs) (Niyonsaba et al., 2006). Without wishing to downplay the importance of the other immune cells of the skin (e.g. Langerhans cells and dermal dendritic cells), this review will concentrate on the role of keratinocytes in protection of the skin.

Keratinocytes are able to sense the presence of pathogens through interaction of their pattern recognition receptors (PRRs) (e.g. TLRs) with microbial associated molecular patterns (MAMPs) (Section 1.1.5.1). Activation of these PRRs triggers signalling pathways that culminate in the release of cytokines from keratinocytes. These may include IL-1, IL-6, IL-8, IL-10, IL-12, IL-15, IL-18, TNF-α, and IFN-α, -ß and -γ to name a few (Nestle et al., 2009, Gröne, 2002). These may be produced either constitutively or induced (Gröne, 2002). Importantly, these cytokines and chemokines provide a link between the innate immune response and the adaptive immune response (Miller and Modlin, 2007).

The proinflammatory cytokines IL-8 and TNF-α are probably the most studied cytokines/chemokines produced by keratinocytes. IL-8, or CXCL8, is known to be a chemoattractant for neutrophils and T-cells (Larsen et al., 1989). TNF-α, meanwhile, has been shown to regulate IL-8 production, adhesion molecule (e.g. ICAM) expression and acts to regulate the movement and activation of dermal cells (Griffiths et al., 1991). In a macroarray study in 2010, it was found that TNF-α application to keratinocytes *in vitro* resulted in modification of the expression of nine genes of interest, including IL-8 (20-fold increase) and human beta defensin-2 (Hbd-2) (125-fold increase) mRNA expression. Protein expression of IL-8 and Hbd-2 was also found to be significantly increased by the application of TNF-α (Guilloteau et al., 2010).

While inflammation is a critical process required for clearance of infections, a reduction in TNF-α and IL-8 levels produced by keratinocytes might correlate with a reduction in inflammation in the skin and so be of use in certain chronic inflammatory diseases of the skin. For example, the use of TNF-α inhibitors (e.g. Infliximab) for the treatment of chronic plaque psoriasis has been evaluated and results are promising though further evidence is needed (Chaudhari et al., 2001). However, anti-TNF-α therapy poses a risk of opportunistic infection, suggesting that a therapy that simultaneously fights infection and reduces excessive inflammation would be of use in treating inflammatory skin disease (Krueger and Callis, 2004).

1.1.5.4 Antimicrobial peptide production (AMP) in the epidermis

Antimicrobial peptides (AMPs) have been isolated in both plants and animals, so it is not surprising that humans also possess these peptides (Schröder and Harder, 2006). Table 1.3 illustrates some of the peptides and other antimicrobial substances produced by cells in the skin. The action of AMPs is thought to be specific for the organism which it is produced in response to.

For example, a study in 2004 showed that psoriasin produced by keratinocytes in response to *E. coli* was ineffective against *S. epidermidis* (Gläser et al., 2005). One mechanism of action of these often cationic or positively charged AMPs is though attraction to the negatively charged groups on bacteria, such as phospholipids (e.g. in lipopolysaccharides or lipoteichoic acid). This results in the formation of a pore, where the membrane integrity is compromised and the cell dies (Lai and Gallo, 2009). It has been suggested that one of the reasons AMPs do not act on mammalian cells is because of differences in the cell membrane structure. For example, bacterial membranes do not possess cholesterol while mammalian cells do. In addition, the composition of membrane phospholipids also differs (Lai and Gallo, 2009).

Table 1.3. Antimicrobial products found in the epidermis.

Source	AMP	Reference
Keratinocytes	Cathelicidin (LL-37)	(Braff et al., 2005a)
Keratinocytes/sweat ducts	Human beta defensin-1 (Hbd-1)	(Fulton et al., 1997)
Keratinocytes	Human beta defensin-2 (Hbd-2)	(Oren et al., 2003)
Keratinocytes	Human beta defensin-3 (Hbd-3)	(Harder et al., 2001)
Keratinocytes	Human beta defensin-4 (Hbd-4)	(Harder et al., 2004)
Stratum corneum	Free fatty acids	(Miller et al., 1988)
Stratum corneum	Sphingolipids	(Bibel et al., 1992)
Keratinocytes	Psoriasin	(Abtin et al., 2008)
Keratinocytes	RNase-7	(Harder and Schröder, 2002)
Sweat glands	Dermicidin	(Schittek et al., 2001)

The intracellular concentration of AMPs often does not match the *in vitro* Minimum Inhibitory Concentrations (MICs) scored for them, and so the question arises how AMPs do in fact kill bacteria *in vivo*. One answer is through synergistic activity with other AMPs (Lai and Gallo, 2009). For example, LL-37 acts synergistically with Hbds-1, 2 and 3 against MRSA (Midorikawa et al., 2003).

It has also been suggested that AMPs play an additional role by mediating the inflammatory response and influencing processes such as cytokine release, wound healing, and cell proliferation (Brown and Hancock, 2006) (Yang et al., 2004, Lai and Gallo, 2009). For example, Hbd-2, LL-37 and psoriasin are neutrophil chemotaxins (Niyonsaba et al., 2004, Tjabringa et al., 2006, Eckert et al., 2003).

1.2 Probiotics and their uses

The discovery of probiotics or "friendly bacteria" arose from studies of the gut microflora, where it emerged that certain bacteria appeared to be beneficial to health, and that microbial imbalance could be related to disease. Probiotics have been defined by the World Health Organisation as "Microorganisms which when administered in adequate amounts confer a health benefit upon the host" (FAO/WHO, 2002). Probiotics are most often lactic acid bacteria such as lactobacilli and bifidobacteria, but can include other bacteria such as *Streptococcus thermophilus* or yeasts such as *Saccharomyces boulardii* (van der Aa Kühle et al., 2005).

To date, most work on probiotics has focussed on the effect of ingestion of probiotics on intestinal health, though recently more studies have emerged exploring the role of probiotics in the mouth, genitourinary tract, throat, and of importance to this study, the skin. What follows is a description of some of the known uses of probiotics to date.

1.2.1 Probiotics for the treatment of gut-associated disorders

Probiotics have been evaluated for the treatment of a range of gastrointestinal diseases in children (Szajewska and Mrukowicz, 2001), adults (Rembacken et al., 1999), and athletes (Cox et al., 2010). Probiotics including *S. boulardii* and lactobacilli have been assessed for use against antibiotic-associated diarrhoea and found to prevent the disease in meta-analyses of published trials on adults and children (D'Souza et al., 2002, Johnston et al., 2011). *Clostridium difficile* associated diarrhoea recurrence may be prevented by *S. boulardii* ingestion, though the evidence to date is not satisfactory to make a definitive conclusion (Parkes et al., 2009). Inflammatory bowel disease (IBD) is a collective term for conditions such as ulcerative colitis (UC) and Crohn's disease (CD) resulting in inflammation of gut mucosa. As the commensal microbiota is thought to be disrupted in IBD (Favier et al., 1997), the use of probiotics for treatment or prevention of IBD has also been assessed.

The probiotic *E. coli* Nissle 1917 has been shown in clinical trials to reduce the risk of remission of UC as effectively as traditional treatments (Rembacken et al., 1999). It is thought that probiotics reduce gut inflammation in IBD through regulation of immune response and/or modification of the gut microbiota (Shanahan, 2004).

There may be a role for probiotics in the elderly. Although there is disparity between studies, the intestinal microflora of the elderly appears to be altered compared to that of the young (Maukonen et al., 2008). Probiotic supplementation may therefore be of benefit for supporting growth of a healthy microflora and associated immune benefits. For example, consumption of a *B. longum* fermented milk was shown to shorten gut transit time in elderly patients (Meance et al., 2001). Meanwhile, probiotics have also been shown to modulate the immune response of elderly people through increased natural killer cell and phagocytic activity after oral ingestion of *L. rhamnosus* or *B. lactis* by patients (Gill et al., 2001).

All of these studies suffer from the fact that different experimental designs, small treatment group numbers and different trial designs mean that a full analysis of the utility of probiotics for gastrointestinal disease is difficult. The use of larger coordinated trials would go some way to rectifying this problem (Parkes et al., 2009, Szajewska and Mrukowicz, 2001, Tuohy et al., 2003).

1.2.2 Alternative uses for probiotics

With current knowledge of the use of probiotics for intestinal health, interest has increased in the possibilities of using non-pathogenic enteric bacteria as a therapy for the prevention of disease elsewhere. Topical application or ingestion of probiotics has long been suggested as a treatment for vaginal candiasis, though clinical trials vary and results conflict (Falagas et al., 2006). However, the evidence is more convincing that probiotics could have a significant effect on the outcome of bacterial vaginosis (BV), a condition whereby the normal lactobacilli population of the vagina is depleted. For example, a clinical trial in 2001 found that 7/11 women with BV ingesting *L. rhamnosus* GR-1 and *L. fermentum* RC-14 reverted to normal vaginal flora within one month. However, whether the vaginal tract was colonised by probiotic strains, or treatment encouraged colonisation by normal commensal lactobacilli was not determined (Reid et al., 2001). A more recent review of the evidence available for the use of probiotics in genitourinary infections however concluded that there were conflicting results for treatment of BV, while the evidence for prevention of urinary tract infections was as yet too little to make conclusions about the efficacy of probiotic supplementation (Barrons and Tassone, 2008).

Several studies have evaluated the role of probiotic lactobacilli in the prevention of dental caries and reduction of *S. mutans* levels in the oral cavity (Çaglar et al., 2006, Nase et al., 2001). *Streptococcus salivarius* K12 is thought to produce bacteriocin like inhibitory substances (BLIS) able to inhibit the pathogenic species that reside in the oral cavity that cause pharyngeal infections and dental caries (Burton et al., 2011, Tagg et al., 2006). However, further work is required to confirm this *in vivo*. Nase *et al* (2001) noted that ingestion of milk containing *Lactobacillus rhamnosus* GG (LGG), a bacterium known to promote gut health and inhibit *Streptococcus* species and acidogenic lactobacilli in the mouth, resulted in a significant reduction of the incidence of and risk of dental caries over a period of seven months. This was measured by lowered numbers of *S. mutans* in the oral cavity. However, the risk of dental caries in this study was complicated by other factors such as dental hygiene and diet, and the period of time was relatively short to observe the formation of dental caries in children (Nase et al., 2001). Çaglar *et al* (2006) demonstrated that *L. reuteri* ATCC 55730 administration by straw or tablet resulted in significant reduction in *S. mutans* levels in the oral cavity of healthy adults over 3 weeks. The dental hygiene company, Sunstar, has recently launched a probiotic lozenge, GUM® periobalance®, which claims to restore the natural dental flora and reduce gum inflammation (Sunstar, 2009). The lozenge contains *L. reuteri* Prodentis™, a combination of *L. reuteri* ATCC PTA 5289 and *L. reuteri* ATCC 55730 (McBain et al., 2009). It is hypothesised that *L. reuteri* ATCC 55730 can adhere to the same agglutinin as *S. mutans*, thereby inhibiting the latter's ability to colonise the mouth (Haukioja et al., 2008). Both strains form biofilms *in vitro* (Jones and Versalovic, 2009). Other hypothesised mechanisms for the action of *L. reuteri* include immunomodulation; for example, *L. reuteri* PTA 5289 was shown to inhibit TNF-α production, while *L. reuteri* ATCC 55730 appeared to be immunostimulatory (Jones and Versalovic, 2009). Another mechanism of action is through the generation of bacteriocins such as Reuterin, or adhesion inhibitors (McBain et al., 2009, Jones and Versalovic, 2009).

Probiotic bacteria have also been applied to the treatment of otitis media, a middle ear infection commonly affecting children. Otitis media is commonly caused by Group A Streptococci, *Haemophilus influenzae, Moraxella catarrhalis and Streptococcus pneumoniae* (Roos et al., 2001). Roos and colleagues performed a randomised double-blind clinical trial in 2001 to assess the impact of an α-streptococcal (non-pathogenic) throat spray on children prone to otitis media infections. The authors found that recolonisation of the eustachian tube with α-streptococci significantly reduced the rate of recurrence of otitis media in children.

However, children were treated with antibiotics for ten days prior to commencement of therapy which may have affected the results of the trial (Roos et al., 2001). The following year another similar study which omitted the use of antibiotics found no significant reduction in the rate of recurrence indicating perhaps that the use of replacement therapy is not straightforward, and the need for more highly adherent strains required (Tano et al., 2002). To date, there are still too few studies to conclude whether probiotics have a significant effect upon incidence and outcome of otitis media (Niittynen et al., 2012).

Probiotics have also been evaluated for the treatment of vernal keratoconjunctivitis, a chronic allergic eye disease. A pilot study in 2008 analysed the effects of treating seven patients with the disease with eye drops containing inactivated freeze-dried probiotic *L. acidophilus* in an attempt to mediate the allergic response. The authors found that treatment resulted in a significant improvement in the symptoms associated with the disease compared to normal values. The treatment appeared to be tolerated well and no side effects were observed. However, further double-blind placebo controlled trials would have to be performed to confirm this result (Iovieno et al., 2008).

1.2.3 Criteria for selection of probiotics

Although many probiotics have been described as generally recognised as safe (GRAS) by the American Food and Drug Administration (FDA), some precautions must be taken with their use. While use of probiotics in immunodeficient patients would be a beneficial replacement for prescribed antibiotics, certain dangers exist in such individuals. Enterococci have been utilised as probiotics, but there is evidence to suggest that some strains can transfer antibiotic resistance to other pathogenic organisms, demonstrating the need for careful assessment of the strains used as probiotics (Noble et al., 1996). There may be an increased potential for probiotic bacteria to cause opportunistic infections in immunodeficient hosts. However, in a study performed by Wolf *et al* (1998), it was found that no significant negative effects of ingestion of *L. reuteri* occurred in HIV patients over the course of 21 days (Wolf et al., 1998). Conversely, other data imply that lactic acid-producing bacteria may pose a risk in the development of endocarditis in immunocompromised persons, though the risk is thought to be low. In addition, in those cases where infection has occurred, the organisms responsible have arisen from the individuals' own flora, and probiotics are thought unlikely to be the culprit (Adams and Marteau, 1995).

Selection of strain-specific probiotics with appropriate properties that are safe for consumption/use is a necessary requirement. Its relative adherence to the target site, acid stability, viability of the final product, absence of transferable resistance genes, and ease of production are all considerations in the making of probiotics (Ouwehand et al., 2002) (Dunne et al., 2001).

1.3 Mechanisms of probiotics

Probiotics may be beneficial through a range of mechanisms. These include 1) inhibition of bacterial growth through the production of inhibitory substances such as acid or bacteriocins, 2) inhibition of bacterial colonisation through competitive exclusion of binding sites, 3) inhibition of pathogenic virulence factors, 4) modulation of host innate immune responses, and 5) antioxidant activity.

Many probiotics are known to produce inhibitory peptides known as bacteriocins. For example, *Lactococcus* sp. HY449 has been shown to produce a bacteriocin (Nisin) capable of inhibiting the growth of several skin bacteria with no apparent cytotoxicity to human cells or irritant side effects (Oh et al., 2006). Nisin is now a common ingredient in cheeses, preventing food spoilage by other bacteria (Hirsch et al., 1951). In addition to the production of specific inhibitory peptides, lactobacilli are also capable of producing acetic and lactic acids, which are inhibitory to the growth of some bacteria. This is a common method of protection against pathogens in the gut. For example, in 2004, *B. breve* strain yakult was shown to protect mice against infection by shiga-toxin producing *E. coli* through a decrease in intestinal pH, inhibiting the toxin (Asahara et al., 2004).

Work *in vitro* has suggested that probiotics use various mechanisms to inhibit pathogens including direct competition for binding sites on epithelial cells (Coconnier et al., 1993). Probiotics may compete for adhesion to the cell through the use of adhesins which may be protein (Heinemann et al., 2000, Miyoshi et al., 2006), carbohydrate (Ruas-Madiedo et al., 2006), lipoteichoic acid (Granato et al., 1999), or S-layer proteins (Chen et al., 2007).

Alternatively, probiotics have been known to alter the adhesive ability of other bacteria through the production of biosurfactants able to modify hydrophobic interactions (Ron and Rosenberg, 2001). For example, in 2002, it was found that a biosurfactant containing collagen binding proteins produced by *L. fermentum* RC-14 was able to inhibit staphylococcal binding to surgical implants (Gan et al., 2002).

Biosurfactants from probiotics have also been assessed for their ability to inhibit adhesion of pathogenic organisms to inert surfaces such as medical devices. For example, in 2006, it was found that a biosurfactant produced by *S. thermophilus* was able to inhibit the adhesion of both *S. aureus* and *S. epidermidis* to silicone rubber used in voice prostheses (Rodrigues et al., 2006).

Some probiotics can affect the gene expression of the bacteria around them. Quorum sensing (a form of bacterial communication) between bacteria is a well-known phenomenon by which bacteria can respond to changes in their environment and changes in population densities through the production of quorum signals. However, the role that probiotics may play in regulating the behaviour of distantly related resident bacteria within the same ecological niche as their own has not been fully elucidated. For example, a recent study in 2011 found that the human vaginal isolate, *L. reuteri* RC-14, could produce small cyclic dipeptides which appeared to have the ability to suppress the expression of virulence associated genes in a TSST (toxic shock syndrome toxin) producing *S. aureus* strain. In particular, these molecules interfered with quorum sensing regulator *agr*, a global regulator of virulence gene expression, resulting in reduction of TSST expression. An investigation into whether the quorum sensing molecule AI-2 or alternative cyclic dipeptides interfered with *S. aureus* quorum sensing concluded that AI-2 did not affect *S. aureus* production of TSST (Li et al., 2011).

Another recent study found that *L. rhamnosus* could inhibit cell death induced in pharyngeal epithelial cells by *Streptococcus pyogenes*. The authors claimed that it could protect epithelial cells using two methods; 1) through lactic acid killing of *S. pyogenes*, and 2) through the action of lactic acid disabling the streptococcal cytotoxin LTA, which is otherwise heat-stable. The probiotic inhibited *S. pyogenes* attachment to epithelial cells, suggesting that degradation of LTA is important in preventing adherence of *S. pyogenes* to cells (Maudsdotter et al., 2011).

Probiotics can also influence the expression of host genes and the innate immune response. For example, *L. rhamnosus* GG and *L. plantarum* 299v can induce up-regulation of genes involved in mucin expression in intestinal epithelia *in vitro*, which in turn inhibits adhesion of enteropathogenic *E. coli* (Mack et al., 1999). In a study in 1999, application of the lactic acid bacterium *S. thermophilus* to keratinocytes *in vitro* and skin *in vivo* resulted in increased ceramide production, lipids important in maintaining the skin barrier (Di Marzio et al., 1999, Di Marzio et al., 2003, Di Marzio et al., 2008).

While not discussed in detail here, probiotic bacteria can influence the expression of tight junction proteins in gut epithelia, and so have a direct effect on barrier function (Liu et al., 2010b). A study in 2010 found that exposure of Caco-2 cells to *L. plantarum* MB452 resulted in an increase in trans-epithelial electrical resistance (TEER -a measure of barrier function) in a dose dependent manner. Gene expression analysis revealed that 19 tight junction related genes were found to change expression levels in response to probiotic exposure. Higher expression of occludin, ZO-1, ZO-2 and cingulin was observed compared to untreated controls (Anderson et al., 2010). However, most studies on probiotic effects on tight junctions have looked at prevention of pathogenic effects on tight junction integrity in disease models, but not in healthy models.

Numerous studies have claimed a relationship between the use of probiotics and the development of improved innate immunity in the gut. Probiotics have a species-specific effect on immune responses by cells of the body, as shown in a recent study comparing the responses of peripheral blood mononuclear cells (PBMC) to three different probiotics, *B. breve, L. rhamnosus* and *L. casei* (Plantinga et al., 2011). The *B. breve* strain induced lower amounts of IFN-γ (a proinflammatory cytokine) than the other two probiotics (Plantinga et al., 2011).

In addition to cytokine stimulation, macrophage activation has been noted in response to intraperitoneal administration of both *Lactobacillus casei* and *Lactobacillus bulgaricus*. However, macrophage activation has been observed in response to oral administration of *L. casei* only. This indicates that these organisms could induce an increased immune response within the gut (Perdigon et al., 1986). Interestingly, *L. rhamnosus* GG has been noted to have different immunomodulatory effects on healthy and allergic individuals in a study where healthy and milk hypersensitive individuals were given milk with or without *L. rhamnosus* GG. In healthy individuals, *L. rhamnosus* GG appeared to have an immunostimulatory effect, while in allergic individuals, the bacterium appeared to down-regulate the inflammatory response to milk (Pelto et al., 1998).

Studies have demonstrated that the production of antimicrobial peptides (AMPs) produced by the epithelial cells of the gut is also influenced by probiotic bacteria. For example, Caco-2 intestinal cells increased production of human beta defensin-2 (Hbd-2) in response to *L. acidophilus, L. fermentum, E .coli* Nissle 1917, *L. paracasei, P. pentosaceus* and VSL#3 (a cocktail of 8 different probiotics) (Schlee et al., 2008).

Finally, some probiotics have antioxidant properties *in vitro*. Oxidative stress and reactive oxygen species can cause significant damage and play roles in ageing, cancer, and other illnesses (Lin and Chang, 2000). Lin and Chang (2000), found that *B. longum* and *L. acidophilus* whole cell and cell-free intracellular extracts were able to inhibit plasma lipid oxidation (a common test for antioxidative activity) and free radical scavenging *in vitro* (Lin and Chang, 2000). This antioxidant activity has been related to the production of exopolysaccharides by probiotic bacteria (Kodali and Sen, 2008) and proteins that could be enhanced by the addition of selenium (Shen et al., 2010).

1.4 Probiotics and the skin

Skin harbours its own diverse microbial community, some of which includes pathogenic species with the potential to cause disease. Since enteric probiotics have been demonstrated to modify bacterial communities not only in the gut but elsewhere, attention has naturally turned to whether there is a potential role for enteric probiotics in skin health/disease. At present, relatively little is known of the effect of enteric commensals and probiotics on the skin. Comparatively few studies have analysed the effects of ingestion or topical application of probiotics and the skin though the majority of these studies have concentrated on the effect of ingested probiotics on the skin.

1.4.1 Oral probiotics and the skin

In 2008, consumption of a fermented milk mixture containing probiotics (*L. casei*, *L. bulgaricus* and *S. thermophilus*), borage oil, green tea, and Vitamin E was shown to improve *stratum corneum* function in volunteers with dry and sensitive skin as judged by measuring the levels of transepidermal water loss (Puch et al., 2008). However, the effects observed could equally have been due to the other components in the fermented mixture. It was suggested that the mixture aided epidermal differentiation and that probiotics may allow systemic immunomodulation and thereby enable an increased effect of other nutrients in the skin (Puch et al., 2008).

It has been suggested that probiotics may have a beneficial effect upon stress induced changes in the skin. The presence of a gut-brain-skin axis has been postulated by Arck *et al* (2010). Oral administration of *L. reuteri* to stressed mice was found to significantly reduce levels of premature termination of active hair growth and stress-induced neurogenic skin inflammation compared to controls (Arck et al., 2010).

1.4.1.1 Immunoregulation and atopic dermatitis

A limited number of studies have found relationships between the ingestion of probiotics and skin immune health. Chapat *et al* (2004) found that consumption of *L. casei* in a murine model of contact dermatitis resulted in down-regulation of the inflammatory response via a reduction in CD8+ T-cell proliferation. It was established that CD4+ regulatory T-cells were required to allow this process to occur, indicating that *L. casei* activates regulatory CD4+ T-cells. Consumption of *L. casei* over a prolonged period of time was required in order to allow systemic immunoregulation. Consumption of *L. casei* cell walls resulted in a similar effect, indicating that cell wall components themselves or bacterial DNA could be responsible, by binding to Toll-like receptors on T-cells and inducing a signalling cascade (Chapat et al., 2004).

Guéniche and colleagues (2006) have examined the role probiotics may play in immunoregulation in relation to the effects of UV skin exposure. UV irradiation can affect the skin's innate immune system by depleting the amount of Langerhans cells present in the epidermis and their ability to act as antigen presenting cells (Stingl et al., 1981). Mice which had ingested *Lactobacillus johnsonii* NCC533 appeared to be resistant to the effects of UV irradiation on Langerhans cell density changes, and maintained IL-10 at levels observed in non-irradiated mice. Gamma irradiated dead *L. johnsonii* also elicited a similar protective effect, (though the results were not shown) while cell-free supernatants did not have a protective effect (Guéniche et al., 2006a). This result was confirmed in 2008 where consumption of *L. johnsonii* accelerated the recovery of skin homeostasis after UV exposure (Peguet-Navarro et al., 2008).

Numerous studies have attempted to relate a beneficial effect of probiotics on the prevention (Kalliomäki et al., 2001, Kopp et al., 2008) and treatment (Isolauri et al., 2000, Rosenfeldt et al., 2003) of pediatric atopic dermatitis, indicating a strong role probiotics play in immune health. Atopic dermatitis is caused by a dysregulation of the T_H1/T_H2 lymphocyte ratio in favour of T_H2 in allergic individuals.

The "hygiene hypothesis" has proposed that this may be due to decreased exposure to antigens in the environment thanks to increased hygiene in developed countries. Exposure to infectious agents appears to direct immune development towards a T_H1 response.

Therefore, probiotics are thought to re-balance the lymphocyte ratio in favour of T_H1, and reduce the production of inflammatory cytokines. However this is much debated, and in one recent meta-analysis of the data, it was concluded that while the evidence is in favour of probiotic prevention of atopic dermatitis, the data are unclear for the treatment of existing atopic disease (Lee et al., 2008).

1.4.2 Topical applications for probiotics

Probiotics have been analysed for their use as a potential topical treatment. There are a limited number of published reports on topical probiotics, but there are numerous patent applications for their uses topically on skin for different applications, suggesting that this is an emerging area of great interest to the scientific community.

Propionibacteria have been assessed for their ability to act as skin specific probiotics and inhibit attachment of pathogenic bacteria to the skin. Ouwehand *et al* (2003) utilised several dairy strains of propionibacteria instead of skin commensal strains (e.g. *P. acnes*) because the latter are associated with disease. The ability of strains to adhere to keratin, produce antimicrobial substances and inhibit adhesion of pathogenic bacteria was assessed *in vitro*. Where bacteria were found to bind to keratin, the ability of the probiotic to inhibit pathogen adhesion to keratin was assessed. However, it was concluded that the propionibacteria tested were unable to inhibit adhesion of pathogenic bacteria to keratins, indicating that probiotics and pathogens utilised different binding sites on keratin. However, this study utilised immobilised keratin as a substrate, so probiotic organisms may exert different effects using a living cell culture system, indicating further examination of this effect is required (Ouwehand et al., 2003).

Probiotic bacteria have been assessed for their inhibitory roles against skin commensals and pathogens. For example, a study in 2009 examined the effect of a number of different lactobacilli and glucomannan hydrolysates (GMH) upon the growth of the skin commensal, *Propionibacterium acnes*. This organism is implicated in the development of *acnes vulgaris* infections (Section 1.1.4.6). The glucomannan hydrolysates were found to promote the growth of lactobacilli and therefore increased the ability of probiotics to inhibit *P. acnes* growth *in vitro* (Al-Ghazzewi and Tester, 2009). In 2009, Kang *et al* found that topical application of a lotion containing the cell-free supernatant from probiotic *Enterococcocus faecalis* significantly reduced the inflammatory lesions in *acne vulgaris* sufferers. The proposed mechanism of action was either through killing of *P. acnes* or direct suppression of inflammation.

Probiotics have also been directly applied to skin in an attempt to improve barrier function in both normal skin and diseased skin. For example, topical probiotic preparations reportedly can induce increased ceramide synthesis in skin. In 1999, it was demonstrated that *S. thermophilus* sonicated lysates induced up-regulation of ceramide in HaCaTs *in vitro* and in the *stratum corneum* of volunteers. It was hypothesised that this was due to the action of bacterial neutral sphingomyelinases able to hydrolyse sphingomyelin into ceramide (Di Marzio et al., 1999). In 2003, the same group tested *S. thermophilus* extracts upon the skin of atopic dermatitis sufferers, hypothesising that decreased ceramide synthesis may contribute to the pathology of this disease, and therefore the bacterium could help regulate and improve ceramide production in atopic skin. The volunteers showed increased production of ceramide and reported a decrease in the symptoms associated with atopic dermatitis (Di Marzio et al., 2003). In 2008 the same group tested the extract upon ageing skin and found that ceramide production, hydration and capacitance were increased after application of the lysate. It was proposed that the probiotic extract could improve the lipid barrier in skin and thus reduce water loss and improve hydration and barrier function in skin (Di Marzio et al., 2008). A patent relating to these studies describes a cream containing sonicated *S. thermophilus* which was found to significantly increase ceramide levels in the skin of individuals (Cavaliere and De Simone, 2001).

While not classed as a probiotic, others have utilised *Vitreoscilla filiformis* (a Gram negative organism found in thermal springs) to improve the skin of atopic dermatitis sufferers. Topical application of the organism reportedly alleviates atopic dermatitis symptoms as measured by the modified eczema area and severity index (mEASI). However, the mechanism of action was not explored further (Guéniche et al., 2006b). Volz *et al* (2006) found that *V. filiformis* extracts interacted with dendritic cells via TLR-2, resulting in cytokine release and immunomodulation *in vitro* (Volz et al., 2006).

Guéniche and colleagues (2010) have demonstrated that a topically applied lysate of *Bifidobacterium longum* subsp. *reuter* in a randomised double-blind placebo trial was able to increase barrier resistance of skin. Barrier resistance was measured by the number of tape strippings required to induce a transepidermal water loss (TEWL) of more than 15g/cm^2/h in skin samples. However, the speed of recovery after barrier disruption was not affected by the probiotic.

Guéniche *et al* (2010) also established that the lysate reduced skin sensitivity and skin dryness, through increased urea levels. Urea acts to increase skin water retention in the *stratum corneum* by creating water binding sites. In addition, an *ex vivo* model was also used to demonstrate the ability of the lysate to inhibit inflammation and decrease TNF-α release (Guéniche et al., 2010). This was the first evidence that a topical preparation of probiotics may regulate barrier function, with several potential roles in wound healing, immunoregulation and allergy treatments.

The use of a probiotic and hesperidin (a flavanone) for the support of barrier function in individuals has been described in a patent by Gueniche and Castiel (2009). Another patent reports the use of *L. paracasei* or extracts of the probiotic alongside *B. longum* for the treatment of sensitive skin such as dry/irritant skin (Guéniche et al., 2006).

Miyazaki *et al* (2003) demonstrated that topically administered *Bifidobacterium*-fermented soy milk products increased production of hyaluronic acid (HA) by epidermal keratinocytes and dermal fibroblasts *in vitro,* and mouse skin *in vivo*. It was suggested that the probiotic was able to ferment the soy milk inactive glycosides into genistein and daidzein, products known to stimulate HA production by cells (Miyazaki et al., 2003). The following year, the same authors used the probiotic fermented soy milk product topically on mouse skin and human skin and found increased HA production, elasticity and hydration of skin (Miyazaki et al., 2004). Similarly, another study in 2006 found that addition of a probiotic-fermented whey product to the culture medium of normal human epidermal keratinocytes resulted in the increased expression of mRNA of markers involved in keratinocyte differentiation. In particular the authors found addition of the fermented whey product increased expression of keratin-10, involucrin and profilaggrin mRNA (Baba et al., 2006). These studies illustrate how probiotic bacteria may produce metabolites (or “post-biotics”) with beneficial effects for the skin.

The development of a probiotic patch with antimicrobial activity has recently been described. The patch contained a lyophilised preparation of *L. fermentum,* suspended in alginate, along with glucose and nitrite salts in order that the probiotic could produce nitric oxide gas. Nitric oxide is reportedly produced in the body in response to microbial infections, and has been shown in animal models to improve the ability to fight pathogenic infections. When the patch was applied to cultures of *E. coli*, *S. aureus*, and *P. aeruginosa,* it was able to kill these organisms within 8h (Jones et al., 2010).

Axillary malodour is caused by the production of odoriferous compounds by skin bacteria such as *Corynebacterium jeikeium*. The direct inhibition of the enzymes that create the malodorous compounds by inhibitors (e.g. phenantroline) is not desirable because many of these inhibitors are themselves harmful. Additionally, commonly used antimicrobials (e.g. Triclosan) in deodorants have the potential to remove all skin commensal bacteria. Therefore, it was proposed that a probiotic approach might be more appropriate to the removal of specific odour-causing bacteria or their by-products (Lang et al., 2007). A patent in 2007 describes the use of probiotic formulations as a replacement for antimicrobials found in deodorants (Lang et al., 2007).

1.4.2.1 Topical probiotics and wound healing

The idea of using probiotic bacteria for the treatment of bacterial infections in wounds is not a new one. The use of topical *L. plantarum* extracts to stimulate the production of antimicrobial peptides in the skin was recently described in a patent. It was suggested that topical *L. plantarum* extracts could be used in the treatment of cuts/wounds, *acne vulgaris* and for reducing skin sensitivity (Sullivan et al., 2009).

A study by Gan *et al* (2002) analysed the use of *L. fermentum* RC-14 and *L. rhamnosus* GR-1 on a silicone surgical implant model in rats. *L. fermentum* and its associated biosurfactant could inhibit surgical implant infection with *S. aureus*, the first evidence that a probiotic strain may be useful in combating infections *in vivo*. The probiotic inhibited *S. aureus* from adhering to the surgical implant and causing infection. The probiotic biosurfactant contained collagen binding proteins that could compete with staphylococcal extracellular matrix binding proteins for binding to host sites. It was also suggested that the biosurfactant could induce the release of signalling molecules from host cells, leading to the inhibition of infection by *S. aureus* (Gan et al., 2002). This was compared to the ability of other probiotics such as *L. rhamnosus* GG and *L. plantarum* 299v to induce production of mucin in intestinal epithelia and inhibit adhesion of enteropathogenic *E. coli* (Mack et al., 1999, Gan et al., 2002). Regarding the use of secreted products from probiotics, care should be taken as resistance can develop to bacterial products, just as seen with antibiotics (Strauss, 2000).

In 2005, Valdez *et al* evaluated the ability of the enteric probiotic, *L. plantarum,* and its products to inhibit *Pseudomonas aeruginosa* infections *in vitro* and in a burned-mouse model. The authors found that *L. plantarum* could inhibit or block quorum sensing molecules produced by *P. aeruginosa*.

It was suggested that blocking of quorum sensing affected the ability of *P. aeruginosa* to form biofilms, produce elastase, and resist treatment with antibiotics *in vitro*. The *in vivo* models revealed that *L. plantarum,* when injected subcutaneously into burned areas following application of *P. aeruginosa,* appeared to increase phagocytosis of *P. aeruginosa* and enhanced tissue regeneration. However, wound healing was delayed in comparison to wounds injected with phosphate buffer solution alone (Valdéz et al., 2005).

The same group have investigated the use of *Lactobacillus plantarum* in the treatment of infected leg ulcers and further examined the effect of the organism on burns (Peral et al., 2009a, Peral et al., 2009b, Valdéz et al., 2005). The authors found that while the organism was not able to easily colonise the skin, when applied it did as well as the conventional SD-Ag (silver sulphadiazine) treatment of burns in bacterial killing and wound healing (Peral et al., 2009b). In chronic ulcers, *L. plantarum* could also promote wound healing and reduce bacterial numbers in wounds. Additionally, *L. plantarum* regulated IL-8 production in patients with chronic infected leg ulcers (Peral et al., 2009a). However, further work must be performed to fully elucidate if topical probiotics can be used for the safe treatment and prophylaxis of wound infections, and what effect they may have upon wound healing.

1.4.3 Probiotics and biofilms

Biofilms are phenotypically distinct microorganisms that grow within an enclosed matrix adhered to one another or to a surface (Costerton et al., 1995). *Proprionibacterium acnes*, which is associated with the pathogenesis of *acne vulgaris*, has been suggested to grow as a biofilm thereby explaining the difficulties in treatment with traditional antibiotics (Burkhart and Burkhart, 2003). There is debate as to whether chronic infected wounds (e.g. diabetic foot ulcers) involve bacterial biofilms (James et al., 2008, Percival and Bowler, 2004). Both *S. aureus* and *P. aeruginosa* are common wound isolates, and their ability to form biofilms is therefore of importance (Bowler et al., 2001). Additionally, medical device infections are often caused by staphylococci growing as biofilms (von Eiff et al., 2005).

Conversely, commensal biofilms may provide a protective role for the surfaces of the body. For example, mixed species biofilms in the vagina can protect against colonisation with pathogenic organisms (Reid et al., 1990, Reid, 2001).

In the mouth dental biofilms of streptococci can antagonise pathogenic *Streptococcus mutans* growth, thereby inhibiting its ability to colonise (Kreth et al., 2008). Consequently, the ability of topical probiotics to form biofilms and inhibit the formation of biofilms by common skin pathogens may be of interest.

1.5 Summary and aims

Many studies have shown the importance of probiotics and their potential therapeutic effects within the gut. However, comparatively few studies have analysed the use of topical probiotics in relation to the skin. Nonetheless, evidence suggests that enteric probiotics may be capable of reinforcing the epidermal barrier through manipulation of the commensal microbiota, tight junction morphology, immunomodulation, epidermal differentiation and *stratum corneum* function. Very little is known of the normal interaction between either commensal skin organisms or enteric probiotics with keratinocytes, necessitating further investigation, which could be extremely valuable to both the health and pharmaceutical industries.

The primary aim of this project is to evaluate the potential of probiotic bacteria to protect against skin infection using a keratinocyte model of the skin, and the model organism *S. aureus*. The particular objectives for this study were to:

- Screen a range of probiotics for their ability to inhibit keratinocyte infection and pathogenesis caused by skin bacteria e.g. *S. aureus.*
- Characterise the probiotics used in this study in terms of their antimicrobial activity *in vitro*.
- Compare the effect topical probiotics may have upon the skin innate immune response through Toll-like receptor expression, AMP and cytokine production with that induced by a skin commensal and a skin pathogen.

The results obtained may have immense therapeutic potential in terms of epidermal wound healing and prevention of infection, in addition to the cosmetics and pharmaceutical industries.

Materials and Methods

2.0 Bacterial culture

Probiotic bacteria were grown routinely to stationary phase in Wilkins-Chalgren Broth (WCB) or Agar (WCA) (Oxoid, Basingstoke, UK) at 37°C in a Mark 3 Anaerobic Work Station (Don Whitley Scientific, Shipley, UK). *S. aureus* and *P. aeruginosa* were grown aerobically at 37°C in Nutrient Broth (Oxoid, Basingstoke, UK). Culture densities were adjusted spectrophotometrically with medium to contain the number of bacteria required. Selective agar was used in some experiments. This was Mannitol Salt Agar – MSA (Oxoid, Basingstoke, UK) or Man-Rogosa Sharpe agar (MRS) for *S. aureus* or Lactobacilli respectively. Both *S. aureus* and coagulase-negative *S. capitis* (CONS) were clinical isolates from chronic wounds donated kindly by Dr. Angela Oates (University of Manchester). Gram stains were performed as described by Cowan (2003), and oxidase (Oxoid, Basingstoke, UK) and catalase (Merck & Co., Inc. Whitehouse station, USA) tests were performed for basic characterisation of organisms, as per the manufacturer's instructions. A Staphylococcus latex kit was used as per the manufacturer's instructions to differentiate between *S. aureus* and *S. capitis* (ProLab Diagnostics, Bromborough, UK).

2.0.1 Preparation of bacteria for experiments utilising mammalian cell culture

For experiments utilising keratinocytes, bacteria were centrifuged at 15,000 x g, washed twice in 0.85% (w/v) NaCl solution, and reconstituted in keratinocyte medium (section 2.2) prior to use in experiments. Medium from keratinocytes was replaced with the medium containing bacteria. For experiments utilising heat-killed *L. reuteri,* bacteria were resuspended in 0.4% (w/v) glucose and heat inactivated by placing in a water bath at 85°C for 45 minutes. Samples were Gram stained to ensure lysis of bacterial cells had not occurred, and plated onto WCA plates to confirm they had been killed. For experiments using a probiotic lysate, 10ml of 10^8 CFU/ml *L. reuteri* was centrifuged, washed, concentrated in 1ml keratinocyte medium and lysed using a bead beater (FastPrep™ FP120, Thermo Electron Corporation). Samples were filter sterilised using a 0.22µm pore filter (Millipore, Billerica, USA) to remove any whole bacteria remaining. Approximately 100µl of this lysate was used to treat keratinocytes. Table 2.1 illustrates the bacteria used in experiments.

Table 2.1. Organisms used in experiments

Probiotic organisms	*Lactobacillus rhamnosus* AC413 *Lactobacillus reuteri* ATCC 55730 *Lactobacillus salivarius* UCC118 *Lactobacillus rhamnosus* ATCC 53103 (LGG)
Commensal/Pathogenic organisms	*Staphylococcus aureus* subsp. *aureus* *S. capitis* *Pseudomonas aeruginosa* PA01

2.0.2 Isolation of bacteria from GUM® periobalance® lozenges

In order to isolate *L. reuteri* Prodentis™ (*L. reuteri* ATCC 55730 and *L. reuteri* ATCC PTA 5289) from GUM® periobalance® lozenges, a lozenge was first aseptically dissolved in 20ml of 0.8% NaCl and incubated anaerobically at 37°C overnight. One ml of this was then spread aseptically onto the surface of a MRS plate and incubated anaerobically overnight at 37°C. Once colonies had grown, two distinct colony types were evident. Subcultures were performed on MRS plates to isolate pure cultures. Gram stains, catalase and oxidase tests were performed to check for the presence of catalase negative, oxidase negative, Gram positive bacilli, as per the manufacturer's instructions. These were then identified to species level by 16S sequencing.

2.0.3 16S Sequencing

A range of organisms were identified to species level using 16S ribosomal DNA sequencing. Bacteria were grown on Wilkins-Chalgren Agar (WCA) overnight and two or three colonies were aseptically removed and homogenized with 100µl of nanopure water. These bacterial suspensions were then heated to 100°C in a boiling water bath for 10 minutes and centrifuged for 10 minutes at 15,000 x g. The supernatants were removed and used as the templates for PCR. Partial 16S rRNA sequences were amplified using primers 8FPL1 (5'-GAG TTT GAT CCT GGC TCA G-3') and 806R (5'-GGA CTA CCA GGG TAT CTA AT-3') at 5 µM each.

Each reaction mixture contained; 24µl of Red Taq DNA polymerase ready mix (Sigma-Aldrich, Gillingham, UK), 2µl each of the forward and reverse primers, 16µl of nanopure water and 5µl of the template DNA. A Perkin-Elmer 480 thermal DNA cycler was used to run 35 thermal cycles (94°C (1min), 53°C (1min) and 72°C (1min). The final cycle incorporated a chain elongation step for 15 minutes at 72°C. Following confirmation of successful amplification by running products on an agarose gel, PCR products were purified using a Qiaquick PCR purification kit according to the manufacturer's instructions (Qiagen, Crawley, UK Ltd, Crawley, West Sussex, UK). These were then sequenced using the reverse primer only by the University of Manchester DNA sequencing facility (www.manchester.ac.uk/dnasequencing). BLAST analysis was performed on the sequences using the EMBL prokaryote database.

2.0.4 Growth curves

Overnight cultures of the organisms in use were diluted 1:100 in medium in a 96 well plate and performed in triplicate. The absorbance of each well was measured every 20 minutes at 686nm over 48-72h and the experiment was repeated three times. A growth curve was then constructed for each of the organisms using Gen 5™ software and a Powerwave XS plate reader (Biotek, Potton, UK) in order to determine the time after inoculation into medium at which each organism entered the stationary phase of growth.

2.0.5 Generation of biosurfactants

For experiments examining whether *L. reuteri* produced a functional biosurfactant, a microplate assay was performed as described by Sen *et al* (2010) based on the interfacial activity of biosurfactants. In brief, 100µl of the test sample was placed in a microtitre plate well and viewed using a backing sheet of grid-lined paper. Biosurfactants would distort the grid, while the negative control (water) would allow the grid to remain undistorted. The positive control was 10% sodium dodecyl sulphate.

2.1 Inhibition of *S. aureus* growth by probiotics

2.1.1 Well-diffusion assays

The ability of *L. reuteri* ATCC 55730, *L. rhamnosus* AC413 and *L. salivarius* UCC118 to inhibit the growth of the skin pathogen, *S. aureus,* was assessed using a well-diffusion assay. The test organisms were grown to stationary phase (approximately 10^8 CFU/ml).

Whole cell cultures (WC) were used, but for some experiments, cells were sedimented in a microfuge at 15,000 x g for 5 minutes and the cell-free supernatant (CFS) removed for use in experiments. In parallel, experiments were performed using cultures of probiotics grown in medium containing 10 x 2mm glass beads to increase available surface area for biofilm growth. *S.aureus* cultures were diluted 1:100 in WCA and 20ml agar plates poured. Once set, 8mm wells were cut aseptically out of the agar and 100µl of the WC or CFS of each probiotic organism put into individual wells. The plates were then incubated at 37°C for 48h anaerobically and the zones of inhibition calculated by measuring the diameter of the zone of inhibition in mm using a ruler. Some inhibition studies were also performed using probiotics grown in an alternate culture medium, Man-Rogosa-Sharpe broth (MRS). In these experiments, the pH of cell-free supernatants from three probiotics was measured before neutralisation with 1M NaOH and inhibition assays performed to compare the original supernatants and the neutralised supernatants.

2.1.2 Spot-on-the-lawn assays

Wilkins-Chalgren Agar (WCA) 10ml plates were spotted with 20µl of an overnight broth (in WCB) of the organisms *L. reuteri* ATCC 55730, *L. rhamnosus* AC413, and *L. salivarius* UCC118. Sterile WCB was used as a negative control. This was incubated at 37°C either aerobically or anaerobically for 48h. An overnight culture of *S. aureus* was diluted 1:100 in semisolid WCA (0.8%) and 10ml of this was poured over the plates containing the spotted probiotics, and incubated aerobically or anaerobically at 37°C overnight. Inhibitory activity was recorded by measuring the diameter of zone sizes in mm using a ruler. Using a microprobe, the pH of the agar near the colony was measured for all organisms grown either aerobically or anaerobically. Additionally, Universal Indicator was added to visualise the change in pH on some plates.

Next, plugs of agar from within the inhibition zones were extracted and either allowed to soak in PBS (Gibco, pH 7.2) or 0.8% NaCl for 1h. The PBS soaked plugs were then washed in 0.8% NaCl. These were then placed onto agar plates seeded with a *S. aureus* lawn and incubated aerobically for 48h to observe whether inhibition of *S. aureus* growth still occurred.

2.1.3 Competition assay

Aliquots (100µl) of overnight cultures of *L. reuteri* and *S. aureus* were inoculated into 10ml broths (both as axenic cultures and as co-cultures). The pH and optical density of cultures was measured at 0 and 48h. At regular intervals (3, 6, 24, 30, and 48h), bacteria were counted by Miles and Misra serial dilution plate counts (Collins et al., 1989) using selective and non-selective agars. At 48h, BacLite ™ Live/Dead stains were performed according to the manufacturer's instructions (Invitrogen, Life Technologies Ltd, Paisley, UK).

2.1.4 Crystal violet assays for biofilm growth

Probiotics were grown overnight in Wilkins-Chalgren Broth (WCB) and 200µl of a 1:100 dilution was added in triplicate to wells of a 96 well microtitre plate. Negative controls consisted of sterile WCB while positive controls were a 1:100 dilution of an overnight broth of *P. aeruginosa* PA01. The plate was incubated at 37°C for 48h with shaking. The plate was shaken over a waste tray and gently washed with water. Following the initial wash, 225µl of a 0.1% crystal violet solution was added to each well and left for 10 minutes. This was then tipped off into a waste tray and the plate washed 3 times. Next, 300µl of ethanol was added to each well of the plate and left for 10 minutes. Each well was pipetted to mix and 125µl of this transferred to a fresh plate. A measure of biofilm growth was determined by recording the optical density of each well at 600nm. Percentage biofilm growth was calculated as follows:

Optical density (OD) of the test organism / OD of P. aeruginosa x 100 / 1.

2.1.5 Biofilm Inhibition

The ability of probiotic bacteria to inhibit the biofilm growth of *S. aureus* was assessed using a slight modification of the crystal violet assay described in Section 2.1.4. Probiotics were grown overnight in Wilkins-Chalgren Broth (WCB) and centrifuged at 15,000 x g for 5 minutes to sediment cells. The cell-free supernatant (CFS) was removed and 200µl of each was placed in wells in a 96 well microtitre plate in triplicate and incubated for 2h at 37°C.

The CFS was then removed and 200µl of a 1:100 dilution of an overnight broth of *S. aureus* was added to each well. The crystal violet assay was performed as previously described in Section 2.1.4.

2.2 Mammalian cell culture

Normal human epidermal keratinocytes (NHEK) (Promocell, Heidelberg, Germany) were maintained in keratinocyte basal medium (Promocell, Heidelberg, Germany) containing a supplement mix (bovine pituitary extract 0.004mg/ml, epidermal growth factor (recombinant human) 0.125ng/ml, insulin (recombinant human) 5µg/ml, hydrocortisone 0.33µg/ml, epinephrine 0.39µg/ml and transferrin (holo-human), 10µg/ml) and 0.06mM $CaCl_2$ (Promocell, Heidelberg, Germany). Medium was substituted twice weekly and cells were cultured at 37^{o}C in a humid atmosphere of 5% CO_2 in 75cm^2 culture flasks. Once cells had grown to 80% confluency, they were washed with 100µl Hanks Buffered Salt Solution (HBSS) per cm^2 of the vessel surface. These were then detached using 100µl of trypsin (0.4%) / EDTA (0.3%) (Promocell, Heidelberg, Germany) per cm^2 of vessel surface until cells had detached, followed by the addition of the same volume of trypsin neutraliser solution (Promocell, Heidelberg, Germany). Cells were then sedimented by centrifugation at 220 x g for 3 minutes. The supernatant was discarded and the cells were resuspended in keratinocyte medium. A viable count was performed on this suspension using trypan blue exclusion assays (Strober, 1997). The cells were then re-seeded into cell culture vessels at approximately 5 x 10^3 cells/cm^2. For experiments using differentiated cells, calcium chloride in the medium was increased to 1.8mM and the cells were grown in this for 24h prior to experimentation.

2.2.1 Culture of NHEK on chamber slides

For some experiments, NHEK were seeded onto 8-well LabTek chamber slides (Nunc ™, Fisher Scientific Ltd, Loughborough, UK) at a density of 5 x 10^4 cells/cm^2. Chamber slides were pre-treated with 150µl of Poly-L-lysine (Sigma-Aldrich, Gillingham, UK) for 20 minutes and washed briefly with PBS (Invitrogen, Life Technologies Ltd, Paisley, UK) prior to the addition of cells.

2.2.2 Measurement of NHEK viability in response to bacteria

NHEK culture medium was removed from confluent keratinocytes growing in 24 well plates and replaced with medium containing 10^8 bacteria (MOI 2000). Cells and bacteria were then incubated for 24h at 37°C in 5% CO_2. Following this keratinocytes were washed twice in PBS and cells were detached as described in Section 2.2. Cell viability was determined by trypan blue exclusion assay (Strober, 1997). Percentage viability of keratinocytes was calculated using the following equation:

Percentage viability = Viable count / Total count x 100/1

2.2.3 *S. aureus* dose and time response assays

Keratinocytes were grown to confluency in a 24 well plate and infected with 10^5, 10^6, 10^7 or 10^8 CFU (MOI of 2, 20, 200, and 2000 respectively) of *S. aureus* in triplicate and incubated at 37°C/5% CO_2 for 24h. Following incubation, keratinocytes were washed twice in PBS to remove planktonic bacteria. Trypan blue exclusion assays were performed to determine keratinocyte viability. To determine at what point 10^6 *S. aureus* began to induce significant cell death in keratinocytes, cells were infected with 10^6 *S. aureus* for 12, 16, and 24h before assessing keratinocyte viability.

2.2.4 *L. reuteri* dose response assay

To determine the ratio of *L. reuteri* to *S. aureus* for use in experiments, confluent NHEK in 24 well plates were exposed to a range of concentrations of *L. reuteri* (10^6, 10^7, and 10^8 CFU/ml), and 10^6 CFU/ml *S. aureus* simultaneously, in triplicate, for 24h before assessing keratinocyte viability using trypan blue exclusion assays.

2.2.5 Measurement of *S. aureus* viability in cell culture

To determine whether *L. reuteri* or NHEK were able to inhibit the growth of *S. aureus* in cell culture, NHEK were grown to confluency in a 24 well plate. These were exposed to either *S. aureus* or *S. aureus* and *L. reuteri* together. In parallel, wells without any cells also had these combinations added to determine the effect of the keratinocytes themselves on staphylococcal viability. After 24h exposure, the medium was removed and centrifuged to pellet extracellular bacteria. The keratinocytes were then trypsinised and 500µl of 0.25% Triton-X-100 (Sigma-Aldrich, Gillingham, UK) in PBS was added for approximately 30 minutes to lyse the cells.

The well contents were then combined with the bacterial cell pellet and serial dilution plate counts using mannitol salt agar (MSA) performed to determine the total number of viable staphylococci.

2.3 Analysis of bacterial adhesion to NHEK

Confluent, differentiating NHEK were exposed to either *L. reuteri* or *S. aureus* for 1h. After incubation, cells were washed three times in PBS (Invitrogen, Life Technologies Ltd, Paisley, UK) to remove non adherent bacteria. The cells were trypsinised and serial dilution plate counts performed using these to assess the number of adherent bacteria. Selective agar was used for growth of staphylococci. In separate experiments, cells were exposed to *L. reuteri* for 1h before the addition of *S. aureus* (exclusion), at the same time (competition) or 30 minutes after *S. aureus* infection had begun (displacement) to determine whether *L. reuteri* could inhibit *S. aureus* adhesion to keratinocytes.

An alternative method for assessing the number of adherent bacteria was utilised for dead preparations of *L. reuteri*. Cells were grown on LabTek chamber slides (Nunc ™, Fisher Scientific Ltd, Loughborough, Leicestershire, UK). Adhesion assays were performed as previously described, using adjusted amounts of bacteria. After the cells were exposed to bacteria, cells were washed twice in PBS and fixed in methanol for 20 minutes before performing a Gram stain (Cowan and Steel, 2003a). The number of adherent bacteria per 100 cells was then assessed using a Keyence All in one Type Fluorescence microscope (Keyence, Milton Keynes, UK).

For experiments analysing the effect of conditioned medium on bacterial adhesion to cells, keratinocytes were exposed to bacteria for 1h. The conditioned medium (CM) from the exposed cells was removed and centrifuged at 15,000 x g for 3 minutes to pellet the bacterial cells. The CM was then filtered through a 0.22µm pore filter (Millipore, Billerica, USA). This CM was then either used to pre-expose cells prior to *S. aureus* infection or combined with 10^6 CFU/ml *S. aureus* and used to infect NHEK. These were incubated at 37°C in 5% CO_2 for 1h. The cells were then washed, trypsinised and bacterial cell counts were performed as described for adhesion assays.

2.3.1 Invasion assays

To determine whether *L. reuteri* could inhibit invasion of keratinocytes by staphylococci, cells were either exposed to *S. aureus* or *S. aureus* and *L. reuteri* for 1h. Cells were washed three times in sterile PBS to remove non-adherent bacteria, and the growth medium replaced with medium containing 100µg/ml gentamicin (Sigma-Aldrich, Gillingham, UK) for 2h. Cells were then washed three times in sterile PBS, trypsinised and lysed in 0.25% Triton-X-100 for 30 minutes to release internalised bacteria. Bacteria in lysates were counted using serial dilutions.

2.3.2 Analysis of mechanisms of bacterial adhesion to cells

Lipoteichoic acid (LTA) from *S. aureus* was obtained from InvivoGen (San Diego, USA). NHEK were exposed to 0.1mg/ml LTA prior to the addition of bacteria in adhesion assays.

An antibody directed against the α5ß1 integrin was used in some experiments. Confluent differentiating NHEK were exposed to three different concentrations (60, 30 and 15 µg/ml) of mouse anti-human integrin α5ß1 JBS5 blocking antibody (Millipore, Billerica, USA) made in PBS for 1h at 37°C in 5% CO_2. Cells were then washed with PBS prior to addition of bacteria in adhesion assays.

2.3.3 Carbohydrate analysis

To determine the role of cell surface carbohydrates in binding of *L. reuteri* and *S. aureus* to NHEK, bacteria were treated with 50mM sodium meta-periodate (Sigma-Aldrich, Gillingham, UK) in 0.1M citrate phosphate buffer (pH 4.5). As a negative control, bacteria were resuspended in citrate phosphate buffer. Cells were incubated at 37°C for 30 minutes and washed twice before resuspending in NHEK medium for infection. Serial dilution counts were performed to check if bacterial viability affected by the sodium meta-periodate.

2.3.4 Protein analysis

To determine whether *L. reuteri* utilised a protein adhesin for adherence to NHEK, *L. reuteri* was centrifuged and resuspended in either proteinase K (20mg/ml) (Qiagen, Crawley, UK) or Trypsin (0.4%) (Promocell, Heidelberg, Germany) for 2h at 37°C, then washed twice in 0.85% saline/trypsin neutraliser and resuspended in keratinocyte medium prior to use in adhesion assays. Serial dilution counts were performed to check if the bacterial viability was affected by treatment with proteases.

2.3.5 Protein extraction and concentration

To ensure proteolysis of *L. reuteri* had occurred following treatment with proteases, untreated and treated bacterial EDTA extracts were compared using SDS-PAGE as described by Smith *et al* (1986). In brief, 20ml cultures were centrifuged at 15,000 x g and resuspended in 2ml of 10mM EDTA in PBS. This was then incubated at 45°C for 1h. The mixture was then centrifuged at 15,000 x g to pellet the cells and the supernatant extracted for use. The protein concentrations of the samples were determined using the Pierce® Bicinchoninic Acid Assay kit (BCA) (Thermo Scientific, Rockford, USA) according to the manufacturer's instructions. A standard curve was constructed using bovine serum albumin (BSA, 2mg/ml) as the standard (Table 2.2). Samples were diluted 1 in 10 with distilled water. In each well of a 96 well plate (Corning, Amsterdam, The Netherlands) 25µl of the diluted sample was added to 200µl of the BCA working reagent. Three replicates for each sample were made and the plate incubated at room temperature for 30-45 minutes. The results were then read on a microtitre plate reader (540-590nm) and the protein concentrations of samples determined by using the standard curve.

Table 2.2. Standard curve standards

Protein (mg/ml)	BSA (2mg/ml)	Distilled water
0.0	-	100µl
0.25	12.5µl	87.5µl
0.50	25.0µl	75.0µl
0.75	37.5µl	62.5µl
1.00	50.0µl	50.0µl
1.50	75µl	25.0µl
2.00	100µl	-

2.3.6 SDS-PAGE

A 10% sodium-dodecyl-sulphate polyacrylamide gel was made (4% stacking gel, 10% separating gel). The EDTA extracts were combined with Laemmli buffer and boiled for 3 minutes to denature the proteins and then loaded in equal concentrations to each of the wells alongside a broad range molecular weight marker (BioRad; Hertfordshire, UK). The proteins were electrophoretically separated for 1-2h at 60V, then at 150V for a further 2-3h till the bromophenol had reached the bottom of the gel. Gels were the silver stained to visualise protein bands.

2.4 Analysis of keratinocyte innate immune responses to bacteria

2.4.1 Immunocytochemistry for Antimicrobial Peptides (AMPs)

Confluent, differentiating keratinocytes were grown in LabTek Chamber slides (Nunc ™, Fisher Scientific Ltd, Loughborough, Leicestershire, UK) as described in Section 2.2.1, and exposed to bacteria for 12h. NHEK were then washed twice in PBS and fixed in 4% Paraformaldehyde for 20 minutes at room temperature. NHEK were washed in PBS and permeabilised by the addition of 0.25% Triton-X-100 for 15 minutes. The cells were washed in PBS and blocked in 5% BSA for 1h. Following this, cells were exposed to the primary antibody overnight at 4°C in the dark (see Table 2.3 for antibodies and concentrations employed). NHEK were washed twice in PBS and the appropriate flurophore-conjugated secondary antibody added for 1h at room temperature in the dark. Following this cells were again washed twice in PBS before the addition of 1µg/ml 4´,6-Diamidino-2-Phenylindole (DAPI) (Sigma-Aldrich, Gillingham, UK) for 30 seconds. Following a final wash in PBS, cells were mounted using Citifluor AF1 solution (Citifluor, UK) and allowed to dry in the dark before analysis. Pictures of cells were taken using oil immersion on an Olympus BX60 microscope using Image Pro Plus. The results were analysed using Image-J, an image processing program, by measuring the pixel intensity of AMP fluorescence adjacent to the nucleus of cells to ensure cytoplasmic expression was measured.

Table 2.3. Antibodies used in immunocytochemistry experiments.

Antibody	Concentration used	Secondary type and concentration
Rabbit anti-human beta defensin-2 (ab63982, Abcam, Cambridge, UK)	1/100	1/400 Goat anti-Rabbit IgG-FITC (Sigma-Aldrich, Gillingham, UK)
Rabbit anti-human beta defensin-3 (ab109572, Abcam, Cambridge, UK)	1/100	1/400 Goat anti-Rabbit IgG-FITC (Sigma-Aldrich, Gillingham, UK)
Rabbit anti-human CAMP (HPA029874, Sigma-Aldrich, Gillingham, UK)	1/100	1/400 Goat anti-Rabbit IgG-FITC (Sigma-Aldrich, Gillingham, UK)
Rabbit anti-human RNAse-7 (HPA005690, Sigma-Aldrich, Gillingham, UK)	1/100	1/400 Goat anti-Rabbit IgG-FITC (Sigma-Aldrich, Gillingham, UK)
Mouse anti-human psoriasin [47C1068] (ab13680, Abcam, Cambridge, UK)	1/50	1/300 Rabbit anti-Mouse IgG-FITC (Sigma-Aldrich, Gillingham, UK)
Mouse IgG1 kappa Isotype control (ab18443, Abcam, Cambridge, UK)	1/50	1/300 Rabbit anti-Mouse IgG-FITC (Sigma-Aldrich, Gillingham, UK)
Rabbit IgG Isotype Control (ab27478, Abcam, Cambridge, UK)	1/100	1/400 Goat anti-Rabbit IgG-FITC (Sigma-Aldrich, Gillingham, UK)

2.4.2 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Confluent differentiating NHEK were exposed either to 10µg/ml LTA (InvivoGen, San Diego, USA) for 24h, or to bacteria for 12h at 37°C in 5% CO_2. Cells were washed twice in PBS to remove any non-adherent bacteria and the total RNA was extracted from cells using Trizol reagent (Invitrogen, Life Technologies Ltd, Paisley, UK). RNA was purified using an Ambion® PureLink® RNA Mini Kit according to the manufacturer's instructions (Invitrogen, Life Technologies Ltd, Paisley). Following this 2µg of RNA was reverse transcribed using a cloned AMV first strand cDNA synthesis kit (Invitrogen, Life Technologies Ltd, Paisley, UK).

The mRNA levels of Hbd-2, Hbd-3, cathelicidin (LL-37), Rnase-7, psoriasin and Toll-like receptor-2 (TLR-2) were examined using pre-developed Taqman assays (Applied biosystems, Weiterstadt, Germany) using the Step One Plus PCR system (Applied Biosystems, Weiterstadt, Germany). To account for any variation during RNA isolation, variations in cell number or changes in the efficiency of reverse transcription/PCR, a GeNorm algorithm (Vandesompele et al., 2002) was used to screen samples across a range of reference genes. These were HPRT1 (hypoxanthine phosphoribosyl transferase I), ACTB (beta-actin), B2M (beta 2 microglobulin), SDHA (succinate dehydrogenase complex subunit A), HMBS (hydroxymethyl-bilane synthase), GAPDH (glyceraldehyde 3 phosphate dehydrogenase), RPL13A (ribosomal protein L13a) and RPL32 (ribosomal protein L32). GeNorm analyses the stability of each transcript and removes the least stable transcript until the two most stable transcripts remain. If the values remained below 0.4 they were considered stable. For this study, SDHA and HMBS were chosen as the stable combination of reference genes because they are genes involved in different pathways (Figure 2.1).

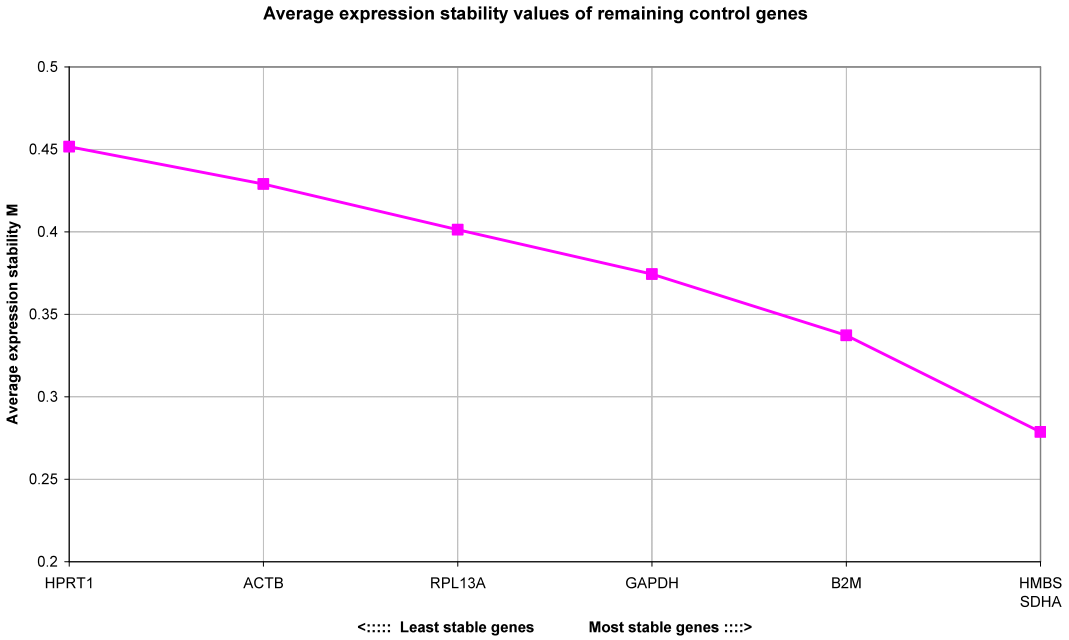


Figure 2.1. Average expression stability (M) of reference genes as determined by GeNorm.

2.4.3 Flow cytometry analysis of Toll-like receptor-2 (TLR-2) expression on NHEK

Confluent differentiating NHEK were exposed to either 10µg/ml LTA (InvivoGen, San Diego, USA) for 24h, or bacteria for 12h at 37°C / 5% CO_2. NHEK were washed twice with PBS to remove non-adherent bacteria and detached using accutase (Promocell, Heidelberg, Germany) according to the manufacturer's instructions. NHEK were centrifuged at 220 x g for 30 seconds and resuspended in PBS. All steps were performed on ice. Cells were stained with fixable viability dye efluor® 450 for 30 minutes in the dark (Ebioscience). NHEK were washed in PBS prior to being fixed for 20 minutes in 4% paraformaldehyde (Sigma-Aldrich, Gillingham, UK).

For intracellular staining, cells were permeabilised in 0.1% saponin (Sigma-Aldrich, Gillingham, UK) for 20 minutes. Non-specific staining of NHEK was blocked using a 1:20 dilution of IgG from human serum (Sigma-Aldrich, Gillingham, UK) made in 5% Bovine Serum Albumin (BSA) (Sigma-Aldrich, Gillingham, UK). NHEK were stained with a 1:100 dilution of rabbit anti-human TLR-2 IgG (ab24192, Abcam, Cambridge, UK) for 20 minutes. Non-specific staining controls were stained with rabbit anti-human TLR-2 IgG (ab24192, Abcam, Cambridge, UK) which had been previously blocked for 30 minutes at room temperature with an equal volume of TLR-2 peptide (ab39869, Abcam, Cambridge, UK). Cells were washed twice in PBS. Following this, cells were incubated with a 1:300 dilution of anti-rabbit IgG-Cy5 secondary antibody (Invitrogen, Life Technologies Ltd, Paisley, UK) for 15 minutes. Secondary only stained controls were also included to account for any non-specific secondary antibody staining. Positive staining for TLR-2 was determined by exposing NHEK to 10µg/ml LTA for 24h as observed by Lew *et al* (2009). Cells were washed twice in PBS before analysis with a Beckman Coulter CyAN™ ADP Flow cytometer. Analysis was performed on cells gated by viability dye, forward scatter, and side scatter profiles. Results were analysed using Summit 4.3 (Beckman Coulter Inc, California, USA).

2.4.4 IL-8 Enzyme Linked Immunosorbent Assays (ELISA)

Confluent differentiating NHEK were grown in 12-well plates and exposed to bacteria for 12h, to evaluate secreted IL-8 production by NHEK in response to bacteria. Following exposure, the cell-free supernatants (CFS) from plates were removed and filtered using a 0.22µm pore filter (Millipore, Billerica, USA) to remove bacteria. Samples were frozen at -20°C until ready for use, avoiding repeated freeze-thaws to maintain integrity of the sample. The experiment was repeated six times.

IL-8 levels were determined using an IL-8 DuoSet ELISA development kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. All samples were assayed in duplicate. The optical density of wells was determined using a Labtek LT400 microplate reader (Labtech International Ltd, Ringmer, UK) set at 450nm.

2.5 Statistical Analysis

All experiments were performed a minimum of three times, with three replicates each time. For experiments comparing two treatments, a students T-test was used. For experiments comparing two or more treatments, a one way ANOVA with blocking by experiment and post-hoc Tukey test was utilised. Dose response and competition assays were analysed using linear regression and 2-way ANOVA respectively. Results were considered significant if $P<0.05$.

Characterisation of bacterial interactions

A preliminary starting point for the evaluation of probiotic bacteria as a therapy for skin was to examine the ability of selected candidate probiotic bacteria to inhibit the growth of skin pathogens such as *S. aureus*. This is a transient coloniser of the skin, and while normally carried asymptomatically, it can cause a variety of diseases ranging from impetigo to septicaemia (Kluytmans et al., 1997, Krut et al., 2003).

Probiotics can compete with pathogenic bacteria for nutrients and produce inhibitory substances such as bacteriocins (Cotter et al., 2005), in addition to modifying the pH of the immediate environment through production of organic acids (Karska-Wysocki et al., 2010). The production of these factors may depend upon the growth phase of the organism (e.g. exponential vs. stationary phase) (Ogunbanwo et al., 2003), or growth mode (e.g. planktonic vs. biofilm growth). For example, *L. salivarius* UCC118 produces a bacteriocin Abp118, which is under the control of a cell-density-dependent quorum sensing mechanism (Flynn et al., 2002). Therefore, the aims of this study were:

- To confirm the identity of the organisms used in this study using 16S sequencing.
- To determine the growth pattern of each organism used.
- To determine whether the probiotics could inhibit the growth of *S. aureus.*
- To determine whether the probiotics produced inhibitory substances.
- To determine whether probiotics could inhibit biofilm formation by *S. aureus.*

3.0 Bacterial characterisation

Before commencing experiments, each of the test bacteria were analysed to confirm their identity. In addition, the identification of probiotic species found within GUM ® periobalance® lozenges was required because this product is purported to contain two probiotic species, *L. reuteri* ATCC PTA 5289 and *L. reuteri* ATCC 55730 (also known as SD2112) (McBain et al., 2009). These were of interest because *L. reuteri* strains reportedly inhibit adhesion of pathogenic bacteria in the mouth (Haukioja et al., 2008) and have immunomodulatory and antimicrobial activity (Jones and Versalovic, 2009). Therefore, it was thought that GUM® lozenges could be a source from which these organisms could be isolated.

GUM® lozenges were dissolved in 0.8% NaCl and 1ml of this spread onto Man-Rogosa-Sharpe (MRS) agar (a selective medium for lactobacilli). Only one colony type was successfully isolated, and further testing was performed to identify which strain of *L. reuteri* this was.

Gram stains, catalase and oxidase tests were performed on all bacteria to assist in confirming their identities. These confirmed the presence of catalase negative, oxidase negative, Gram positive rods for lactobacilli species and catalase positive, oxidase negative, Gram positive cocci for staphylococci species. A staphylococcus latex kit was used as per the manufacturer's instructions to differentiate between *S. aureus* and coagulase-negative staphylococci (CONS) (Section 2.0). The CONS was isolated from a chronic wound (Section 2.0), and the most common CONS associated with wound infection is *S. epidermidis* (Cowan and Steel, 2003b, Sewell et al., 1982). However, growth of colonies of the CONS on mannitol salt agar (MSA) changed the medium yellow, indicating it fermented mannitol, and hence suggesting the organism was not *S. epidermidis*, but another staphylococcal species.

16S sequencing (Section 2.0.3) is a rapid technique for the accurate determination of bacterial species. The 16S rRNA gene contains conserved sequences common to all bacteria and also variable regions specific for bacterial species. Therefore, to confirm the identity of the organisms under test, this technique was utilised to identify each of the organisms to species level (Table 3.1). Full sequences can be found in Appendix A. Sequencing revealed that the organism isolated from the GUM lozenges had 99% similarity to *L. reuteri* ATCC 55730 and the CONS had 99% similarity to *Staphylococcus capitis*.

Table 3.1. Basic Local Alignment Sequence Tool (BLAST) results for each organism sequenced.

Probiotic bacteria	*Lactobacillus rhamnosus* AC413
	Lactobacillus salivarius UCC118
	Lactobacillus reuteri ATCC 55730
Skin pathogens	*Staphylococcus* subsp. *aureus*
	Pseudomonas aeruginosa PA01
Skin commensal	*Staphylococcus capitis* - (CONS)

Growth curves for each of the probiotic strains and both the staphylococci were constructed in order to determine the time after inoculation into Wilkins-Chalgren Broth (WCB) at which each organism entered the stationary phase of growth. Stationary phase can be described as the phase at which the total cell absorbance (as measured by optical density) ceases to increase. This is important because some probiotics may produce bacteriocins in their exponential and stationary phases of growth, and bacteriocin production may peak at stationary phase (Ogunbanwo et al., 2003). Other genes involved in colonisation may also be expressed during these growth phases. For example, *L. salivarius* UCC118 expresses genes involved in adhesion at their highest levels during stationary phase (Van Pijkeren et al., 2006).

Overnight cultures of the organisms in use were diluted 1:100 in Wilkins-Chalgren Broth (WCB) in a 96 well plate and performed in triplicate. The absorbance of each well was measured every 20 minutes at 686nm over 48h and the experiment was repeated three times. The graphs were constructed using the mean values obtained. Error bars have been shown only at selected time points to allow clarity. In all cases the maximum standard error has been stated in figure legends (Figure 3.1 - Figure 3.5).

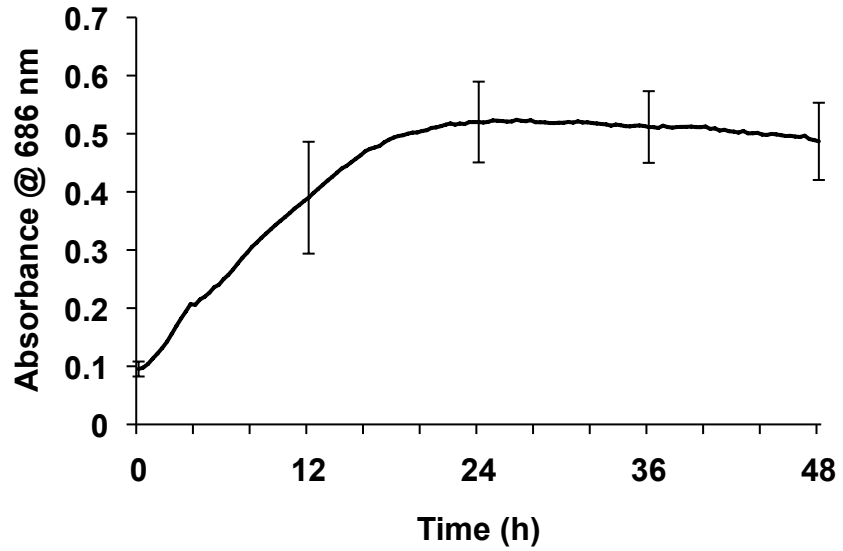


Figure 3.1. Growth curve for *S. aureus* in batch culture.
Stationary phase begins at approximately 22h post inoculation (Max SE=0.1) (n=3).

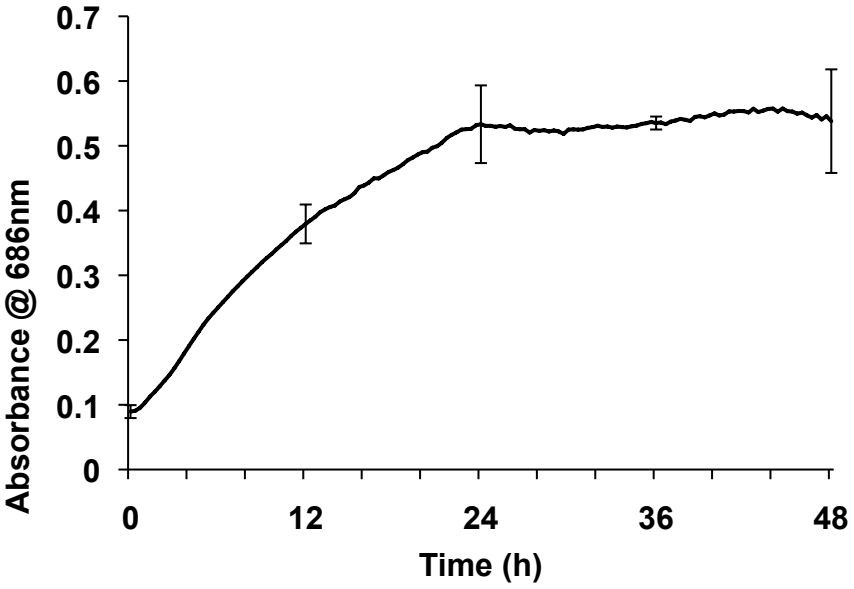


Figure 3.2. Growth curve for *S. capitis* in batch culture.
Stationary phase begins at approximately 22h post inoculation (Max SE=0.08) (n=3).

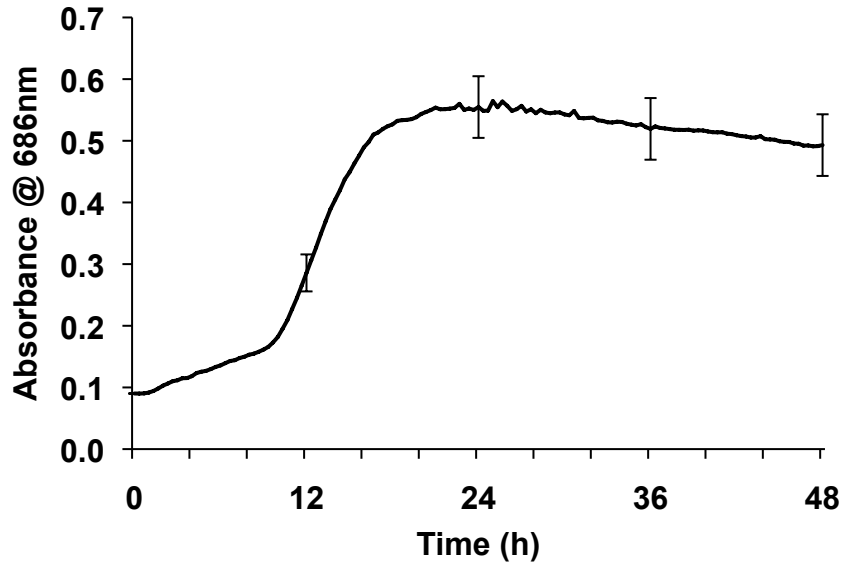


Figure 3.3. Growth curve for *L. reuteri* ATCC 55730 in batch culture.
Stationary phase begins at approximately 20h post inoculation (Max SE=0.06) (n=3).

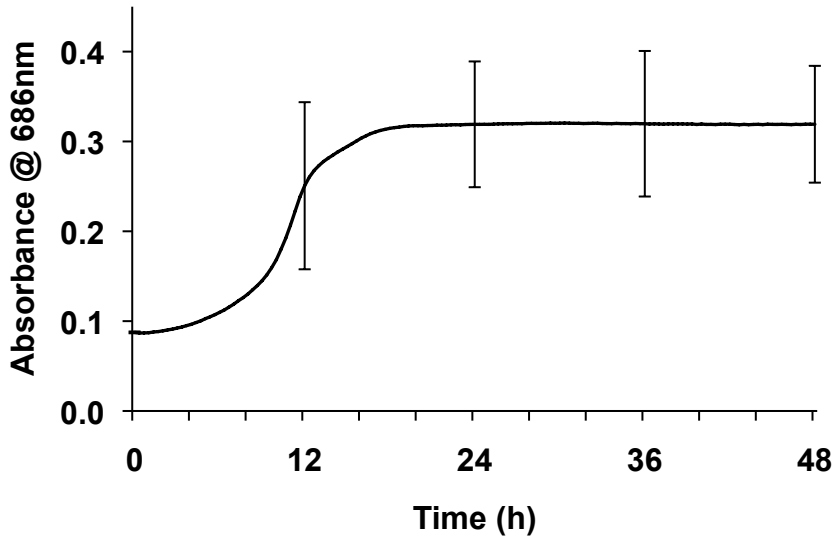


Figure 3.4. Growth curve for *L. rhamnosus* AC413 in batch culture.
Stationary phase begins at approximately 20h post inoculation (Max SE=0.09) (n=3).

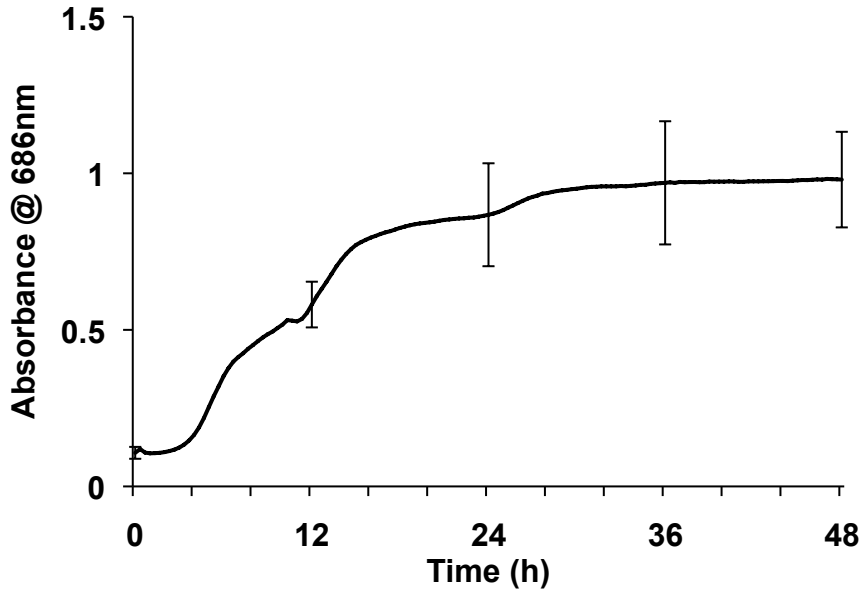


Figure 3.5. Growth curve for *L. salivarius* UCC118 in batch culture.
Stationary phase begins at approximately 27h post inoculation (Max SE=0.2) (n=3).

3.1 Well-diffusion Assays

Probiotics are known to produce a variety of substances that can inhibit the growth of other organisms (Section 1.3). Therefore, the ability of probiotics to directly inhibit growth of the skin pathogen *S. aureus* was assessed using well-diffusion assays. *S. aureus* cultures were diluted 1:100 in Wilkins-Chalgren Agar (WCA) and 20ml plates poured. WCA is a general purpose medium for the growth of anaerobic bacteria. Once set, 8mm wells were cut aseptically out of the agar and 100µl of the test probiotic added to the wells. The plates were incubated at 37°C anaerobically for 48h and zones of inhibition calculated by measuring the diameter using a ruler. Test probiotics used were either stationary phase whole cell cultures (WC) or cell-free supernatants (CFS) to determine the antimicrobial activity of CFS or the direct inhibitory activity of whole cell cultures (Section 2.1.1). Parallel experiments were performed using cultures that had been grown in medium containing glass beads to increase the available surface area because production of bacteriocins may be regulated by cell-density-dependent mechanisms (Section 2.1.1).

Zones of inhibition were not observed when *S. aureus* was challenged with WC cultures or CFS of probiotic organisms grown in WCB, a medium commonly used for growth of anaerobic species. However, other studies have reported inhibitory activity of lactobacilli following growth in Man-Rogosa Sharpe (MRS) broths (Karska-Wysocki et al., 2010).

MRS is a selective medium for the growth of lactobacilli. Therefore, the experiment was repeated using WC and CFS from cultures grown in Man-Rogosa Sharpe (MRS) broth (Figure 3.6). Zones of inhibition were visible for each of the probiotics tested (Table 3.2, Figure 3.6). There was no significant difference (P>0.05) in the size of zones of inhibition produced by organisms when grown in the presence vs. absence of glass beads (Table 3.2).

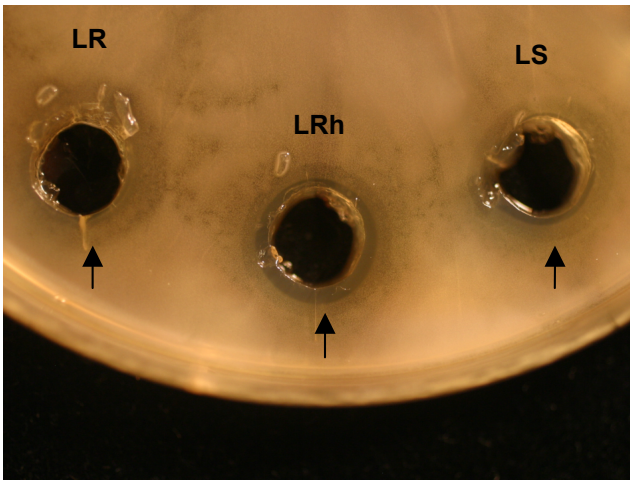


Figure 3.6. Well-diffusion assay.
Representative image of one experiment demonstrating inhibition of *S. aureus* growth by CFS derived from cultures grown in MRS broth (*L. reuteri* = LR, *L. rhamnosus* = LRh, *L. salivarius* = LS).

Table 3.2. Zones of inhibition (ZOI) of bacteria grown in MRS medium with/without beads (n=3).

Organism	***L. reuteri*** **ZOI (mm)**	***L. rhamnosus*** **ZOI (mm)**	***L. salivarius*** **ZOI (mm)**
Without beads -WC	13 ± 1	14 ± 1	14 ± 2
With beads -WC	11 ± 3	14 ± 2	14 ± 1
Without beads -CFS	13 ± 2	13 ± 1	12 ± 1
With beads -CFS	13 ± 2	13 ± 1	13 ± 1

To test whether the inhibition was due to the production of organic acids, the pH of CFS of the three probiotics (*L. reuteri, L. rhamnosus* and *L. salivarius*) grown as 48h broth cultures in both MRS and WCB was measured. MRS cultures were significantly more acidic than WCB cultures (Table 3.3).

Table 3.3. The pH of CFS of cultures grown in different media (n=3).

	WCB	MRS	P-value
L. reuteri	5.6 ± 0.1	3.26 ± 0.3	P=0.013
L. rhamnosus	3.87 ± 0.2	2.74 ± 0.03	P=0.015
L. salivarius	3.81 ± 0.01	2.66 ± 0.3	P=0.001

The CFS was then neutralised using 1M NaOH to determine whether the inhibitory activity against *S. aureus* was due to the presence of acids or other inhibitory substances. When the CFS of cultures grown in MRS were neutralised, no inhibition zones were observed (Figure 3.7).

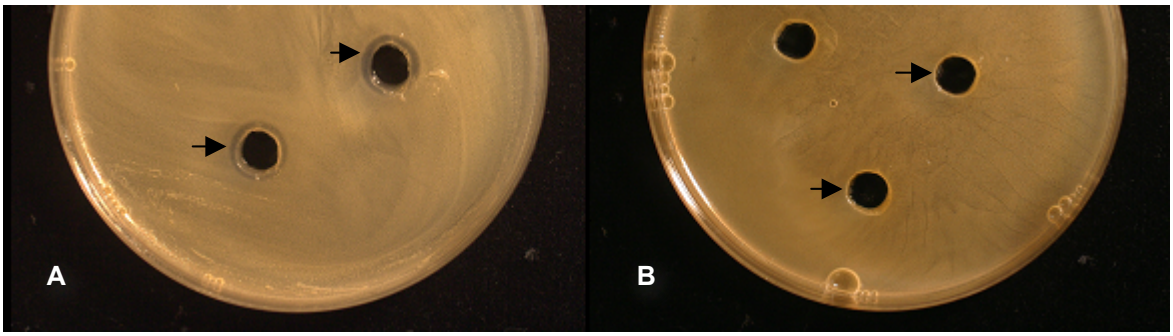


Figure 3.7. Neutralisation of CFS from probiotics resulted in loss of inhibition.
(A) Before neutralisation of CFS with 1M NaOH. Arrows indicate the zones of inhibition. (B) Well-diffusion assay using neutralised CFS. Arrows indicate the absence of zones of inhibition.

3.2 Spot-on-the-lawn Assays

Another mechanism by which probiotics have been shown to inhibit pathogens is through the production of bacteriocins. These are antimicrobial peptides active against a range of other, mostly Gram positive bacteria. Since bacteriocin production may be controlled in a cell-density-dependent manner (Section 1.3), another method for testing the ability of probiotics to release inhibitory substances was adopted. The "Spot-on-the-lawn" assay (Section 2.1.2) was used because it allowed the growth of a high concentration of probiotic organisms on agar before the addition of *S. aureus*. The experiment utilised Wilkins-Chalgren medium. A coagulase-negative staphylococcus (*S. capitis*) was included as a comparison because some coagulase-negative staphylococci are also able to produce bacteriocins (Augustin et al., 1992). The diameter of the resultant zone of inhibition was measured using a ruler.

The organisms tested did not grow well, nor could any zones of inhibition be observed under aerobic conditions. Under anaerobic conditions, *L. rhamnosus* and *L. salivarius* had inhibitory activity against *S. aureus* while *S. capitis* and *L. reuteri* did not inhibit *S. aureus* growth (Table 3.4). The zones of inhibition produced by *L. rhamnosus* and *L. salivarius* were considerably larger than those observed using well-diffusion assays (Figure 3.8).

Table 3.4. Diameters of inhibition zones for Spot-on-the-lawn assays (n=3).

	Mean ± SD (Aerobic)	Mean ± SD (Anaerobic)
S. capitis	-	-
L. reuteri	-	-
L. rhamnosus	-	20.33 ± 0.35
L. salivarius	-	20.33 ± 0.78

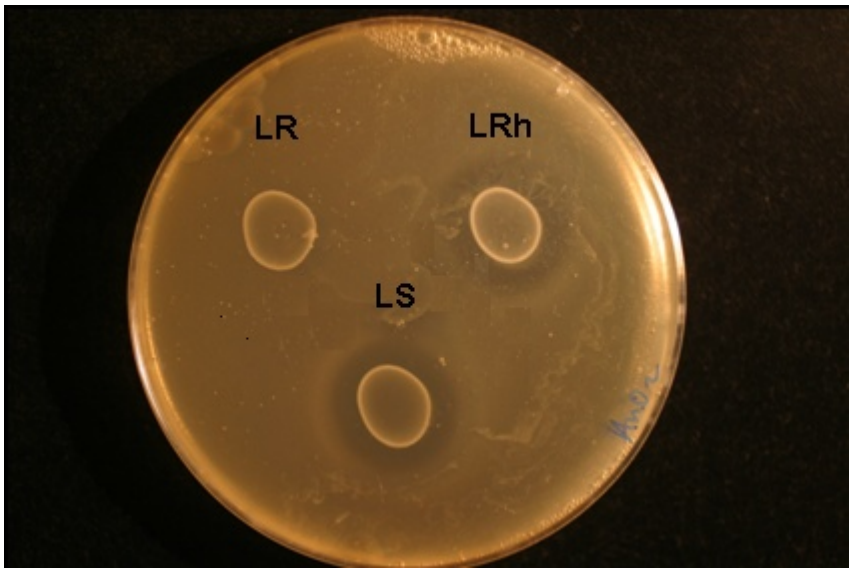


Figure 3.8. Spot-on-the-lawn assay.
Spot-on-the-lawn assay demonstrating zones of inhibition produced by *L. rhamnosus* (LRh) and *L. salivarius* (LS), but not *L. reuteri* (LR).

To determine whether acid production by the probiotics under aerobic and anaerobic conditions was responsible for the inhibition of *S. aureus*, a microprobe was used to measure the pH of the agar in the areas adjacent to the colony growth. The pH was at least two logs lower in all plates incubated anaerobically (Table 3.5).

Table 3.5. Mean pH values around colony growth (n=3).

	Aerobic	Anaerobic
L. reuteri	7.80 ± 0.10	5.46 ± 0.02
L. rhamnosus	7.85 ± 0.04	5.19 ± 0.08
L. salivarius	7.82 ± 0.16	5.25 ± 0.25
S. capitis	7.75 ± 0.03	5.49 ± 0.02

When Universal Indicator was added to *L. salivarius* and *L. rhamnosus* plates, a clear change in the pH was observed in the zones of inhibition between plates incubated both aerobically and anaerobically (an example is illustrated in Figure 3.9a and b). Addition of Universal Indicator to *L. reuteri* and *S. capitis* plates showed no change in the pH of the agar close to colony growth.

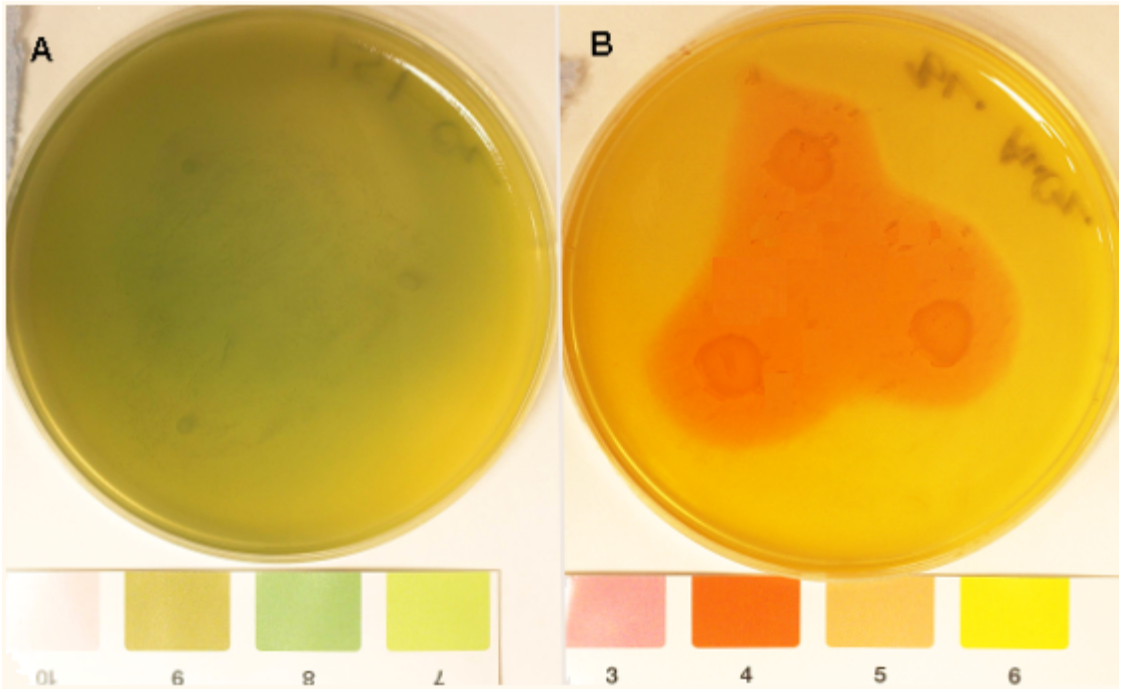


Figure 3.9. Universal Indicator stained Spot-on-the-lawn assay for *L. salivarius*.
A) Aerobically grown *L. salivarius* Spot-on-the-lawn assay. No inhibition of *S. aureus* was observed and the pH remained neutral. **B)** Anaerobically grown *L. salivarius* Spot-on-the-lawn assay. The pH of agar away from *L. salivarius* colonies was approximately pH 5-6 while there was a clear shift to pH 4 from the outer edges of the colonies in the zones of inhibition.

To identify whether acid production or bacteriocin production in *L. rhamnosus* and *L. salivarius* was responsible for the ability to inhibit *S. aureus* growth, plugs from the zones of inhibition were extracted and either left to soak in peptone buffered saline (PBS) (pH 7.2) or 0.8% saline for 1h.

These plugs were then placed on a fresh WCA agar plate streaked with *S. aureus* and incubated overnight at 37°C. PBS treatment resulted in the loss of inhibition against *S. aureus*, while saline treatment had no effect on the ability of *L. rhamnosus* or *L. salivarius* to inhibit *S. aureus* growth (Figure 3.10).

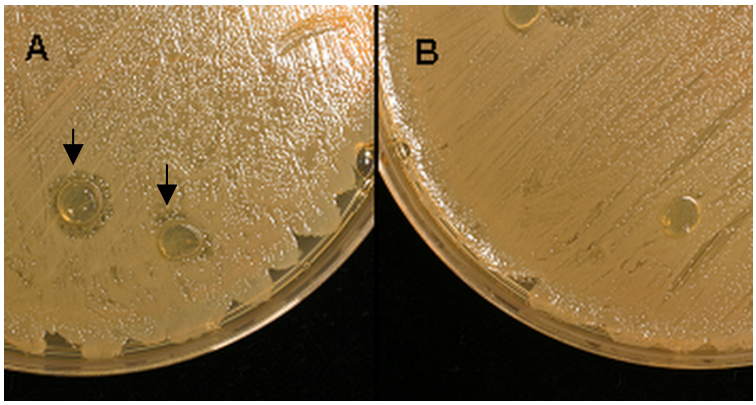


Figure 3.10. Plug assay demonstrating loss of inhibition after soaking agar plugs in PBS. A) Plate with a lawn of *S. aureus* showing zones of inhibition (arrows) around plugs extracted from an *L. salivarius* Spot-on-the-lawn plate, soaked in saline for 1h. B) Plugs extracted from an *L. salivarius* Spot-on-the-lawn plate, soaked in PBS for 1h lost their ability to inhibit *S. aureus* growth.

3.3 Crystal Violet Assays for Biofilm Growth

Biofilms are phenotypically distinct microorganisms that grow within an enclosed matrix adhered to one another or to a surface (Costerton et al., 1995). The ability to form biofilms allows bacteria to attach to inert or biological surfaces efficiently and may therefore be an indication of their ability to colonise epithelia after initial attachment and form biofilm communities (Lebeer et al., 2007). Therefore, it was decided to assess whether the probiotics used in these experiments were able to form biofilms *in vitro*. A crystal violet assay was utilised since bacteria adherent to and forming biofilms on microtitre plates will stain with crystal violet. This is a well recognised method of measuring biofilm-forming capacity *in vitro* (Merritt et al., 2005). The biofilm forming organism, *P. aeruginosa*, was also investigated in this assay as a positive control.

In this assay, bacterial cells grown in microtitre plates for 48h were washed to remove planktonic bacteria. Adherent cells remaining were stained with crystal violet dye, which was solubilised and the optical density measured to give an indication of the relative level of biofilm production compared to the positive control (Section 2.1.4). All three probiotics could produce biofilms. *L. reuteri* was the most efficient biofilm producer (Figure 3.11).

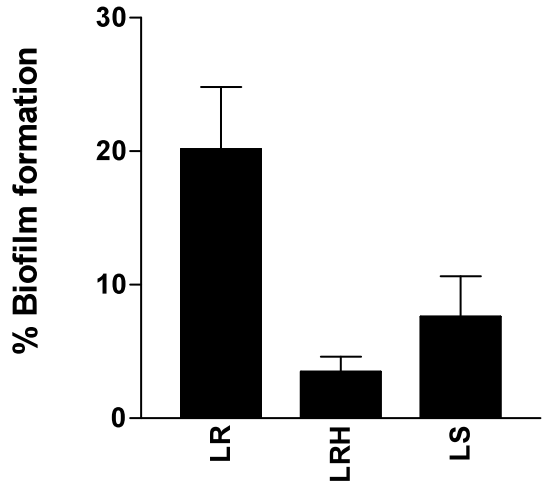


Figure 3.11. Biofilm growth by probiotic bacteria relative to *P. aeruginosa* positive control. *L. reuteri* (LR) had approximately 20.2 ± 4.6%, *L. rhamnosus* (LRH) had 3.5 ± 1.1% and *L. salivarius* (LS) had 7.6 ± 3% biofilm growth relative to *P. aeruginosa* PA01 (100%). Results are shown as the mean ± SEM (n=4).

3.4 Biofilm Inhibition

Some staphylococcal skin infections (e.g. impetigo) involve the organism growing in a biofilm (Akiyama et al., 2003). Since biofilms can be up to 500 times less susceptible to antimicrobial treatment than their planktonic counterparts (Costerton et al., 1995), the use of agents that inhibit biofilm formation would be extremely useful. Some lactobacilli can disrupt biofilm formation by pathogenic bacteria; for example, *L. rhamnosus* GR-1 can inhibit the growth of *Gardnerella vaginalis* biofilms (McMillan et al., 2011). Therefore, the ability of cell-free supernatants (CFS) from probiotic bacteria to inhibit biofilm growth of *S. aureus* was analysed. The experiment was performed as follows: wells of a microtitre plate were pre-treated with CFS from probiotic cultures for 2h prior to the experiment. The CFS was removed and a 1:100 dilution of a *S. aureus* broth placed into wells and the crystal violet assay performed as previously described. Biofilm inhibition assays demonstrated that none of the three test probiotic supernatants could significantly inhibit the biofilm growth of *S. aureus* ($P>0.05$) (Figure 3.12).

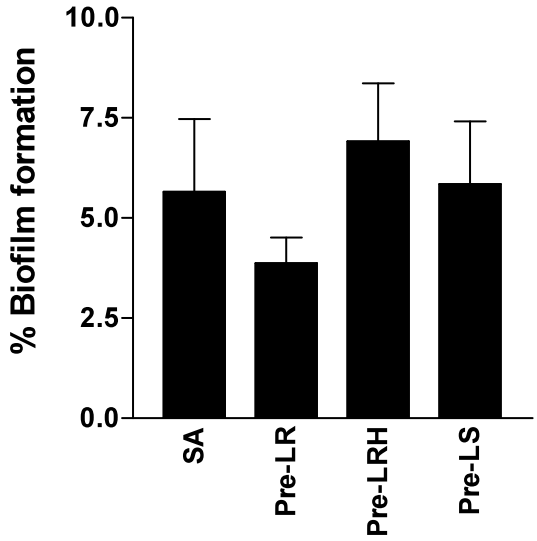


Figure 3.12. Inhibition of *S. aureus* biofilm growth by probiotic supernatants.
None of the probiotic supernatants were able to significantly affect the ability of *S. aureus* to form biofilms (P>0.05). *S. aureus* (untreated) had 5.7 ± 1.8% biofilm growth while *S. aureus* in wells pre-exposed to *L. reuteri* (Pre-LR) had 3.9 ± 0.6%, wells pre-exposed to *L. rhamnosus* (Pre-LRH) had 6.9 ± 4.5% and wells pre-exposed to *L. salivarius* (Pre-LS) had 5.8 ± 1.6% biofilm growth. Results are expressed as the mean ± SEM (n=3).

3.5 Discussion

As a starting point for studying the use of probiotics as a topical therapy for skin, the potential ability of probiotic bacteria to inhibit the growth of a common skin pathogen, *S. aureus* was investigated. Probiotics can inhibit the growth of some pathogenic bacteria as demonstrated in a number of *in vitro* studies (Karska-Wysocki et al., 2010, Hasslöf et al., 2010). For example, *L. acidophilus* and *L. casei* have previously been demonstrated to inhibit the growth of a methicillin resistant *S. aureus* strain *in vitro* (Karska-Wysocki et al., 2010).

The majority of *in vitro* studies demonstrating inhibition of pathogenic organisms by lactobacilli have utilised probiotics cultured in Man-Rogosa-Sharpe medium, a sugar-rich selective medium for lactobacilli. In the current investigation, well-diffusion assays were performed using probiotics grown in two types of medium, Wilkins-Chalgren broth (WCB) and Man-Rogosa-Sharpe (MRS) medium (Section 3.1). WCB is a general purpose medium for the cultivation of anaerobic microorganisms, with relatively low levels of fermentable carbohydrates. Probiotics grown in WCB did not inhibit the growth of *S. aureus*, while probiotics grown in MRS did (Figure 3.6) (Table 3.2).

This suggests that the ability of probiotics to inhibit *S. aureus* growth is dependent upon the growth conditions of the organism. The pH of cell-free supernatants (CFS) from broths of probiotics grown in the two different media were compared and the pH was significantly lower in MRS medium, suggesting that acid production was responsible for the inhibitory effect observed (Table 3.3).

All three of the probiotics used in this study are heterofermentative species; where the by-products of glucose metabolism are lactate (lactic acid), ethanol and CO_2 (Årsköld et al., 2008, Claesson et al., 2006, Morita et al., 2009). The ability of all three probiotics to inhibit *S. aureus* when grown in MRS medium may be due to the higher glucose concentration in the medium compared to WCB medium. MRS medium has approximately 20g/l glucose, while WCB medium has a lower concentration at 1g/l. Therefore, production of organic acids from glucose is lessened in WCB medium, explaining why no inhibition was observed using this medium.

The ability of lactobacilli to inhibit the growth of pathogenic bacteria has previously been related to their ability to produce organic acids in culture (Simark-Mattsson et al., 2009). In this study, neutralisation of the CFS resulted in loss of the inhibitory effect against *S. aureus*, suggesting that the lowered pH of CFS of probiotics grown in MRS was responsible for the inhibitory effect of these organisms (Figure 3.7). This pH dependent inhibition of growth has been noted before in experiments where inhibition of growth of *Salmonella enterica* serovar *typhimurium* by lactobacilli was lost when the pH of CFS was adjusted to pH 6.5 (Fayol-Messaoudi et al., 2005)

Acid production by probiotics is a well-known method of protection against pathogens in the gut (Asahara et al., 2004) and urogenital tract (Boskey et al., 1999). Several mechanisms exist by which acid may inhibit the growth of pathogenic organisms. For example, a study in 2004 found that *Bifidobacterium breve* strain yakult protected mice against infection by shiga-toxin producing *E. coli.* Colonisation of mice with *B. breve* induced a decrease in intestinal pH, prior to infection with *E. coli*, and it was concluded that release of organic acids by *B. breve* inhibited the shiga-like toxin produced by the *E. coli* (Asahara et al., 2004). In addition, Zmantar *et al* (2010) found that reduction in pH correlated with a reduced ability of *S. aureus* to form biofilms. This was thought to affect staphylococcal enzyme production thereby preventing their attachment and ability to form biofilms *in vivo* (Zmantar et al., 2010). Studies have also suggested that lowering the pH of wounds inhibits their colonisation by pathogenic organisms (Schreml et al., 2009).

Finally, a direct effect of acids has also been noted; undissociated lactic acid can permeabilise Gram negative bacteria by penetrating the Gram negative outer membrane and lowering the cytoplasmic pH (Alakomi et al., 2000).

Hydrogen peroxide production is a further mechanism utilised by probiotics to prevent the growth of pathogenic bacteria. For example, H_20_2-producing lactobacilli in the vagina are of importance in preventing bacterial vaginosis. Measurement of H_20_2 production can be performed using colorimetric assays (Rabe and Hillier, 2003). Hydrogen peroxide was not measured in the present study, because MRS medium is not thought to be suitable for the detection of H_20_2 (Rodríguez et al., 1997). Additionally, lactobacilli were incubated anaerobically, thereby inhibiting formation of H_20_2.

Another mechanism by which lactobacilli may inhibit the growth of pathogenic organisms is through the production of other inhibitory substances such as bacteriocins, which are antimicrobial substances active against other bacteria. The producers of such bacteriocins are normally immune to their own bacteriocins (Cotter et al., 2005). These may be produced in a cell-density-dependent manner. Therefore, the production of inhibitory substances was measured using two methods. Firstly, the well-diffusion assay was repeated using probiotics grown in medium containing glass beads to increase the available surface area for growth. No significant difference was found in the inhibitory activity of organisms grown in the presence vs. absence of beads, suggesting that a cell-density-dependent mechanism was not responsible for the inhibitory effect (Table 3.2). Secondly, a Spot-on-the-lawn assay was chosen because it allowed the growth of the probiotics for 48h prior to the addition of *S. aureus*. *L. reuteri* did not produce any zones of inhibition, while both *L. rhamnosus* and *L. salivarius* were able to produce clear inhibition zones anaerobically (Table 3.4, Figure 3.8). Measurement of the pH of the agar using a Universal Indicator demonstrated a clear shift in the pH of the agar in the zones of inhibition (Figure 3.9). In order to determine whether additional inhibitory substances were present other than organic acids, two plugs of agar from the zones of inhibition from *L. rhamnosus* and *L. salivarius* plates were removed and allowed to soak in PBS or 0.8% NaCl for 1h. Plugs soaked in PBS (pH 7.2) lost their ability to inhibit *S. aureus* (Figure 3.10), suggesting that the inhibitory effect was due to acid production. However, this test is not conclusive; the ability of these organisms to produce non-lactic acid inhibitory components such as bacteriocins in addition to acids cannot be ruled out.

Both *L. reuteri* ATCC 55730 (Axelsson et al., 1989) and *L. salivarius* UCC118 (Corr et al., 2007) can produce bacteriocins. Although some studies have suggested that some strains of *L. rhamnosus* are able to produce bacteriocins (Sarika et al., 2010, Todorov and Dicks, 2005), *L. rhamnosus* AC413 has not yet been shown to produce a bacteriocin.

Lactobacillus reuteri produces the bacteriocin reuterin (3-hydroxypropionaldehyde, 3-HPA) (Axelsson et al., 1989) which possesses broad-spectrum antimicrobial action. Reuterin production can be encouraged by incubation of the organism with glycerol (Talarico et al., 1988). Suggested mechanisms by which reuterin acts include i) the aldehyde group may inactivate proteins containing thiol groups or amines, or ii) it may act to block the enzyme ribonucleotide reductase, an enzyme required for DNA synthesis (Schaefer et al., 2010). Production of reuterin by *L. reuteri* ATCC 55730 has been demonstrated using HPLC analysis of cell-free supernatants (Cleusix et al., 2008).

L. salivarius UCC118 produces a broad-spectrum antimicrobial, known as Abp118, which is responsible for its anti-infective activity (Corr et al., 2007, Flynn et al., 2002). Abp118 production is dependent on cell-density-dependent quorum sensing mechanisms. *L. salivarius* possesses a gene cluster which incorporates an induction peptide (AbpIP), a histidine protein kinase (AbpK) and a response regulator (AbpR). Production of Abp118 is due to autoinduction by AbpIP (Flynn et al., 2002). Production of Abp118 by *L. salivarius* UCC118 has been demonstrated by purification using an ammonium sulphate method (Flynn et al., 2002).

It is possible that bacteriocin production in this study was not observed because the growth conditions did not encourage its production. For example, Flynn *et al* (2002) found that Abp118 production was inhibited in the presence of wort because the autoinducer was not produced under these conditions. Addition of AbpIP induced bacteriocin production (Flynn et al., 2002).

3.5.1 Biofilm formation

Biofilms have been defined as phenotypically distinct microorganisms that grow within an enclosed matrix adhered to one another or to a surface (Costerton et al., 1995). Many pathogenic organisms also grow as biofilms, and as they can be several orders of magnitude less susceptible to antibiotic treatments than their planktonic counterparts, their eradication can be particularly problematic (Costerton et al., 2003). Skin diseases associated with *S. aureus* (such as atopic dermatitis and impetigo) involve the organism growing in a biofilm mode of growth (Akiyama et al., 2003).

Figure 3.11 demonstrates that each of the probiotic organisms used in this study could form biofilms. *L. reuteri* appeared to be the most efficient biofilm former at approximately 20.2 ± 4.6% biofilm formation relative to *P. aeruginosa* (100%). *L. rhamnosus* and *L. salivarius* were also able to form biofilms with biofilm formation of 3.5 ± 1.1% and 7.6 ± 3%, respectively. *L. reuteri* ATCC 55730 can form biofilms *in vitro* (Jones and Versalovic, 2009) and has been shown to colonise the stomach, duodenum and ileum when administered orally (Valeur et al., 2004).

L. rhamnosus strains have previously been shown to form biofilms *in vitro* (Lebeer et al., 2007) The *L. rhamnosus* isolate used in this study has been demonstrated to coaggregate well with other oral isolates, a process implicated in the development of biofilms in the oral cavity (Ledder et al., 2008). *L. salivarius* UCC118 can also efficiently form biofilms *in vitro* (Raftis et al., 2010). The organism possesses extensive regions of DNA coding for extracellular polysaccharide production (EPS), a substance that is utilised in biofilm formation (Claesson et al., 2006).

Figure 3.12 demonstrates that the probiotic cell-free supernatants (CFS) could not significantly inhibit *S. aureus* biofilm formation. Whole cell cultures were not used for biofilm inhibition assays because they were shown to form biofilms themselves (Figure 3.11) and could therefore interfere with the results. Probiotics were grown in WCB medium, suggesting this result may be due to the absence of organic acids in this medium (Table 3.3, Figure 3.6).

For example, it has been demonstrated that *L. reuteri* ATCC 55730 grown in MRS medium can inhibit *Streptococcus mutans* biofilm formation *in vitro.* However, pH adjustment of *L. reuteri* broths resulted in a loss of inhibitory activity against *S. mutans* (Söderling et al., 2011). *S. aureus* biofilm formation *in vitro* has previously been shown to be affected by the pH by Zmantar *et al* (2010). However, Zmantar *et al* found that while biofilm production by *S. aureus* was inhibited at low pH values for most strains, some strains appeared to increase biofilm production at lowered pH, suggesting pH may act as a selective pressure for the development of resistant biofilms (Zmantar et al., 2010).

In summary, while it was found that each of the probiotics used in this study was capable of inhibiting *S. aureus* growth *in vitro*, this was shown to be growth medium-dependent. The ability of each of the organisms to adhere to microtitre plates and form biofilms suggested that they would be able to attach to keratinocytes *in vitro*. Therefore, it was decided to assess whether probiotics could inhibit *S. aureus* in a cell culture model of the skin.

The potential of probiotics to protect keratinocytes from the toxic effects of a skin pathogen, *S. aureus*

Chapter 3 investigated whether probiotics could inhibit the growth of *S. aureus* directly. In this chapter, investigations into whether probiotics can protect keratinocytes from the toxic effects of *S. aureus* are described. In the gut, probiotics are purported to protect epithelia from pathogenic bacteria through a number of mechanisms, including inhibition of bacterial colonisation and modulation of host immune responses (Section 1.3). In the present study, normal human epidermal keratinocytes (NHEK) in culture were used as a model system with which to screen probiotic bacteria for their possible protective effects. The aims of this investigation were to demonstrate:

- The effects of *S. aureus* on NHEK viability.
- Whether probiotics are well tolerated by keratinocytes.
- Whether probiotics can protect against *S. aureus* pathogenicity of NHEK.

4.0 *S. aureus* induces cell death in NHEK in a time- and dose-dependent manner.

As a starting point for experiments, the effects of *S. aureus* on keratinocyte viability were investigated. For this study, trypan blue exclusion was used as an endpoint. Trypan blue is a membrane-impermeable dye that cannot penetrate cells with an intact cell membrane, allowing discrimination between viable and dead cells. To begin with, the effects of *S. aureus* on NHEK viability were investigated by incubating keratinocytes for 24h in the presence of the pathogen.

S. aureus exhibited a significant dose-dependent effect upon the viability of keratinocytes (P>0.0001) (Figure 4.1) such that at a concentration of 10^8 CFU *S. aureus* (MOI 2000), only 3.3% of the keratinocytes were viable following 24h incubation. At the lower concentration of 10^5 CFU (MOI 2), 49.4% of keratinocytes remained viable after *S. aureus* exposure.

It has been suggested that non-healing skin wounds are clinically infected (rather than contaminated) if levels of bacteria exceed 10^5 organisms (Bowler et al., 2001). Therefore, 10^6 *S. aureus* was chosen for use in further experiments as a physiologically relevant concentration to use. This concentration induced significant cell death after 16h incubation with *S. aureus* (P=0.03) (Figure 4.2).

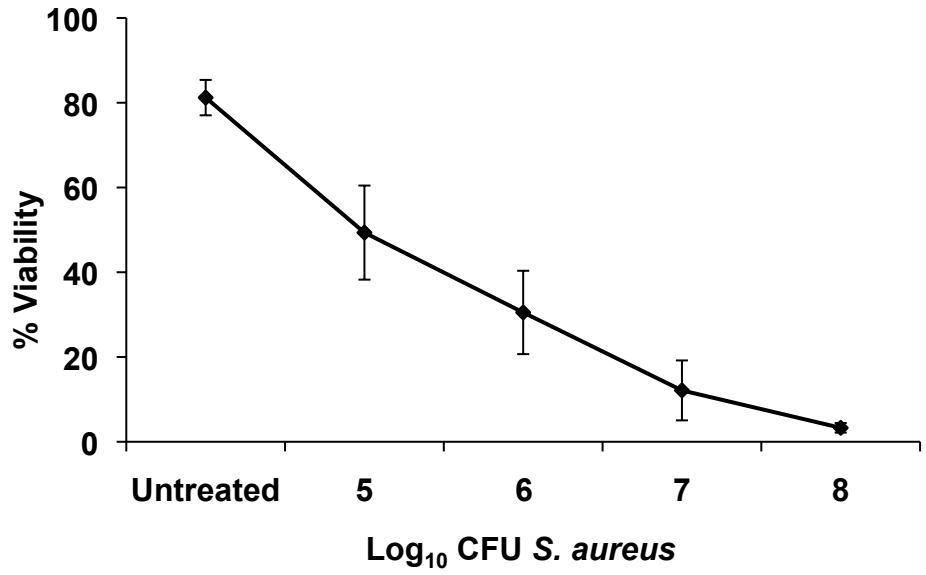


Figure 4.1. *S. aureus* had dose dependent effects on keratinocyte viability.
After 24h incubation, 81.2 ± 4.1% of untreated cells were viable. The viability of NHEK was reduced in a dose-dependent manner with infection of NHEK with different concentrations of *S. aureus*. Exposure to 10^5 CFU/ml *S. aureus* resulted in 49.4 ± 11.1% of cells remaining viable, while 10^6 CFU/ml *S. aureus* reduced the viability of cells to 30.5 ± 9.8%. At 10^7 CFU/ml *S. aureus*, 12.1 ± 1.1% remained viable and this was reduced to 3.3 ± 1.1% in cells exposed to 10^8 CFU/ml *S. aureus.* Linear regression analysis confirmed a linear relationship between concentration and percentage viability (P< 0.001). Results are expressed as mean ± SEM (n=3).

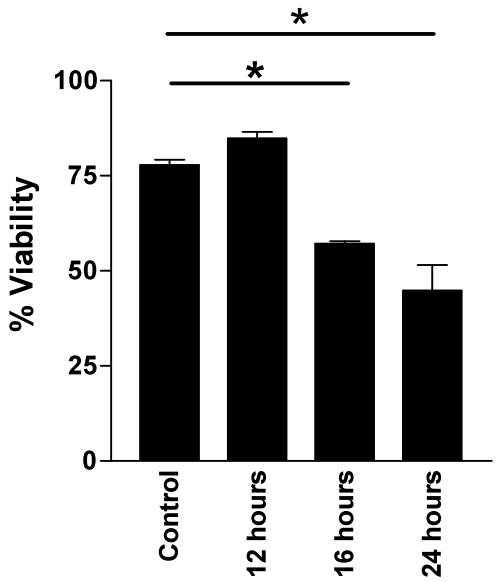
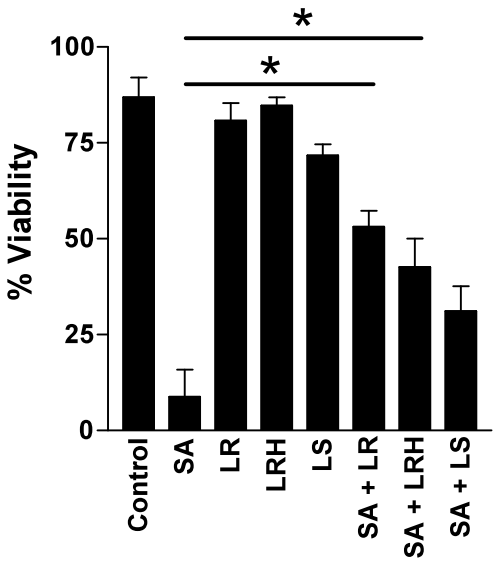


Figure 4.2. *S. aureus* induced keratinocyte cell death between 12h and 16h of infection. Untreated cells had a total viability of 77.8 ± 1.5%. There was no significant difference in the viability of keratinocytes exposed to 10^6 *S. aureus* for 12h (84.8 ± 1.7%). The viability of NHEK exposed to *S. aureus* for 16 and 24h was significantly reduced to 57.1 ± 0.7% (P=0.03*) and 44.8 ± 6.7% (P=0.003*) respectively, compared to untreated controls. Results are expressed as the mean ± SEM (n=3).

4.1 *L. reuteri* protects NHEK from *S. aureus*-induced cell death

Since no reports exist in the literature as to whether probiotics are toxic to keratinocytes, an experiment was performed to assess the viability of keratinocytes in the presence of *L. reuteri* ATCC 55730, *L. rhamnosus* AC413 and *L. salivarius* UCC118, all at 10^8 CFU. As observed in Section 4.0, *S. aureus* (10^8 CFU) induced significant cell death in keratinocytes (P<0.0001). By contrast, exposure of NHEK to the lactobacilli did not result in any significant cell death, compared to untreated cells (P>0.05, Figure 4.3). The ability of the three strains of lactobacilli to protect keratinocytes from the effects of *S. aureus* was next investigated. NHEK were exposed to a combination of *S. aureus* and probiotic simultaneously. Figure 4.3 demonstrates that *S. aureus* (10^8 CFU) reduced the viability of NHEK to 8.8 ± 7.1% but that in contrast, 53.1 ± 4.2% of NHEK remained viable after exposure to *S. aureus* and *L. reuteri* (10^8 CFU) simultaneously (P=0.0001). Likewise, 42.7 ± 7.4% of NHEK were viable after exposure to *S. aureus* and *L. rhamnosus* (10^8 CFU) (P=0.00012). However, NHEK exposed to *S. aureus* and *L. salivarius* had a similar viability to NHEK infected with *S. aureus* alone (P>0.05) (Figure 4.3). Since the protective effect afforded by *L. reuteri* was the greatest, this organism was selected for further experiments.

(A)



(B)

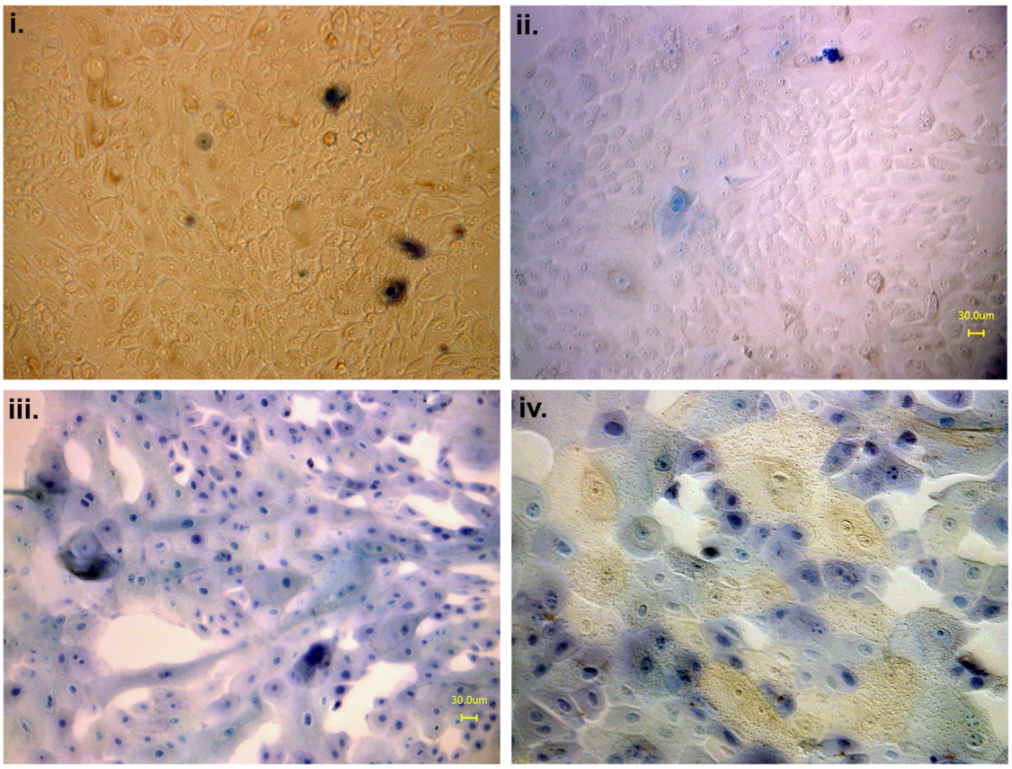


Figure 4.3. Lactobacilli protect keratinocytes from the cytotoxic effects of *S. aureus*.
(A) Percentage viability for uninfected NHEK (86.9 ± 5.1%) and NHEK infected with 10^8 CFU *S. aureus* (SA) (8.8 ± 7.1%), *L. reuteri* (LR), (80.8 ± 4.5%), *L. rhamnosus* (LRH) (84.8 ± 2.1%), *L. salivarius* (LS) (71.7 ± 2.9%), *S. aureus* & *L. reuteri* (SA+LS) (53.1 ± 4.2%), *S. aureus* & *L. rhamnosus* (SA+LRH) (42.7 ± 7.4%), and *S. aureus* & *L. salivarius* (SA+LS) (31.1 ± 6.5%) (n=3). **(B)** Representative images of infected cells stained with trypan blue. i) No treatment, ii) exposed to 10^8CFU/ml *L. reuteri*, iii) infected with 10^8 CFU/ml *S. aureus* or iv) infected with 10^8 CFU/ml *S. aureus* and 10^8 CFU/ml *L. reuteri* simultaneously. Images were optimised for contrast and exposure using Adobe Photoshop 3.

4.2 *L. reuteri* dose response

In order to determine whether the protective effect observed with *L. reuteri* was dose dependent, the protective effect was investigated using three different amounts of *L. reuteri* (10^6, 10^7, 10^8 CFU) with 10^6 *S. aureus*. However, there was no significant difference in the level of protection afforded to keratinocytes against *S. aureus* infection with different quantities of *L. reuteri* ($P>0.05$) (Figure 4.4).

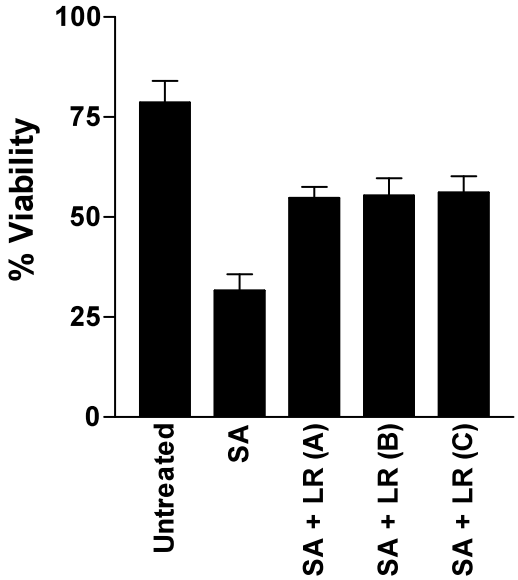


Figure 4.4. *L. reuteri* protects keratinocytes at all quantities used.
Approximately 31.6 ± 4.1% of cells remained viable after infection with 10^6 *S. aureus* for 24h. In contrast, 54.8 ± 2.7% of NHEK were viable after exposure to *S. aureus* and 10^6 *L. reuteri* (A), 55.4 ± 4.3% of cells were viable after exposure to *S. aureus* and 10^7 *L. reuteri (B)* and 56.2 ± 4.1% of cells were viable after exposure to *S. aureus* and 10^8 *L. reuteri* (C). Results are expressed as the mean ± SEM (n=3).

4.3 Comparison of protective effect of *L. reuteri* between undifferentiated and differentiating NHEK

Recent evidence suggests that keratinocytes mount a response to pathogens that is partially dependent on the differentiation status of the cells (Lai et al., 2010). Undifferentiated NHEK (grown in low calcium conditions) are more representative of the keratinocytes present in the *stratum basale* of the epidermis (Section 1.1.1). Therefore, to compare possible differences in response, a model of differentiating keratinocytes was set up. This involved growing NHEK in medium containing 1.8mM $CaCl_2$ for 24h prior to experimentation and is a well characterised method of producing differentiating keratinocytes (Chadebech et al., 2003, Lai et al., 2010).

Figure 4.5 shows that there was no significant difference in the protection afforded by *L. reuteri* in undifferentiated (i.e. grown in medium with 0.06mM calcium) and cells allowed to differentiate by growing in medium containing 1.8mM calcium for 24h (P>0.05). In further experiments, differentiated NHEK were utilised.

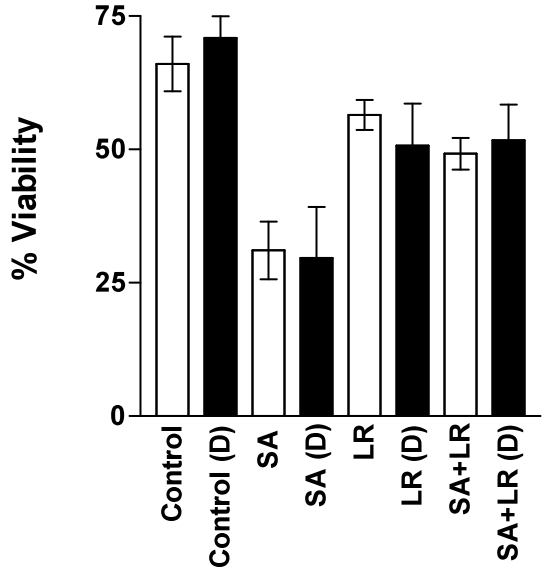


Figure 4.5. Differentiation of cells did not affect the ability of *L. reuteri* to protect NHEK against *S. aureus.*
The viability of control undifferentiated cells was similar to cells allowed to differentiate (D) for 24h (66 ± 0.8% and 70.9 ± 2.8% respectively). The viability of S. *aureus* (SA) (10^6 CFU) treated cells was similar in both undifferentiated and differentiating cultures (31.1 ± 3.3% and 29.6 ± 9.9% respectively, P>0.05). The viability of *L. reuteri* (LR) treated cells was similar in both undifferentiated and differentiating cultures (56.5 ± 3.2% and 50.7 ± 10.6% respectively, P>0.05). The viability of *S. aureus* and *L. reuteri* (SA+LR) treated cells was similar in both undifferentiated and differentiating cultures (49.2 ± 1.4% and 51.7 ± 7.5% respectively, P>0.05). Results are expressed as the mean ± SEM (n=3).

4.4 The protective effect of *L. reuteri* is time dependent

The timing of *L. reuteri* application relative to *S. aureus* infection was next investigated. NHEK were either pre-exposed to *L. reuteri* or post-exposed 1, 2 or 4h following infection with *S. aureus*. The graph in Figure 4.6a shows that there was no significant difference between the viability of NHEK pre-exposed to *L. reuteri,* and co-infected NHEK. However, the viability of keratinocytes exposed to *L. reuteri* 1, 2 or 4h after *S. aureus* infection was similar to cells exposed to *S. aureus*. Therefore, post-exposure resulted in the loss of the protective effect of *L. reuteri* (P>0.05) (Figure 4.6b).

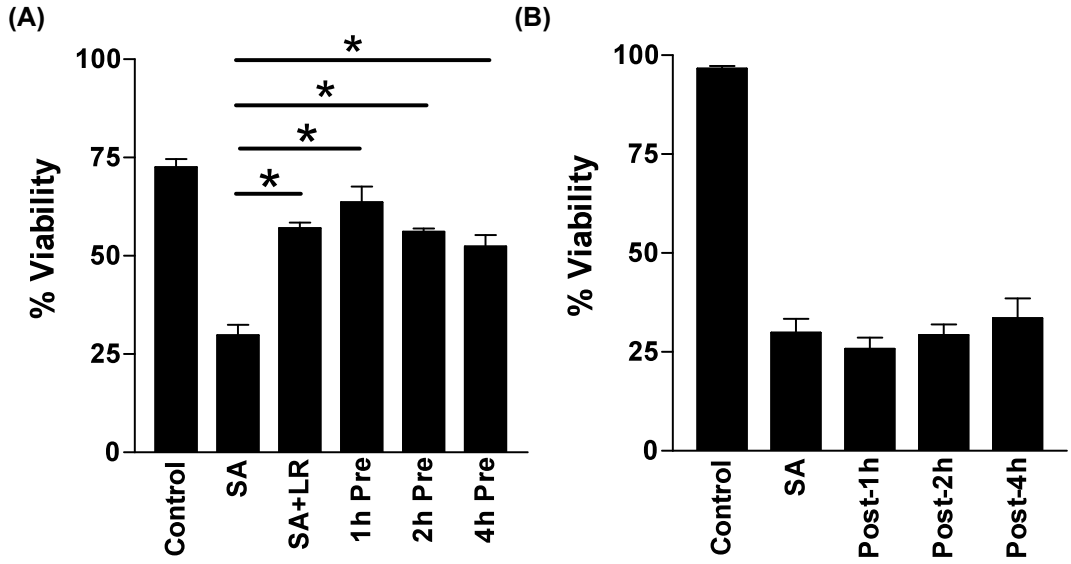


Figure 4.6. *L. reuteri* protects keratinocytes only if added prior to infection with *S. aureus*. **(A)** Percentage viability was significantly higher in cells that were co-infected (SA+LR) (57.1 ± 2%, P=0.002*) and pre-exposed for 1h (1h Pre) (63.6 ± 5.6%, P=0.0003*), 2h (2h Pre) (56.1 ± 1.2%, P=0.002*) and 4h (4h Pre) with *L. reuteri* (52.4 ± 4.1%, P=0.007*) compared to *S. aureus* (SA) infected cells (29.8 ± 3.8%). **(B)** There was no significant difference between the viability of cells treated with *L. reuteri* 1h (Post-1h) (25.8 ± 0.6%), 2h (Post-2h) (29.3 ± 2.6%), or 4h (Post-4h) (33.57 ± 4.9%) after infection had begun and cells that had been infected with *S. aureus* (SA) alone (29.9 ± 3.4%) (P>0.05). Results are expressed as the mean ± SEM (n=3).

4.5 Lysates of, but not heat-killed *L. reuteri* protect NHEK

Since some probiotic lysates can be beneficial when applied topically to the skin (Section 1.4.2), an assessment of whether lysates of *L. reuteri* and heat-killed preparations of *L. reuteri* could protect NHEK was performed. This would elucidate whether a heat-labile component, or viable bacteria were required for the protective effect to occur. Figure 4.7 illustrates that although heat-killed *L. reuteri* did not protect NHEK from *S. aureus* (P>0.05), an *L. reuteri* lysate afforded a significant protective effect (P=0.01).

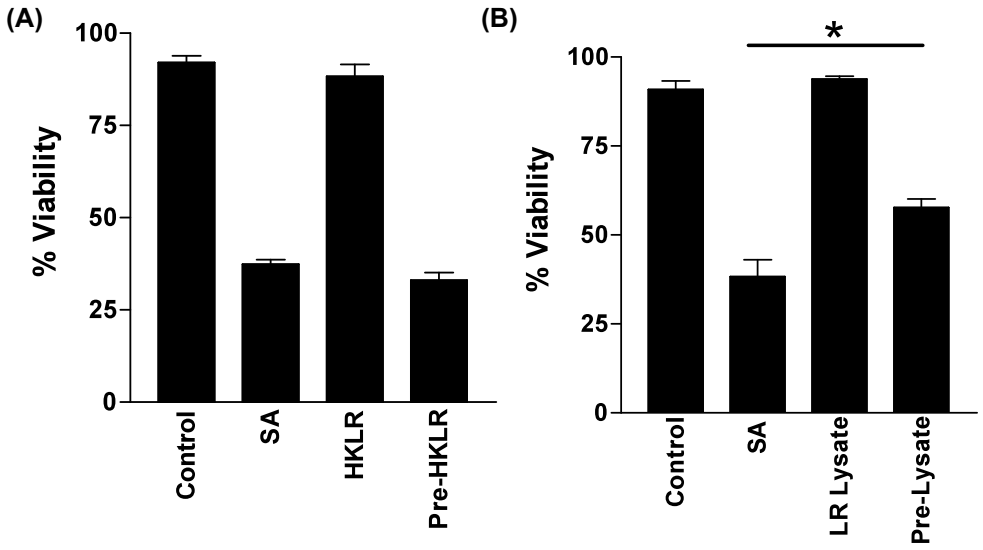


Figure 4.7. Lysates of *L. reuteri* protect NHEK, but not heat-killed *L. reuteri*.
(A) NHEK exposed to 10^6 *S. aureus* (SA) had reduced viability (37.4 ± 1.3 %) compared to controls (92 ± 1.9 %) while keratinocytes exposed to heat-killed *L. reuteri* (HKLR) were unaffected compared to controls (88.3 ± 3.3 %, P>0.05). NHEK pre-exposed to heat-killed *L. reuteri* (Pre-HKLR) had similar viabilities to *S. aureus* exposed keratinocytes (33 ± 2.1 %). **(B)**. Cells exposed to *S. aureus* (SA) alone had significantly lower viability (38.3 ± 4.7 %) than cells pre-exposed to a lysate of *L. reuteri* (Pre-Lysate) (57.7 ± 2.4 %, P=0.01*). Results are expressed as mean ± SEM (n=3).

4.6 *L. reuteri* does not inhibit the growth of *S. aureus*

Although the investigation performed in Section 3.1 demonstrated that *L. reuteri* did not inhibit *S. aureus* growth unless the lactobacilli had been grown in medium containing high levels of glucose (Figure 3.6), it was considered possible that the probiotics could nevertheless be having a direct antimicrobial effect on *S. aureus*. Therefore, competition assays were performed in which the growth of both strains was measured in both axenic and co-cultures grown in medium.

There was no significant reduction in either *L. reuteri* or *S. aureus* viability during co-culture for 48h compared to axenic cultures (P>0.05). A Live/Dead analysis of the co-culture at 48h also demonstrated no significant *S. aureus* killing (Figure 4.8).

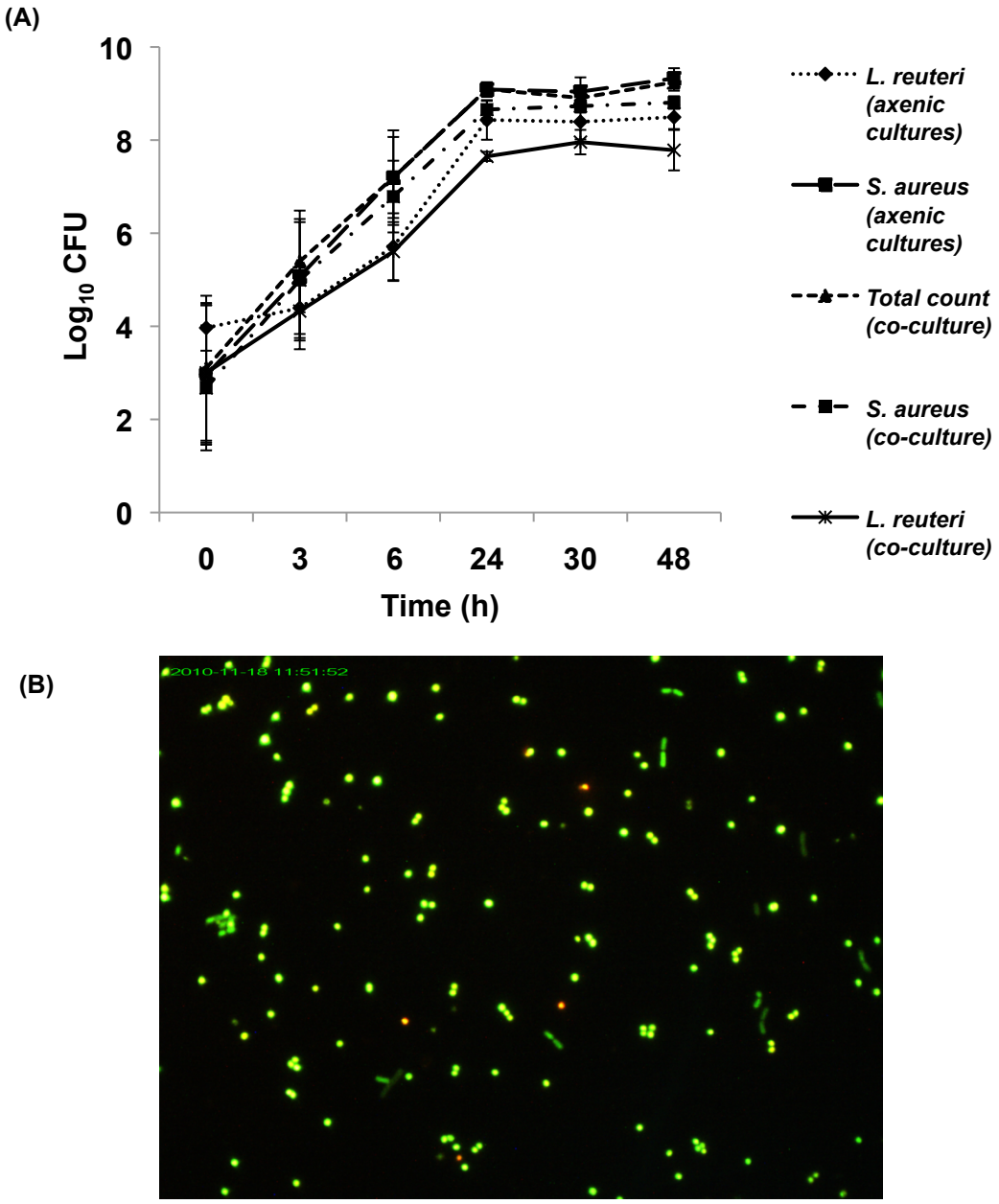


Figure 4.8. *L. reuteri* does not inhibit the growth of *S. aureus* in co-culture.
(A) Competition assay revealing no significant difference between groups over time (P=0.146). Results are expressed as the mean ± SEM (n=3). **(B)** Live/Dead stain of 48h co-culture. Green cells are viable while red cells are dead.

Since organic acids produced by lactobacilli can inhibit the growth of *S. aureus* (Section 1.3), the production of organic acids in keratinocyte medium was investigated by measuring the pH of cell cultures in wells that were i) untreated, ii) exposed to *S. aureus*, iii) *L. reuteri*, or iv) both together. No significant difference was found in the pH of NHEK culture exposed to *L. reuteri* or exposed to *S. aureus* and *L. reuteri* concomitantly compared to uninfected cell cultures (P>0.05) (Figure 4.9).

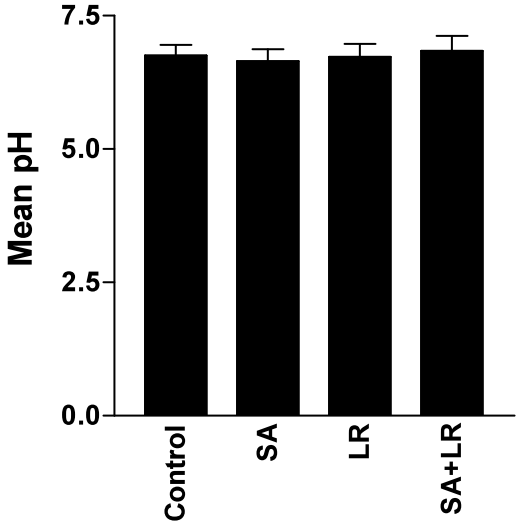


Figure 4.9. *L. reuteri* did not change the pH of cell culture supernatants.
Mean pH of cell cultures that were untreated (6.8 ± 0.2), infected with 10^6CFU/ml *S. aureus* (SA) (6.7 ± 0.2), 10^8CFU/ml *L. reuteri* (LR) (6.8 ± 0.3) and cultures infected with both (SA+LR) (6.9 ± 0.3). There was no significant difference between groups (P>0.05). Results are expressed as the mean ± SEM (n=3).

Since keratinocytes produce their own antimicrobial peptides, (Section 1.1.5.4) an experiment was performed to assess whether *S. aureus* growth was inhibited by NHEK *per se*. *S. aureus* grown in the absence of cells had a viable count of 8.0 (log_{10} CFU). *S. aureus* grown in the presence of keratinocytes had a viable count of 8.6 ± 0.2 (log_{10} CFU). *S. aureus* co-incubated with *L. reuteri* in the absence of cells had a viable count of 8.4 ± 0.4 (log_{10} CFU) while *S. aureus* co-incubated with *L. reuteri* in the presence of cells had a viable count of 8.0 ± 0.2 (log_{10} CFU). The presence of keratinocytes, *L. reuteri* or both did not have any significant effect on the number of viable staphylococci in the experiment (P>0.05, n=3).

4.7 Conclusions

This work explored whether an enteric probiotic could potentially be used to protect keratinocytes from the pathogenic effects of *S. aureus* using a keratinocyte cell culture model. Preliminary experiments to determine the effect of *S. aureus* on keratinocyte viability demonstrated a dose- and time-dependent reduction in the number of viable keratinocytes in the presence of the pathogen (Figure 4.1 and Figure 4.2). This is in agreement with other reports of the toxic effects of *S. aureus* on keratinocytes. However, the exact level of toxicity varies between studies. For example, Nuzzo and colleagues (2000) found that 24h exposure of NHEK to 10^8 *S. aureus* resulted in a reduced viability of 58% compared to just 3.3% in the present study (Nuzzo et al., 2000). This variation in the levels of *S. aureus* induced cell death could be explained by strain differences or potential differences in handling the keratinocytes. NHEK can grow through multiple passages and up to 15-20 population doublings before senescence occurs. Cell cultures at older passages may be more susceptible to microbial infection and change their genotypic and phenotypic features as they reach senescence. In the present study, only NHEK in passages 3-5 were utilised, making this model very defined.

In contrast to *S. aureus*, none of the probiotic strains significantly affected the viability of keratinocytes during 24h incubation, suggesting that probiotics are well tolerated by keratinocytes. Incubation of cells with both *S. aureus* and *L. reuteri* resulted in a protective effect as observed by a significant increase in the number of viable NHEK in comparison to keratinocytes infected with *S. aureus* alone (Figure 4.3). This protective effect was species-specific, as demonstrated by *L. rhamnosus*, which afforded less protection than *L. reuteri*. By contrast, *L. salivarius* was unable to protect NHEK from *S. aureus* induced cell death (Figure 4.3). Furthermore, the differentiation status of the keratinocyte made no difference to the protective effect suggesting that protection was a function only of the probiotic and probably not due to e.g. an immune response mounted by the keratinocytes.

The species/strain-specific effects of probiotics have been well documented and are thought to be due to the variation in probiotic surface structures. A study in 2005 showed both *in vitro* and *in vivo* that probiotic lipoteichoic acid (LTA) could modulate host immune responses through modification of its teichoic acids.

A Dlt$^-$ mutant of *L. plantarum* with a defect in the D-Ala content of its LTA was shown to be more anti-inflammatory than its wild type counterpart (Grangette et al., 2005). Another study in 2008 suggested that the distribution of polysaccharide molecules on *L. rhamnosus* GG was different to a mutant with impaired adherence to gut epithelia and impaired exopolysaccharide (EPS) production and subsequent biofilm production (Francius et al., 2008). However, whether or how surface structures are involved in the protective mechanism observed in this study is yet to be determined.

The timing of application of *L. reuteri* affected the degree of protection conferred by the probiotic to protect NHEK against *S. aureus* induced cell death. The graphs in Figure 4.6 demonstrate that NHEK pre- or co-exposed to *L. reuteri* were protected from *S. aureus* induced cell death. In contrast, post-exposed NHEK had similar viabilities to cells infected with *S. aureus* alone. Taken together, these results suggest that *L. reuteri* may not be used in the treatment of existing *S. aureus* infections, but could be a used as a prophylactic measure (e.g. in barrier creams and soaps) to prevent *S. aureus* colonisation and infection from occurring.

The protective effect of *L. reuteri* did not require viable bacteria because a lysate also afforded protection of NHEK from *S. aureus*, albeit at a reduced level (Figure 4.7a). However, heat-killing the probiotic resulted in loss of the protective mechanism (Figure 4.7b). These observations suggest that a heat-labile molecule is responsible for the protective effect.

The protective effect observed for *L. reuteri* is probably not due to the direct inhibition of *S. aureus* growth by probiotic bacteria. Three lines of evidence support this view. Firstly, Section 3.1 (Figure 3.6) demonstrated that *L. reuteri* inhibited the growth of *S. aureus* if the lactobacilli had been grown in medium containing levels of glucose that could encourage the production of organic acids. Secondly, competition assays demonstrated that *L. reuteri* had no effect upon *S. aureus* growth, and Live/Dead stains of 48h cultures confirmed no significant reduction in bacterial viability in cultures (Figure 4.8). Thirdly, the pH of cell cultures exposed to *S. aureus*, *L. reuteri*, or both simultaneously was no different from the pH of media containing no bacteria (Figure 4.9). This suggests that the production of organic acids by *L. reuteri* does not occur in NHEK medium.

However, as NHEK medium is buffered, this does not rule out the possibility of acid production by *L. reuteri*, but suggests that it is not produced in sufficient amounts to change the pH of the culture medium. Maudsdotter *et al* (2011) utilised a BioProfile FLEX chemical cell culture analyser to measure lactic acid production under cell culture conditions, suggesting this could be a useful method for determining if *L. reuteri* can produce organic acids under cell culture conditions.

Since NHEK are known to produce antimicrobial peptides (AMPs) (Section 1.1.5.4) experiments were also performed to determine whether *S. aureus* viability changed in the presence vs. absence of NHEK and the presence vs. absence of *L. reuteri*. The presence of NHEK, *L. reuteri* or both concomitantly resulted in no significant change in the viability of *S. aureus* after 24h exposure (Section 4.6).

This is not the first example of a probiotic organism protecting epithelial cells from the harmful effects of a pathogenic organism. Enterocytes may also be protected from *Clostridium difficile* pathogenicity by *L. delbruekii* cell-free supernatants (Banerjee et al., 2009). A recent study found that *L. reuteri, L. rhamnosus* and *L. oris* protect pharyngeal epithelial cells from *Streptococcus pyogenes* induced cell death. Lactic acid produced by lactobacilli disabled *S. pyogenes* lipoteichoic acid thereby protecting pharyngeal epithelial cells (Maudsdotter et al., 2011). However, to my knowledge there are no published reports of a protective effect for probiotic bacteria on epidermal keratinocytes.

Taken together, these data suggest that the protective effect of *L. reuteri* does not involve loss of *S. aureus* viability or significant inhibition of *S. aureus* growth by factors produced either by the probiotic or the keratinocytes. Since adhesion is often considered the first step in pathogenesis by *S. aureus*, inhibition of adhesion could be a possible mechanism. For that reason, the ability of *L. reuteri* and *S. aureus* to adhere to keratinocytes was investigated, and this is described in Chapter 5.

Mechanism of protection of keratinocytes by *L. reuteri*

The data in Chapter 4 demonstrated that *L. reuteri* could protect human keratinocytes from the toxic effects of *S. aureus*. In the present chapter, the potential mechanisms underlying this effect were investigated. The ability of *S. aureus* to induce cell death in keratinocytes is a complex process. In general, it is thought that adhesion to keratinocytes prior to invasion is a critical step that initiates a cascade of events eventually leading to cell death (Kintarak et al., 2004a). Since the protective mechanism by which *L. reuteri* prevents pathogen induced cell death does not appear to be due to inhibition of *S. aureus* growth or direct killing of the bacteria, inhibition of *S. aureus* adhesion is a putative mechanism of protection. Therefore, the aims of this investigation were:

- To determine whether *S. aureus* and *L. reuteri* adhere to normal human epidermal keratinocytes (NHEK).
- To determine whether *L. reuteri* inhibits *S. aureus* adhesion by competition for, exclusion of or displacement from binding sites.
- To investigate the mechanisms underlying any observed effects of *L. reuteri* on *S. aureus* adhesion.

5.0 Adhesion of bacteria to NHEK

Initial investigations were performed to evaluate whether *S. aureus* and *L. reuteri* adhere to NHEK. Adhesion assays were performed as described in Section 2.3. The data presented in Figure 5.1a demonstrate that both organisms adhered to NHEK after 1h exposure to the cells. Since heat-killed *L. reuteri* did not protect NHEK from the pathogen (Section 4.5), the ability of heat-killed *L. reuteri* to adhere to NHEK was investigated. Although heat-killed *L. reuteri* were adherent to some extent, the numbers of adherent bacteria were significantly less compared to viable *L. reuteri* (P=0.04) (Figure 5.1b). Importantly, *L. salivarius*, which did not protect NHEK from *S. aureus* induced cell death (Section 4.1), also failed to adhere to NHEK as efficiently as *L. reuteri* (Figure 5.1c).

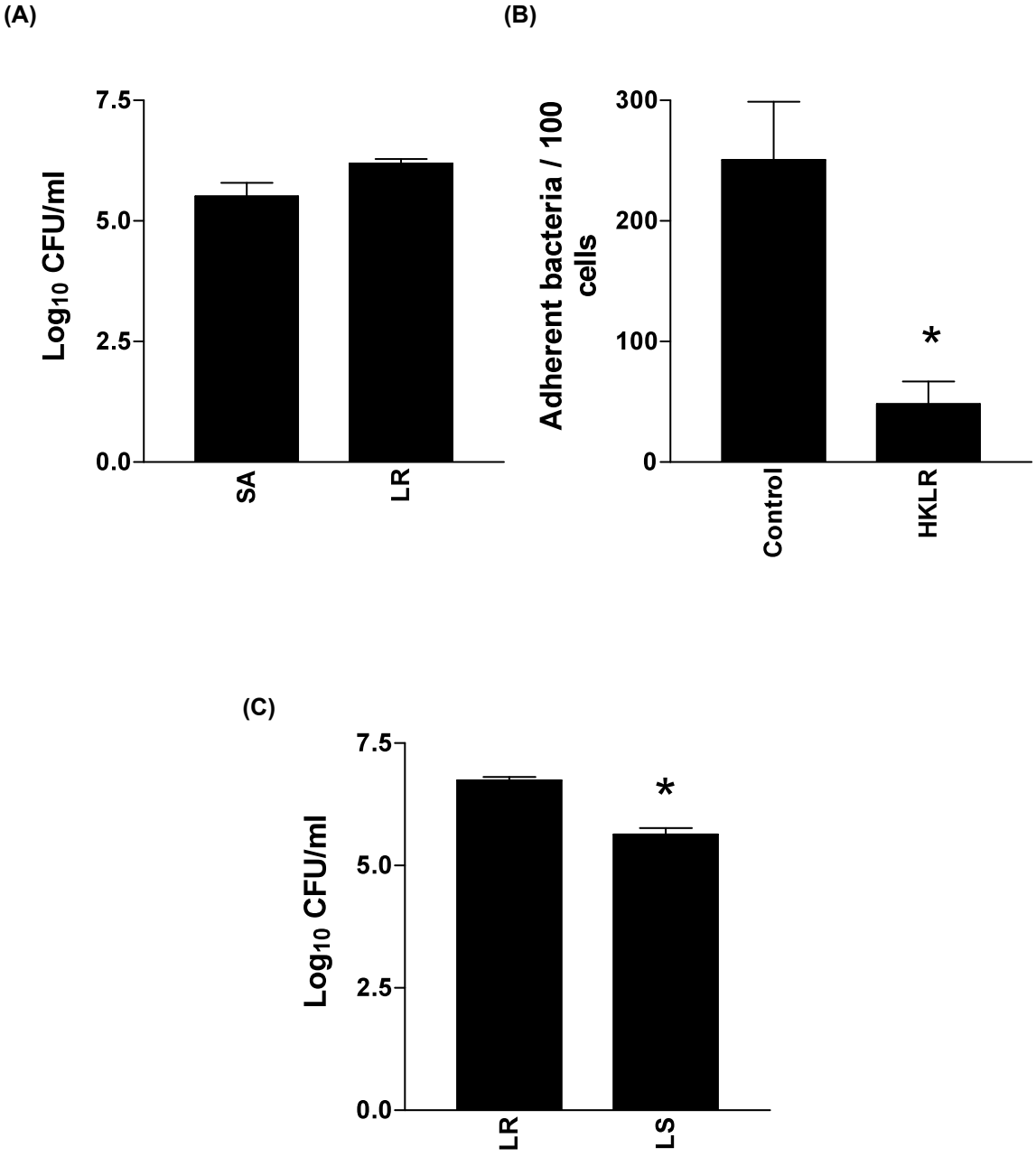


Figure 5.1. Ability of bacteria to adhere to NHEK.
(A) *S. aureus* (SA) (10^6), when applied to NHEK for 1h before washing, adhered to cells at approximately 5.5 ± 0.3 $\log_{10}$ CFU/ml, while *L. reuteri* (LR) (10^8) adhered at approximately 6.2 ± 0.1 $\log_{10}$ CFU/ml. **(B)** Untreated *L. reuteri* (Control) adhered to NHEK at approximately 251 ± 48 bacteria/100 cells, while heat-killed *L. reuteri* (HKLR) adhered significantly less well (P=0.04*) at approximately 48 ± 19 bacteria/100 cells. **(C)** *L. reuteri* (LR) adhered significantly better (6.7 ± 0.1 $\log_{10}$ CFU/ml) than *L. salivarius* (LS) (5.6 ± 0.1 $\log_{10}$ CFU/ml, P=0.005). Results are expressed as the mean ± SEM (n=3).

5.1 *L. reuteri* inhibits *S. aureus* adhesion and invasion of NHEK

In the gut, probiotics are believed to protect the gut epithelium through the inhibition of pathogenic adhesion to gut epithelial cells (Section 1.3). For this reason, it was decided to investigate whether *L. reuteri* inhibits *S. aureus* adhesion to keratinocytes, and whether this is dependent upon the timing of addition of *L. reuteri* to cells; i.e. whether the probiotic can exclude, displace or compete for binding sites on NHEK with the pathogen. Figure 5.2a and b demonstrate that if cells were pre-exposed to *L. reuteri* (exclusion) or co-exposed to *L. reuteri* alongside *S. aureus* infection (competition), the binding of *S. aureus* to cells was inhibited (P=0.03 and P=0.008 respectively). However, as demonstrated in Figure 5.2c, if *L. reuteri* was applied to cells following *S. aureus* infection, it was not able to prevent *S. aureus* from adhering (displacement) (P>0.05).

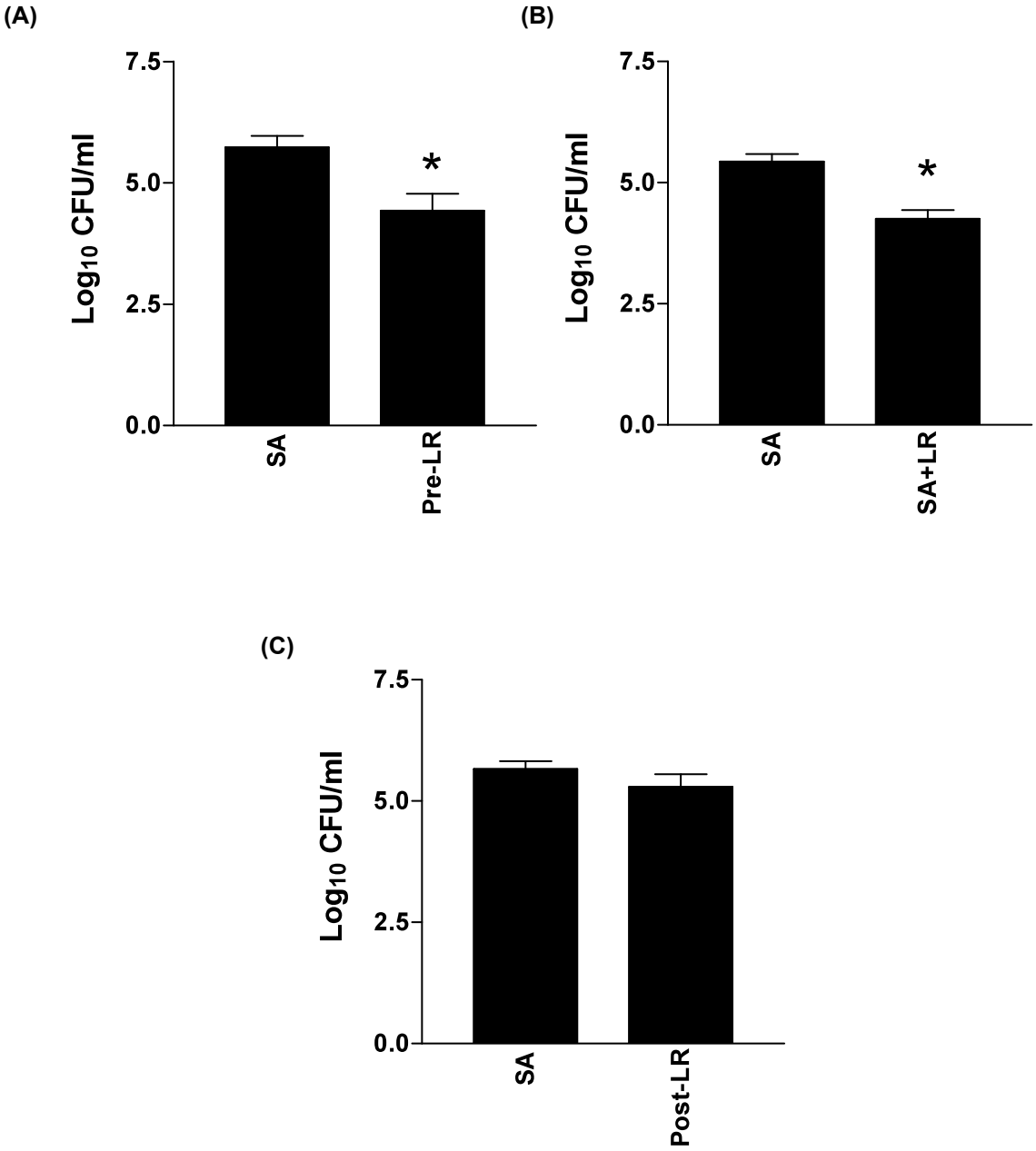


Figure 5.2. *L. reuteri* can inhibit *S. aureus* from adhering to NHEK.
(A) Exclusion. Cells pre-exposed to *L. reuteri* before *S. aureus* (Pre-LR) infection had significantly less staphylococci adhered to them (4.4 ± 0.4 $\log_{10}$ CFU/ml) compared to cells infected with *S. aureus* (SA) alone (5.7 ± 0.2 $\log_{10}$ CFU/ml) (P=0.03*). **(B) Competition**. Cells co-infected with *L. reuteri* (SA+LR) had significantly less staphylococci adhered to them (4.4 ± 0.4 $\log_{10}$ CFU/ml) compared to cells infected with *S. aureus* (SA) alone (5.6 ± 0.1 $\log_{10}$ CFU/ml) (P=0.008*). **(C) Displacement.** There was no significant difference in the number of adherent staphylococci on cells exposed to *L. reuteri* after *S. aureus* infection had begun (Post-LR) (5.3 ± 0.5 $\log_{10}$ CFU / ml), compared to cells infected with *S. aureus* (SA) alone (5.7 ± 0.3 $\log_{10}$ CFU/ml, P=0.47). Results are expressed as the mean ± SEM (n=3).

Since the ability of *S. aureus* to invade cells is dependent on adhesion to NHEK, the ability of *L. reuteri* to inhibit the invasion of NHEK by *S. aureus* was next investigated. Gentamicin protection assays (Section 2.3.1) demonstrated that *L. reuteri* significantly inhibited invasion of NHEK by *S. aureus* (P=0.009, Figure 5.3).

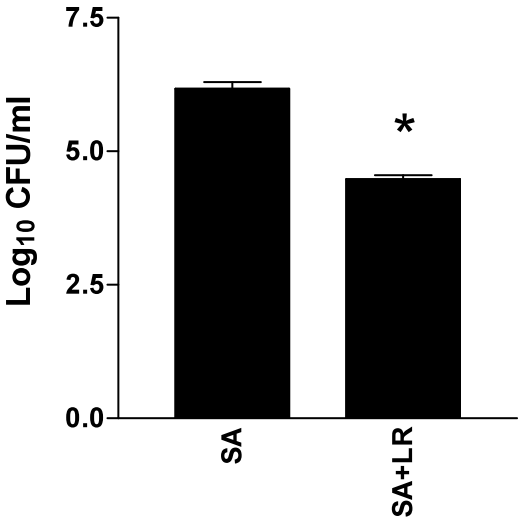


Figure 5.3. *L. reuteri* inhibits *S. aureus* invasion of NHEK.
Cells co-exposed to *L. reuteri* at the same time as *S. aureus* infection (SA+LR) had significantly fewer internalised staphylococci (4.5 ± 0.1 log CFU/ml) than cells infected with *S. aureus* alone (SA) (6.1 ± 0.1 log CFU/ml). Results are expressed as the mean ± SEM (n=3).

5.1.1 Lysates of *L. reuteri* inhibit *S. aureus* adhesion to NHEK

In Section 4.5, the ability of *L. reuteri* lysates to protect NHEK was shown. Therefore, in order to further confirm a link between adhesion and protective function, the ability of the lysate to inhibit *S. aureus* adhesion to NHEK was next investigated. Lysates of *L. reuteri* significantly reduced the number of staphylococci bound to NHEK (P=0.032), although not to the same extent as viable *L. reuteri* (P=0.0002, Figure 5.4a). By contrast, heat-killed *L. reuteri* did not significantly inhibit *S. aureus* adhesion to NHEK (Figure 5.4b).

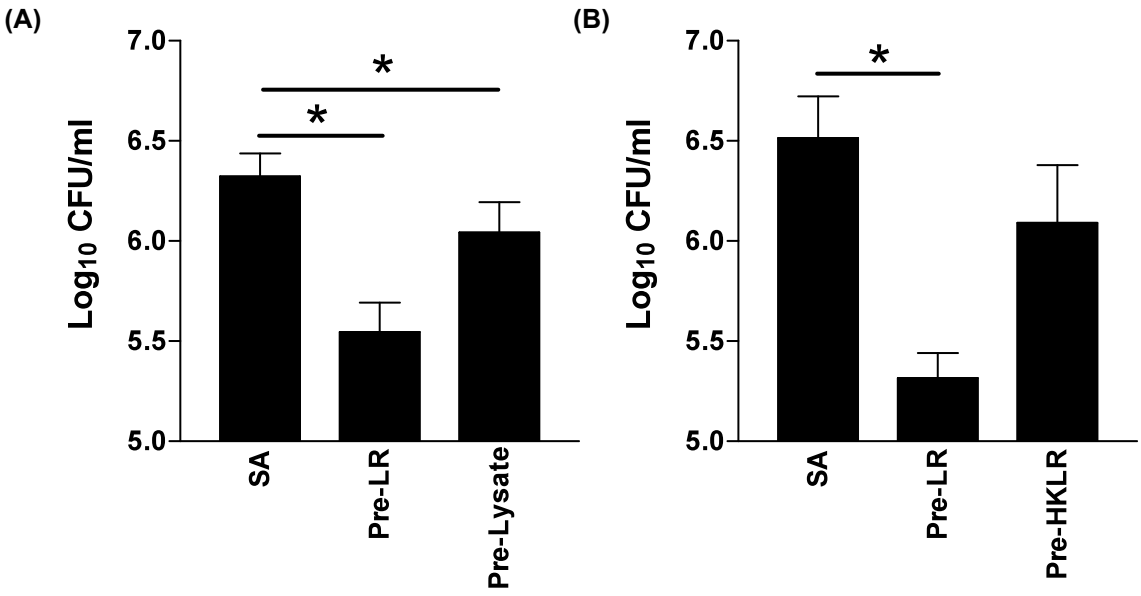


Figure 5.4. Lysates of *L. reuteri* could inhibit *S. aureus* adhesion to NHEK, but heat-killed *L. reuteri* could not.
(A) The number of staphylococci adherent to NHEK when applied alone (SA) (6.3 ± 0.1 $\log_{10}$ CFU/ml), was significantly more than when added after NHEK were pre-exposed to viable *L. reuteri* (Pre-LR) (5.6 ± 0.2 $\log_{10}$ CFU/ml, P=0.0002*), and when pre-exposed to lysates of *L. reuteri* (Pre-Lysate) (6.0 ± 0.2 $\log_{10}$ CFU/ml, P=0.032*) (n=4). **(B)** The number of staphylococci adherent to NHEK when applied alone (SA) (6.5 ± 0.2 $\log_{10}$ CFU/ml), was significantly different when NHEK were pre-exposed to viable *L. reuteri* (Pre-LR) (5.3 ± 0.1 $\log_{10}$ CFU/ml, P=0.003*). There was no significant difference between the number of adherent staphylococci on cells infected with *S. aureus* and on cells pre-exposed to heat-killed *L. reuteri* (Pre-HKLR*)* (6.1 ± 0.3 $\log_{10}$ CFU/ml, P>0.05) (n=3). Results are expressed as the mean ± SEM.

5.1.2 *L. salivarius* does not inhibit *S. aureus* adhesion to NHEK

To further confirm a link between adhesion and the protection against *S. aureus* toxicity, the ability of *L. salivarius*, which was not protective (Section 4.1), to inhibit *S. aureus* adhesion was investigated. Figure 5.5 demonstrates that *L. salivarius* did not inhibit *S. aureus* binding to NHEK (P>0.05).

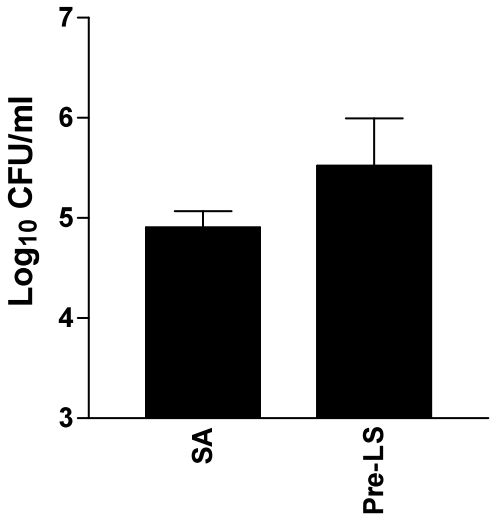


Figure 5.5. *L. salivarius* did not inhibit staphylococcal adhesion to NHEK.
Keratinocytes pre-exposed to *L. salivarius* (Pre-LS) had a similar number of adherent staphylococci (5.5 ± 0.5 log_{10} CFU/ml) compared to cells exposed to *S. aureus* alone (SA) (4.9 ± 0.2 log_{10} CFU/ml). Results are expressed as the mean ± SEM (n=3).

5.2 The principal mechanism of protection is the inhibition of adhesion

Thus far, the data suggest a link between the adhesion of lactobacilli and the protection against the pathogenic effects of *S. aureus*. *S. aureus* utilises a fibronectin binding protein (FnBP) to adhere to extracellular fibronectin, which in turn binds to the α5ß1 integrin on keratinocytes (Kintarak et al., 2004a, Kintarak et al., 2004b). In order to confirm this observation, a blocking antibody to the α5ß1 integrin was used to test the effects on *S. aureus* adhesion to NHEK. The data in Figure 5.6a demonstrate that the α5ß1 antibody inhibited binding of *S. aureus* to NHEK (P=0.007). To confirm the link between adhesion and the protective effect of *L. reuteri*, the possibility that blocking the α5ß1 integrin may protect against pathogenesis was investigated. Figure 5.6b demonstrates that blocking protected keratinocytes from the pathogenic effects of *S. aureus* (P=0.03).

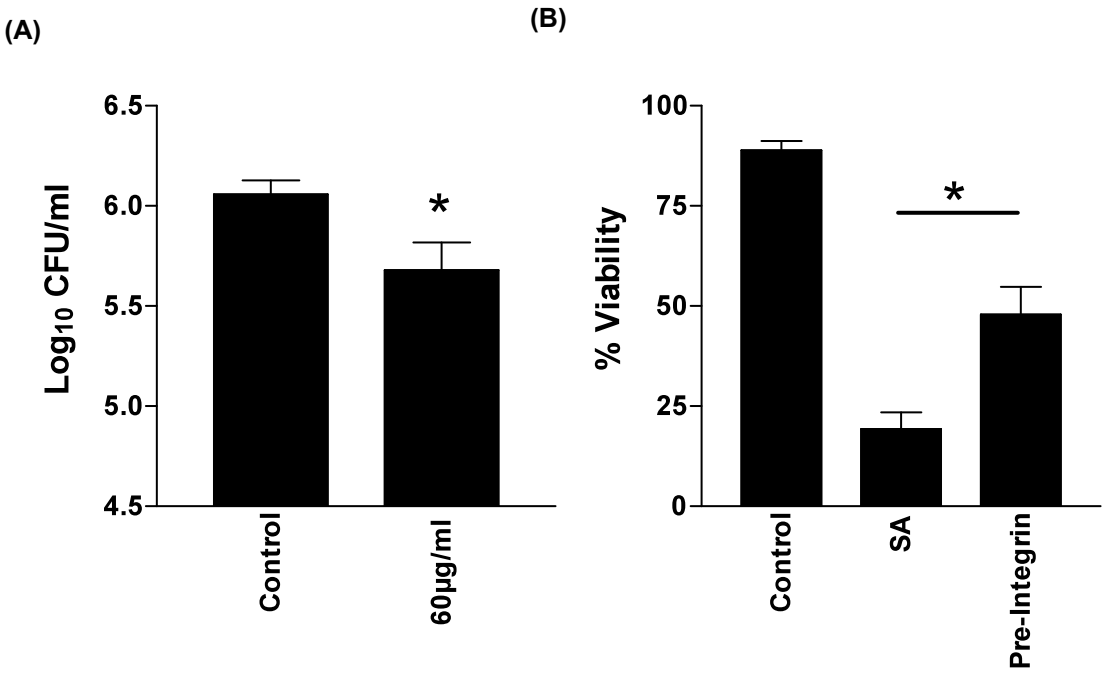


Figure 5.6. Blocking of the α5β1 integrin is sufficient to protect NHEK from *S. aureus*.
(A) Significantly fewer staphylococci adhered to cells treated with 60µg/ml blocking antibody (5.7 ± 0.1 log_{10} CFU/ml) compared to untreated NHEK (Control) (6.1 ± 0.1 log_{10} CFU/ml, P=0.007). **(B)** Significantly more keratinocytes were viable after infection with *S. aureus* for 24h if pre-exposed to an anti-α5ß1 integrin antibody (Pre-Integrin) prior to infection (47.9 ± 6.0%) compared to keratinocytes infected with *S. aureus* alone (SA) (19.3 ± 2.0%, P=0.03*). Results are expressed as the mean ± SEM (n=3).

5.3 Mechanism of bacterial adhesion to NHEK

5.3.1 *L. reuteri* does not aggregate with *S. aureus* on NHEK

Having established a link between adhesion and the ability of *L. reuteri* to protect keratinocytes from *S. aureus* pathogenesis, the potential mechanisms underlying these effects were next investigated. It has been reported that lactobacilli can co-aggregate with pathogenic bacteria thereby reducing their attachment to and subsequent colonisation of epithelia. For example, *L. acidophilus*, *L. gasseri* and *L. jensenii* purportedly co-aggregate with uropathogenic *E. coli* and *G. vaginalis*, preventing their colonisation of the vaginal epithelium (Boris et al., 1998). Therefore, Gram stains of adherent bacteria on NHEK exposed to both bacteria individually or pre-exposed to *L. reuteri* before *S. aureus* infection were performed to assess whether *L. reuteri* co-aggregates with *S. aureus*. Visualisation indicated that *L. reuteri* appeared to autoaggregate but did not co-aggregate with *S. aureus* (Figure 5.7).

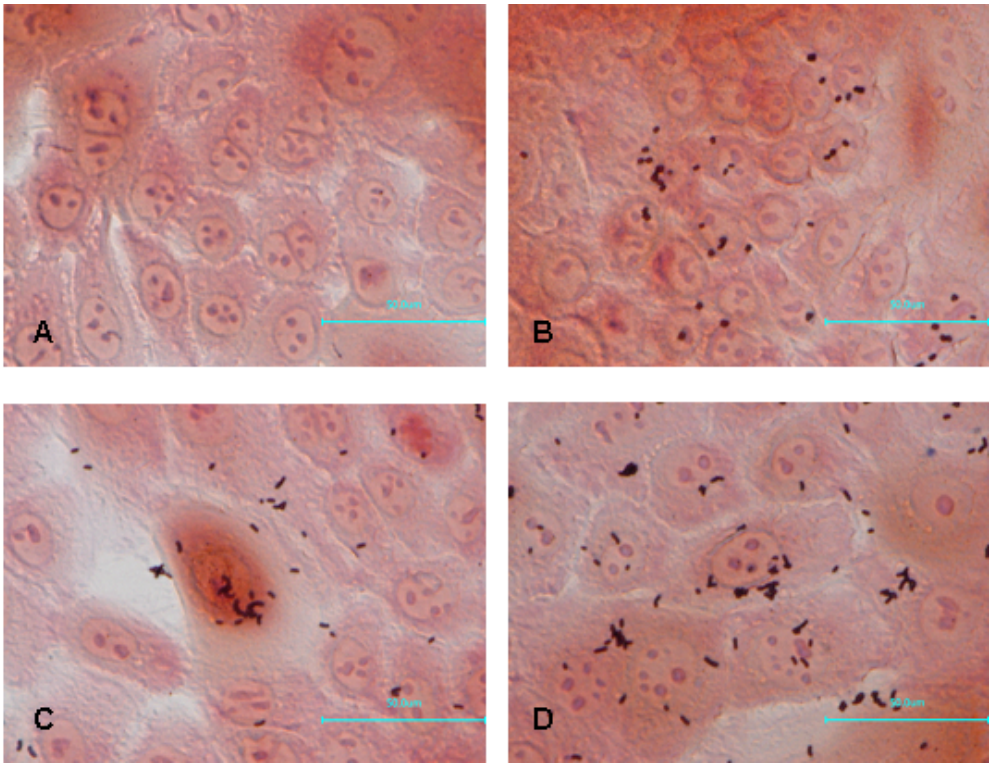


Figure 5.7. *L. reuteri* did not co-aggregate with *S. aureus* on NHEK.
(A) Untreated NHEK. **(B)** *S. aureus* infected NHEK. **(C)** *L. reuteri* treated NHEK. **(D)** NHEK pre-treated with *L. reuteri* before *S. aureus* exposure.

5.3.2 *L. reuteri* does not secrete substances that inhibit the adhesion of *S. aureus* to NHEK

Probiotic bacteria may secrete biosurfactants (Ron and Rosenberg, 2001) and release surface components that are involved in adhesion to surfaces. Biosurfactants include glycolipids and lipopolysaccharides which may play a role in selective attachment of bacteria to surfaces (Ron and Rosenberg, 2001). Therefore, the possible production of a functional biosurfactant by *L. reuteri* was investigated.

Biosurfactant was extracted using the method described in Section 2.0.5. Biosurfactant activity was measured by adding 100µl into a microtitre plate placed onto a grid-lined piece of paper. Functional biosurfactants cause optical distortion of the grid because they form a concave fluid surface (Sen et al., 2010). However, no evidence of biosurfactant production by *L. reuteri* could be detected using these assay conditions (Figure 5.8).

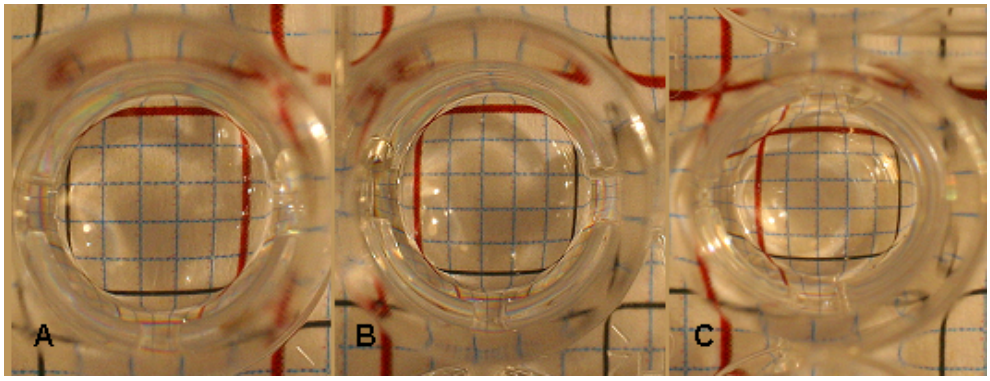


Figure 5.8. *L. reuteri* did not appear to produce a biosurfactant.
Microtitre plate assay demonstrating **(A)** negative control (water) **(B)** putative biosurfactant isolated from *L. reuteri* and **(C)** positive control (10% SDS). The positive control exhibited distortion of the grid behind the plate while neither the negative control nor the sample biosurfactant distorted the grid.

Some bacteria can secrete proteins involved in adhesion to mammalian cells (Spurbeck and Arvidson, 2010, Gan et al., 2002). Therefore, the conditioned medium from cells exposed to *L. reuteri* was investigated for its ability to inhibit *S. aureus* adhesion to cells. Conditioned media (CM) from *L. reuteri*-exposed NHEK did not inhibit *S. aureus* adhesion when NHEK were pre- or co-exposed to the CM (P>0.05, Figure 5.9a and b).

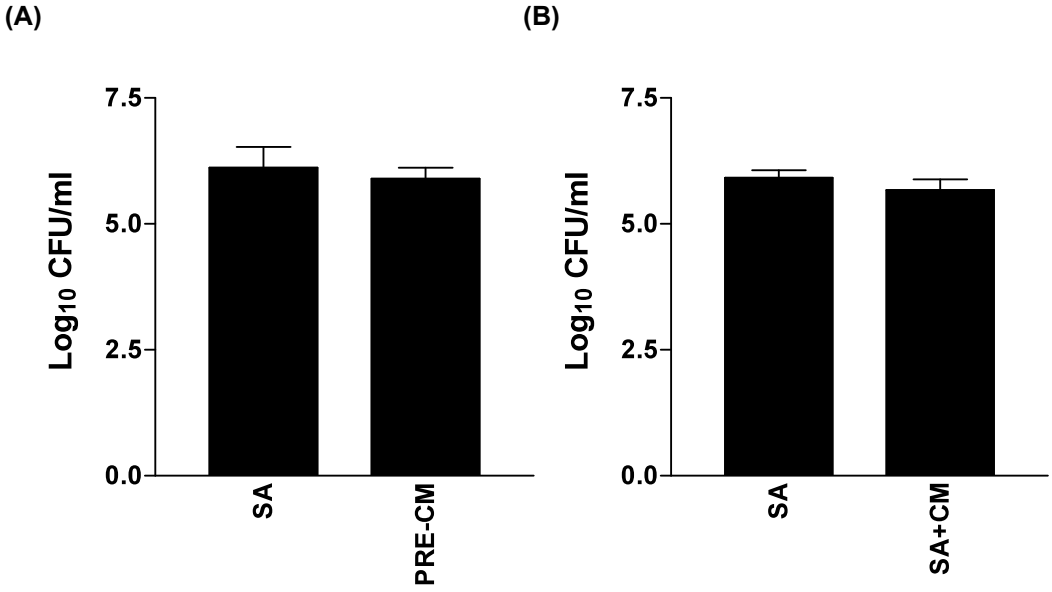


Figure 5.9. Conditioned media from cells exposed to *L. reuteri* did not inhibit *S. aureus* adhesion.
(A) There was no significant difference (P>0.05) in the number of adherent *S. aureus* on NHEK pre-exposed to conditioned media (PRE-CM) (5.9 ± 0.2 $\log_{10}$ CFU/ml) from cells infected with *S. aureus* (SA) (6.1 ± 0.4 $\log_{10}$ CFU/ml). **(B)** Likewise, there was no significant difference in the quantity of adherent *S. aureus* on NHEK when infected with *S. aureus* (SA) (5.9 ± 0.2 $\log_{10}$ CFU/ml) or simultaneously exposed to the conditioned media (SA+CM) (5.7 ± 0.2 $\log_{10}$ CFU/ml). Results are expressed as the mean ± SEM (n=3).

5.3.3 *L. reuteri* does not utilise fibronectin binding proteins to adhere to NHEK

As shown in Figure 5.6a, *S. aureus* utilised fibronectin binding proteins (FnBPs) to adhere to extracellular fibronectin and bind to the α5ß1 integrin on keratinocytes. Hence, the ability of *L. reuteri* to adhere to the α5ß1 integrin was explored. However, blocking of the α5ß1 integrin had no effect on *L. reuteri* adhesion to NHEK (Figure 5.10, P>0.05).

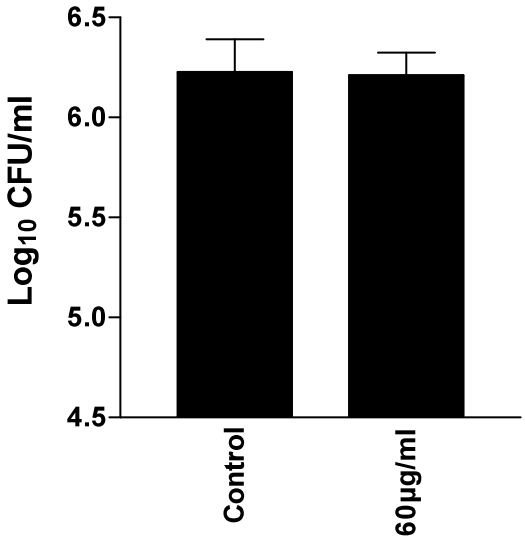


Figure 5.10. *L. reuteri* did not require the α5ß1 integrin to adhere to NHEK.
The blocking antibody had no significant effect on the ability of *L. reuteri* to adhere to NHEK (6.2 ± 0.1 $\log_{10}$ CFU/ml) compared to untreated NHEK (6.2 ± 0.2 $\log_{10}$ CFU/ml). Results are expressed as the mean ± SEM (n=4).

5.3.4 Effect of protease treatment of *S. aureus* and *L. reuteri* on adhesion to NHEK

Lactobacilli may express carbohydrate-binding proteins that they use to adhere to surfaces (Heinemann et al., 2000). For that reason, an investigation into whether *L. reuteri* ATCC 55730 and *S. aureus* possess cell surface proteins involved in adhesion was carried out. Experiments were performed in which *S. aureus* and *L. reuteri* were pre-treated with two different proteases and their ability to adhere to keratinocytes assessed. Proteinase-K is a broad spectrum protease which cleaves peptide bonds of aliphatic, aromatic and hydrophobic residues (Ebeling et al., 1974) while trypsin is a more specific protease known to cleave at the C-terminal side of lysine or arginine in peptides (Walsh et al., 1970). Figure 5.11a and b confirmed that *S. aureus* uses a protein adhesin to adhere to keratinocytes because both trypsin and proteinase-K treatment led to a significant loss in its ability to adhere to NHEK (P=0.006 and P=0.046 respectively). Conversely, Figure 5.12a and b demonstrate that neither trypsin nor proteinase-K had any significant effect on adhesion of *L. reuteri* to NHEK (P>0.05).

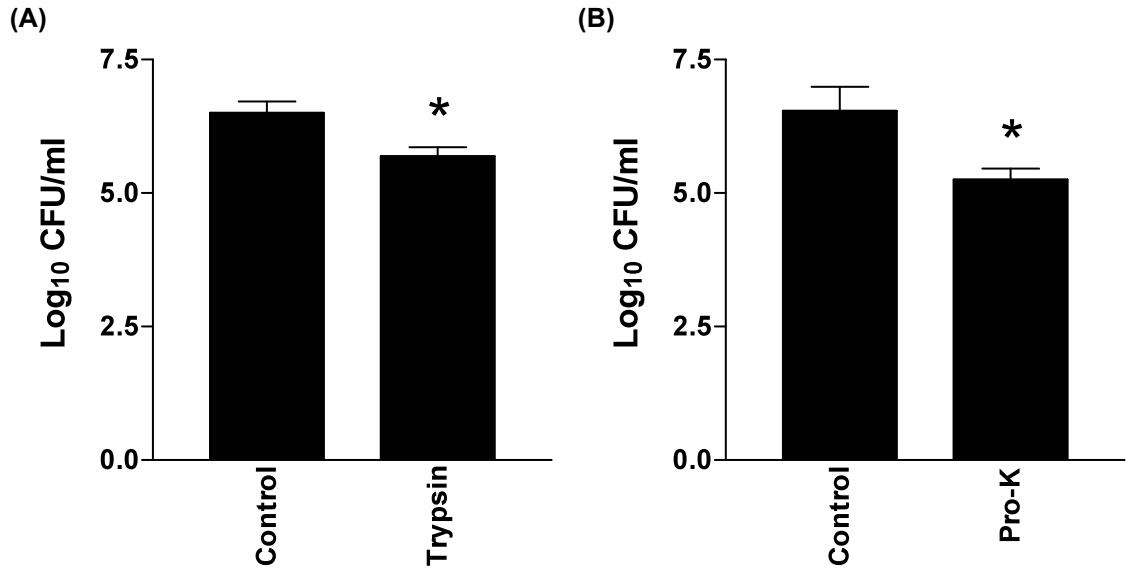


Figure 5.11. *S. aureus* utilises a protein adhesin to adhere to keratinocytes.
(A) *S. aureus* treated with trypsin was not able to adhere as well (5.7 ± 0.2 $\log_{10}$ CFU/ml) as untreated control *S. aureus* to NHEK (6.5 ± 0.2 $\log_{10}$ CFU/ml) (P=0.006*). **(B)** *S. aureus* treated with proteinase-K was not able to adhere as well (5.3 ± 0.2 $\log_{10}$ CFU/ml) as untreated *S. aureus* to NHEK (6.5 ± 0. $\log_{10}$ CFU/ml) (P=0.046). Results are expressed as the mean ± SEM (n=3).

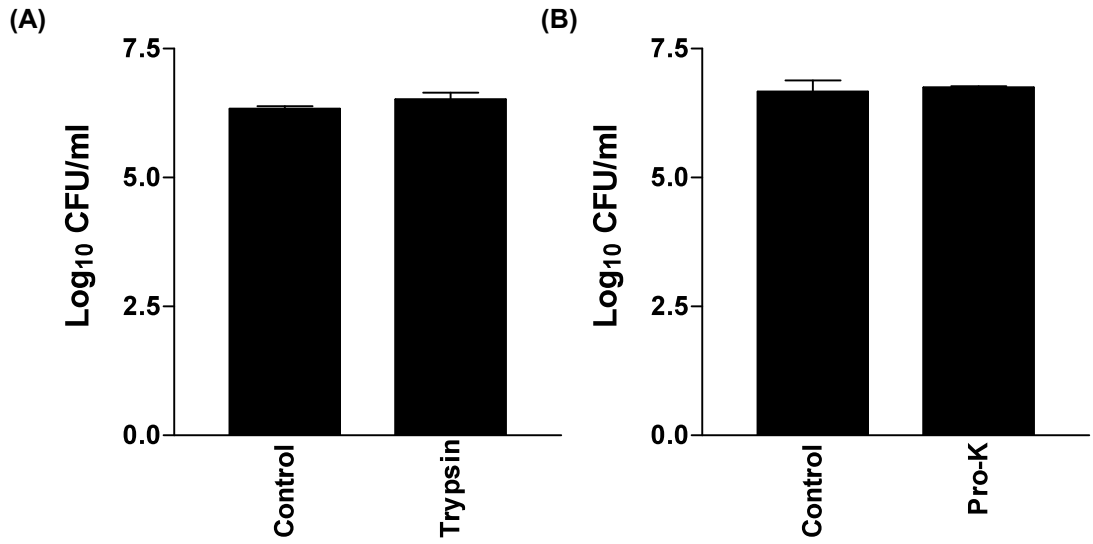


Figure 5.12. *L. reuteri* does not use a protein adhesin to adhere to keratinocytes.
(A) Treatment of *L. reuteri* with trypsin had no significant effect on the number of *L. reuteri* able to adhere to NHEK (6.5 ± 0.1 $\log_{10}$ CFU/ml) compared to untreated *L. reuteri* (6.3 ± 0.1 $\log_{10}$ CFU/ml). **(B)** Treatment of *L. reuteri* with proteinase-K had no significant effect on the amount of *L. reuteri* able to adhere to NHEK (6.8 ± 0.02 $\log_{10}$ CFU/ml) compared to untreated *L. reuteri* (6.7 ± 0.2 $\log_{10}$ CFU/ml). Results are expressed as the mean ± SEM (n=3).

In order to confirm that the proteases had worked, an EDTA extraction of cell surface proteins was performed (Section 2.3.5) on untreated and protease-treated bacteria. The extracts were analysed using silver staining of SDS-PAGE gels to visualise if protease treatment cleaved cell surface proteins effectively. Figure 5.13 demonstrates that protease treatment resulted in the disappearance of proteins when assessed by gel electrophoresis.

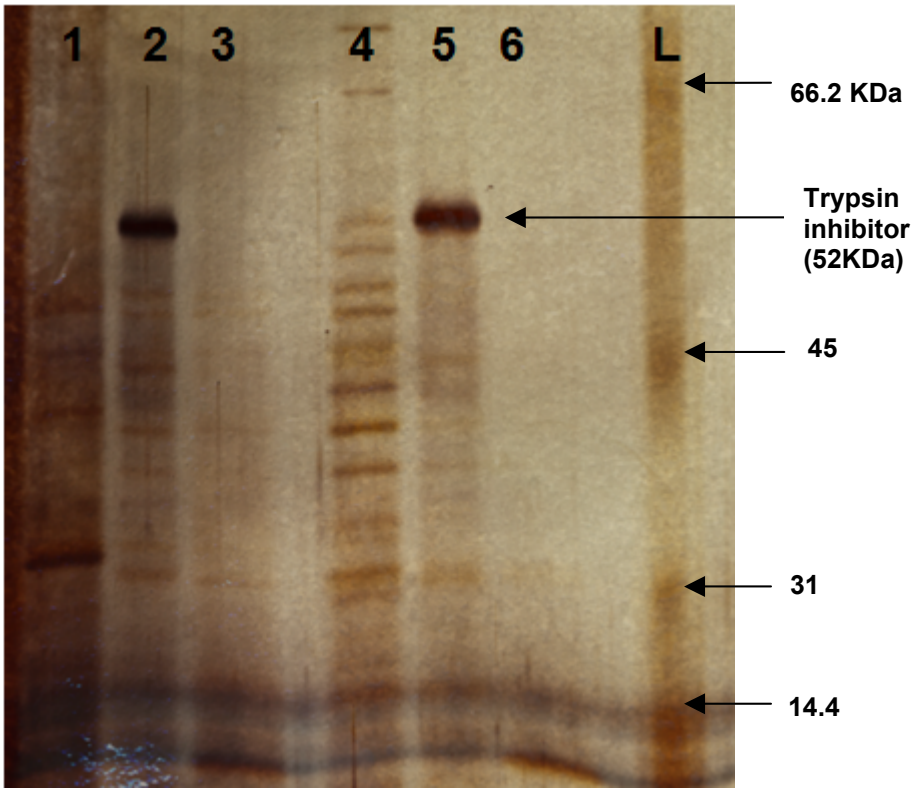


Figure 5.13. Protease treatment cleaved bacteria cell surface proteins.
SDS-PAGE of bacterial cell surface extracts. Lane 1=*S. aureus* (untreated). Lane 2=trypsin treated *S. aureus*. Lane 3=proteinase-K treated *S. aureus*. Lane 4=*L. reuteri* (untreated). Lane 5=trypsin treated *L. reuteri*. Lane 6=proteinase-K treated *L. reuteri*. L= ladder.

It was considered possible that while *L. reuteri* may not utilise a cell surface protein to adhere to NHEK directly, a protein could still be responsible for the ability of *L. reuteri* to inhibit *S. aureus* adhesion. Therefore, adhesion experiments were performed using protease treated *L. reuteri*. Figure 5.14 demonstrates that neither trypsin nor proteinase-K had any significant effect on the ability of *L. reuteri* to inhibit *S. aureus* adhesion ($P>0.05$).

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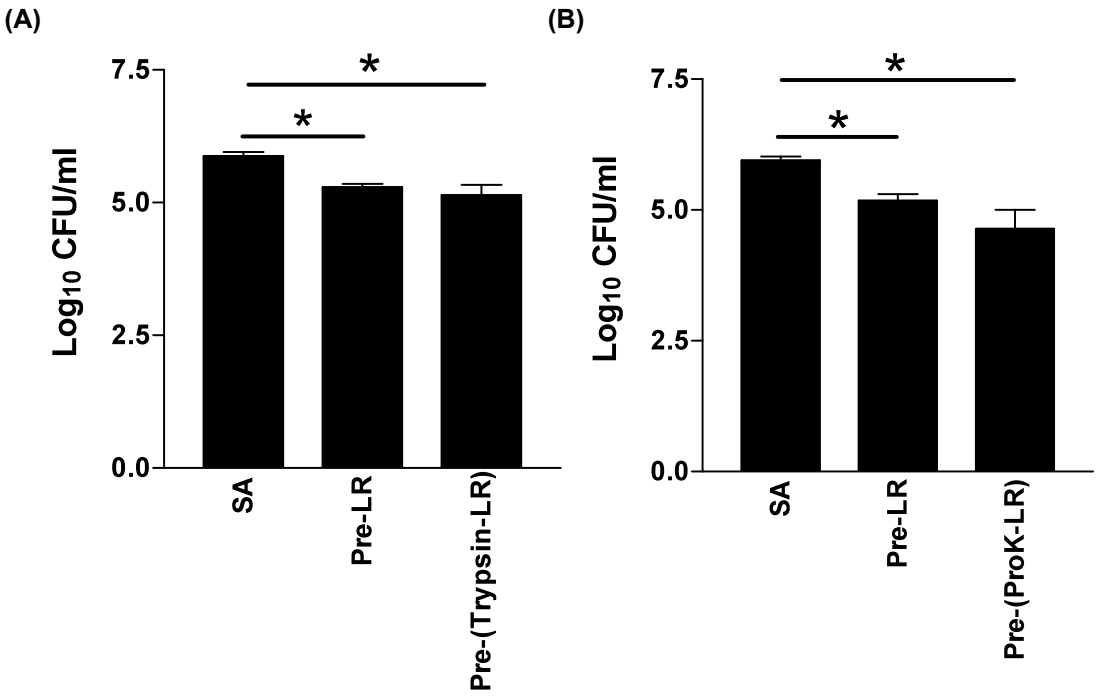


Figure 5.14. Protease treatment of *L. reuteri* did not affect its ability to inhibit *S. aureus* adhesion to NHEK.
(A) There was no significant difference between the number of staphylococci adhered to NHEK pre-exposed to untreated *L. reuteri* (Pre-LR) (5.3 ± 0.1 $\log_{10}$ CFU/ml) and trypsin treated *L. reuteri* (Pre-(Trypsin-LR)) (5.1 ±0.2 $\log_{10}$ CFU/ml) (P>0.05). NHEK pre-exposed to untreated *L. reuteri*, and trypsin treated *L. reuteri*, had significantly fewer *S. aureus* adhered to them (P=0.048* and P=0.004* respectively) compared to NHEK infected with *S. aureus* alone (SA) (5.9 ± 0.1 $\log_{10}$ CFU/ml). **(B)** NHEK pre-exposed to proteinase-K treated *L. reuteri* (Pre-(ProK-LR)) had significantly fewer *S. aureus* adhered to them (4.6 ± 0.4 $\log_{10}$ CFU/ml, P=0.004*) compared to NHEK infected with *S. aureus* alone (SA) (6 ± 0.1 $\log_{10}$ CFU/ml). NHEK pre-exposed to untreated *L. reuteri* (Pre-LR) also had significantly less staphylococci adhered to them (5.2 ± 0.1 $\log_{10}$ CFU/ml, P=0.048*) compared to *S. aureus* infected cells. There was no significant difference between the number of staphylococci adhered to NHEK exposed to untreated *L. reuteri* and proteinase-K treated *L. reuteri* (P>0.05). Results are expressed as the mean ± SEM (n=4).

5.3.5 Lipoteichoic acid (LTA) and carbohydrate adhesins

Some strains of *S. aureus* can utilise lipoteichoic acid (LTA) to adhere to nasal epithelial cells (Aly et al., 1980). Therefore, the involvement of LTA in either *S. aureus* or *L. reuteri* adhesion to NHEK was investigated. Pre-treatment of NHEK with 100µg LTA resulted in a significant reduction in the number of staphylococci adherent to NHEK (P=0.014, Figure 5.15a). However, pre-treatment of NHEK with LTA had no significant effect on the ability of *L. reuteri* to adhere to NHEK (P>0.05, Figure 5.15b).

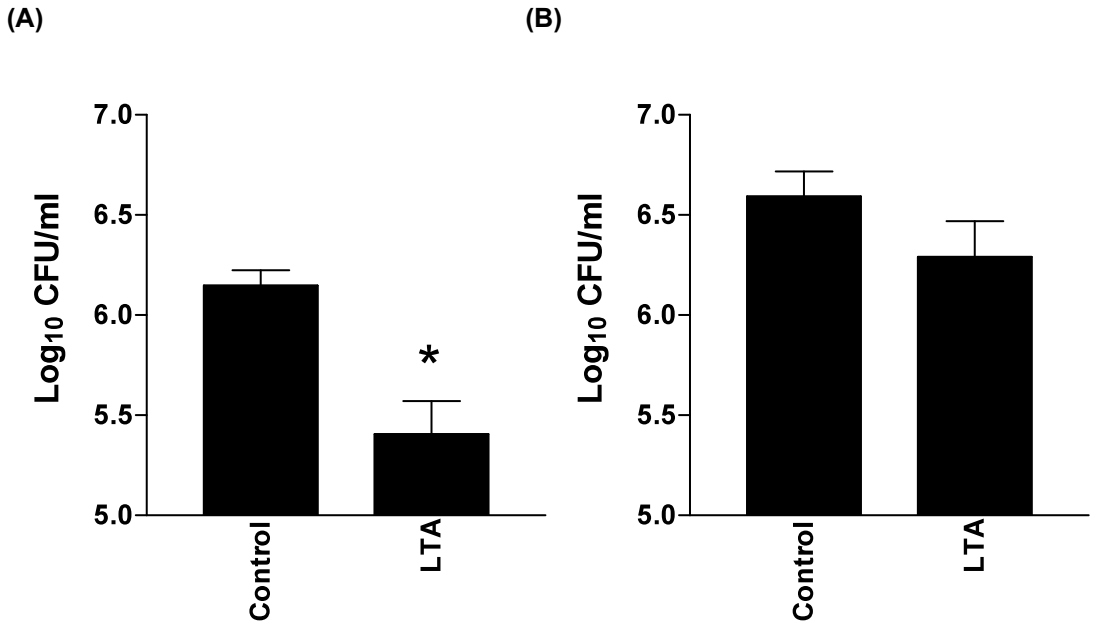


Figure 5.15. *S. aureus* uses LTA to bind to NHEK, but *L. reuteri* does not.
(A) Significantly fewer staphylococci adhered to cells treated with 100µg lipoteichoic acid (LTA) (5.4 ± 0.2 $\log_{10}$ CFU/ml) compared to untreated control keratinocytes (6.2 ± 0.1 $\log_{10}$ CFU/ml, P=0.014). **(B)** There was no significant difference in the number of lactobacilli adhered to untreated control NHEK (6.6 ± 0.1 $\log_{10}$ CFU/ml) and LTA treated NHEK (LTA) (6.3 ± 0.2 $\log_{10}$ CFU/ml). Results are expressed as the mean ± SEM (n=4).

The possibility that a cell surface carbohydrate was involved in the mechanism of *L. reuteri* adhesion was next examined by treating *L. reuteri* with sodium meta-periodate. This is a chemical that oxidises cell surface carbohydrates, and has been used in studies examining probiotic adhesion to cells (Greene and Klaenhammer, 1994, Boris et al., 1998). However, experiments demonstrated that periodate treatment affected bacterial viability (data not shown), so a direct microscopy method (Section 2.3) was chosen for comparison of untreated *L. reuteri* and periodate treated *L. reuteri* adhesion to NHEK (Figure 5.16). Periodate treatment significantly reduced the adhesion of *L. reuteri* to NHEK (P=0.0005).

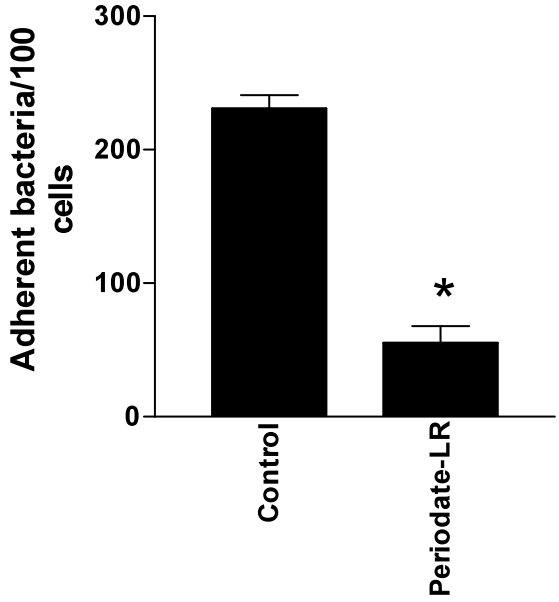


Figure 5.16. Periodate treatment affected *L. reuteri* ability to adhere to NHEK.
Untreated control *L. reuteri* adhered to keratinocytes significantly better (231 ± 10 bacteria/100 cells) than periodate treated *L. reuteri* (55 ± 12 bacteria/100 cells), P=0.0005. Results are expressed as the mean ± SEM (n=3).

5.4 Conclusions

S. aureus occurs intermittently on the skin of up to 60% of people, while 20% of people are stably colonised by the organism. It is most often found in the moist regions of the body, such as the axilla and anterior nares (Kluytmans et al., 1997). The organism does not usually infect intact skin, but once it has breached the *stratum corneum* of the epidermis (as observed in skin wounds), it may initiate infections such as impetigo and boils (Krut et al., 2003).

The mechanism by which *S. aureus* induces cell death in keratinocytes is complex and involves the expression of a variety of virulence factors. Initiation of this process is dependent on the ability of the organism to adhere prior to invasion of the cell (Kintarak et al., 2004b). *S. aureus* utilises adhesins to adhere to epithelial cells, including fibronectin binding proteins (FnBPs) (Kintarak et al., 2004a, Mempel et al., 2002), protein A, clumping factors and coagulase (Mempel et al., 1998).

In the gut, probiotics have been shown to protect the epithelium from colonisation by pathogenic bacteria through a number of mechanisms including inhibition of pathogen growth and adhesion to the epithelial cells (Section 1.3). In Section 3.1 it was shown that *L. reuteri* inhibited the growth of *S. aureus* through production of inhibitory substances. However, this was dependent on the culture conditions. Section 4.1 demonstrated that *L. reuteri* protected NHEK against *S. aureus* induced cell death. However, neither *L. reuteri* nor NHEK influenced staphylococci viability in cell culture assays. Additionally, the same protective effect was observed regardless of the concentration of *L. reuteri* used (Section 4.2). Therefore, one possibility is that the protective effect observed for *L. reuteri* could involve competitive exclusion of binding sites on NHEK. Consequently the ability of *L. reuteri* and *S. aureus* to adhere to NHEK, and the mechanisms responsible for the protective effect observed in Chapter 4 were investigated.

The data strongly suggests that the protective effects observed in Section 4.1 rely on inhibition of the binding of *S. aureus* to NHEK. Several lines of evidence support this view; i) inhibition of *S. aureus* adhesion using a blocking antibody reduced its toxicity to NHEK (Figure 5.6), ii) probiotics that could not bind to NHEK e.g. *L. salivarius,* (Figure 5.5a) were not protective, iii) heat-killed *L. reuteri* does not adhere to NHEK (Figure 5.1) and does not protect keratinocytes from *S. aureus* induced cell death (Section 4.5, Figure 4.7).

The timing of application of *L. reuteri* is critical to its ability to inhibit adhesion since addition of the probiotic after infection with *S. aureus* did not reduce the binding of the pathogen to NHEK (Figure 5.2c). However, others have found that displacement may take longer to occur than competition and exclusion (Lee et al., 2003), suggesting that a longer exposure to *L. reuteri* may be required. Taken together, the data suggests that under the experimental conditions used in the present study, the mechanism of protection by *L. reuteri* is one of competitive exclusion.

Competitive exclusion of pathogens from binding sites is a common mechanism by which probiotics protect the gut epithelium. For example, the ability of *L. acidophilus*, *L. paracasei*, *L. salivarius* and *L. crispatus* to inhibit the adhesion of *S. aureus* to vaginal epithelial cells (VEC) has been shown to occur by exclusion and competition. Additionally, these probiotics could displace adherent *S. aureus* to differing degrees, indicating species-dependent efficacy. Interestingly, none of these organisms significantly affected the ability of *E. coli* to adhere to VEC (Zárate and Nader-Macias, 2006). Furthermore, in the present study, species-specific effects were observed because *L. salivarius* does not adhere to NHEK as efficiently as *L. reuteri* and cannot protect cells from pathogenesis (Figure 5.5).

There are a number of mechanisms by which lactobacilli may adhere to epithelia and prevent colonisation by other bacteria. These include; i) manipulation of hydrophobic surface properties through biosurfactant production, ii) secretion of adhesins, iii) cell wall protein or carbohydrate adhesins and iv) influencing the production of adhesive factors on epithelia and v) autoaggregation and co-aggregation.

Autoaggregation and co-aggregation of *L. reuteri* with other bacteria could be a method for prevention of *S. aureus* adhesion because aggregation with the pathogen may block it from adhering to its cellular targets. Indeed, aggregation has been used as a property with which to screen potential probiotics (Collado et al., 2008). However, Figure 5.7 demonstrates that while *L. reuteri* appeared to autoaggregate on the surface of NHEK, it did not co-aggregate with *S. aureus* on NHEK.

Probiotics can produce biosurfactants for regulating attachment to surfaces through modification of the net hydrophobicity and surface charge. In 2002, Gan and colleagues showed that *L. fermentum* RC-14 produced a biosurfactant containing collagen binding proteins, and this could inhibit staphylococcal binding to surgical implants (Gan et al., 2002).

The current study, however, did not find any evidence of biosurfactant production by *L. reuteri* using a microtitre plate assay (Figure 5.8). Additionally, Figure 5.9 illustrates that the conditioned medium had no effect on the ability of *S. aureus* to adhere, demonstrating that *L. reuteri* did not secrete any adhesins in the presence of NHEK responsible for the inhibition of *S. aureus* adhesion.

A bioinformatic analysis of the draft genome sequence of *L. reuteri* ATCC 55730 for putative cell surface or secreted proteins indicates that the organism possesses a number of secreted and cell surface proteins, some of which could putatively be involved in adhesion to epithelia (Båth et al., 2005). For example, the protein Lr0793 found in *L. reuteri* ATCC 55730 is homologous to CnBP, a collagen binding protein in *L. reuteri* NCIB 11951 (Båth et al., 2005, Roos et al., 1996). However, treatment of *L. reuteri* with proteases had no effect on the ability of the organism to adhere to NHEK (Figure 5.12), nor did it affect the ability of *L. reuteri* to inhibit *S. aureus* adhesion to NHEK (Figure 5.14). Conversely, protease treatment of *S. aureus* inhibited its ability to adhere to NHEK, demonstrating the requirement of *S. aureus* cell surface proteins for adhesion. This is in agreement with other studies also showing that *S. aureus* uses multiple mechanisms for binding including cell surface components such as fibronectin binding proteins (Kintarak et al., 2004a) and lipoteichoic acid (Aly et al., 1980).

L. reuteri does not appear to utilise the α5β1 integrin (Figure 5.10b), or lipoteichoic acid (Figure 5.15b) to adhere to NHEK because specific blockade of these sites on NHEK did not affect adhesion of *L. reuteri*.

Successful probiotics are required to survive passage through the low pH in the stomach and be able to grow and/or persist the in the intestine. As a result, many commensal strains of lactobacilli have evolved to resist the action of proteolytic enzymes and glycosidases found throughout the digestive tract and bile fluids. *L. reuteri* ATCC 55730 is an indigenous coloniser of the human gastrointestinal tract, therefore, the ability to resist the action of enzymes may be a mechanism for survival (Connolly, 2004). Consequently, it is perhaps not so surprising that in this study *L. reuteri* was resistant to proteases.

Treatment with sodium meta-periodate affected the ability of *L. reuteri* to adhere to NHEK (Figure 5.16). However, it also affected bacterial viability. Sodium meta-periodate acts to oxidise carbohydrates and therefore may have effects other than removal of cell surface polysaccharides.

For example, the structural molecule peptidoglycan could be oxidised, disrupting the cell wall and killing the organism. Since periodate-treated bacteria were non-viable, it cannot confidently be concluded that adhesion of *L. reuteri* to NHEK was dependent on cell surface carbohydrates. On the other hand, lysates of *L. reuteri* are protective (Section 4.5), potentially demonstrating that viable bacteria are not a requirement for adhesion or for the protection of NHEK. Interestingly, heat-killed *L. reuteri* could not protect keratinocytes against *S. aureus* induced cell death. This suggests that whatever the mechanism *L. reuteri* uses to adhere to keratinocytes, it involves a heat-labile molecule.

To date, there is no published work on the ability of probiotic bacteria to adhere to epidermal keratinocytes. However, in the gut, collagen binding proteins (Heinemann et al., 2000), mucus binding proteins (Miyoshi et al., 2006), lipoteichoic acids (Granato et al., 1999) and S-layer proteins (Chen et al., 2007, Deepika et al., 2010) have been demonstrated to mediate adhesion of probiotic bacteria to epithelia. Therefore, it is possible that *L. reuteri* employs multiple mechanisms to adhere to NHEK and prevent *S. aureus* from adhering.

In summary, the data presented in this chapter suggest that *L. reuteri* inhibits the attachment of *S. aureus* to keratinocytes by a mechanism involving competitive exclusion of the pathogen from binding sites on NHEK. *L. reuteri* could be used as a prophylactic therapy against skin colonisation with *S. aureus,* as a component in barrier creams or soaps. Having attempted to uncover potential bacterial mechanisms involved in inhibition of pathogenesis, in the next chapter the innate immune response of keratinocytes is explored to determine whether probiotics can induce production of antimicrobial peptides or cytokines by keratinocytes.

Characterisation of the innate immune response of keratinocytes to probiotics

The epidermis of the skin acts as a barrier against invasion by microorganisms. The keratinocytes are the first living cells of the skin to come into contact with exogenous microorganisms. Whereas commensal bacteria are well tolerated on the surface of the skin, keratinocytes mount a rapid and robust innate immune response in response to infecting pathogens. This is conducted via pattern recognition receptors, such as Toll-like receptors (TLRs) expressed by keratinocytes which are triggered by microbial associated molecular patterns (MAMPs), leading to subsequent production of antimicrobial peptides (AMPs) and cytokine/chemokine signalling by keratinocytes to surrounding tissues and cells (Section 1.1.5.1).

Chapters 3 and 4 demonstrated that a probiotic, *L. reuteri*, can protect keratinocytes from a skin pathogen, *S. aureus*, and the mechanism behind that protection was partially uncovered as competitive exclusion of *S. aureus* binding to NHEK. However to date, no studies have investigated the innate immune response of keratinocytes to probiotics. The stimulation of the innate immune response by probiotics may also play a role in limiting infection of the skin. However, equally, probiotics themselves may not be well tolerated. Therefore, in this chapter a comparison of the response of keratinocytes to the skin pathogen *S. aureus*, a skin commensal, *S. capitis*, and *L. reuteri* ATCC 55730 was performed. For comparison, the response of skin cells to a second probiotic, *L. rhamnosus* GG (ATCC 53103) was also investigated. In the gut, *L. reuteri* has been demonstrated to produce an immunostimulatory effect (Valeur et al., 2004, Jones and Versalovic, 2009), while *L. rhamnosus* GG is believed to elicit an immunomodulatory effect (Zhang et al., 2005). Therefore, the response of keratinocytes to these organisms may be different.

The aims of this investigation were:

- To compare the effects of probiotic lactobacilli to those of *S. aureus* and *S. capitis* on TLR-2 expression in NHEK.
- To compare the effects of probiotic lactobacilli to those of *S. aureus* and *S. capitis* on antimicrobial peptide (AMP) expression in NHEK.
- To compare the effects of probiotic lactobacilli to those of *S. aureus* and *S. capitis* of skin bacteria on the secretion of the neutrophil attractant chemokine IL-8 by NHEK.

6.0 Time response assay

Section 4.1 demonstrated that the viability of NHEK was unaffected when they were incubated with *L. reuteri* for up to 24h (Figure 4.3). However, *S. aureus* induced significant cell death in NHEK within 12h to 16h post-infection (Figure 4.2). Therefore, for experiments investigating the effects of bacteria upon NHEK innate immune responses, a 12h infection period was chosen.

A concentration of 10^4 CFU/ml *S. capitis* was used in experiments because this was a physiologically relevant number of bacteria to use (Leyden et al., 1987). A concentration of 10^8 CFU/ml of *L. rhamnosus* GG was used to enable comparison with *L. reuteri*, which has been used at this concentration in previous studies (Chapters 4 and 5). Likewise, a concentration of 10^6 CFU/ml *S. aureus* was used in experiments as in previous studies (Section 4.0).

Previous studies have suggested that the commensal microflora may act to boost innate immune responses to pathogens (Wanke et al., 2011). Therefore, NHEK were pre-exposed to probiotics or *S. capitis* (a skin commensal) prior to *S. aureus* infection to determine whether probiotics could influence innate immune responses to *S. aureus.* For that reason, it was necessary to determine whether *S. capitis*, *L. rhamnosus* GG (LGG) or pre-exposure to *L. reuteri, L. rhamnosus* or *S. capitis* prior to *S. aureus* infection induced any significant cell death to NHEK. Time response assays demonstrated that at 12h exposure, neither *S. capitis*, nor *L. rhamnosus* GG caused significant cell death in NHEK (Figure 6.1a and b, $P>0.05$). However, *S. capitis* induced significant cell death after 24h ($P=0.0004$, Figure 6.1a). Additional experiments demonstrated that there was no cytotoxicity in NHEK pre-exposed to *L. reuteri*, *L. rhamnosus* or *S. capitis* prior to *S. aureus* infection for 12h (Figure 6.1c).

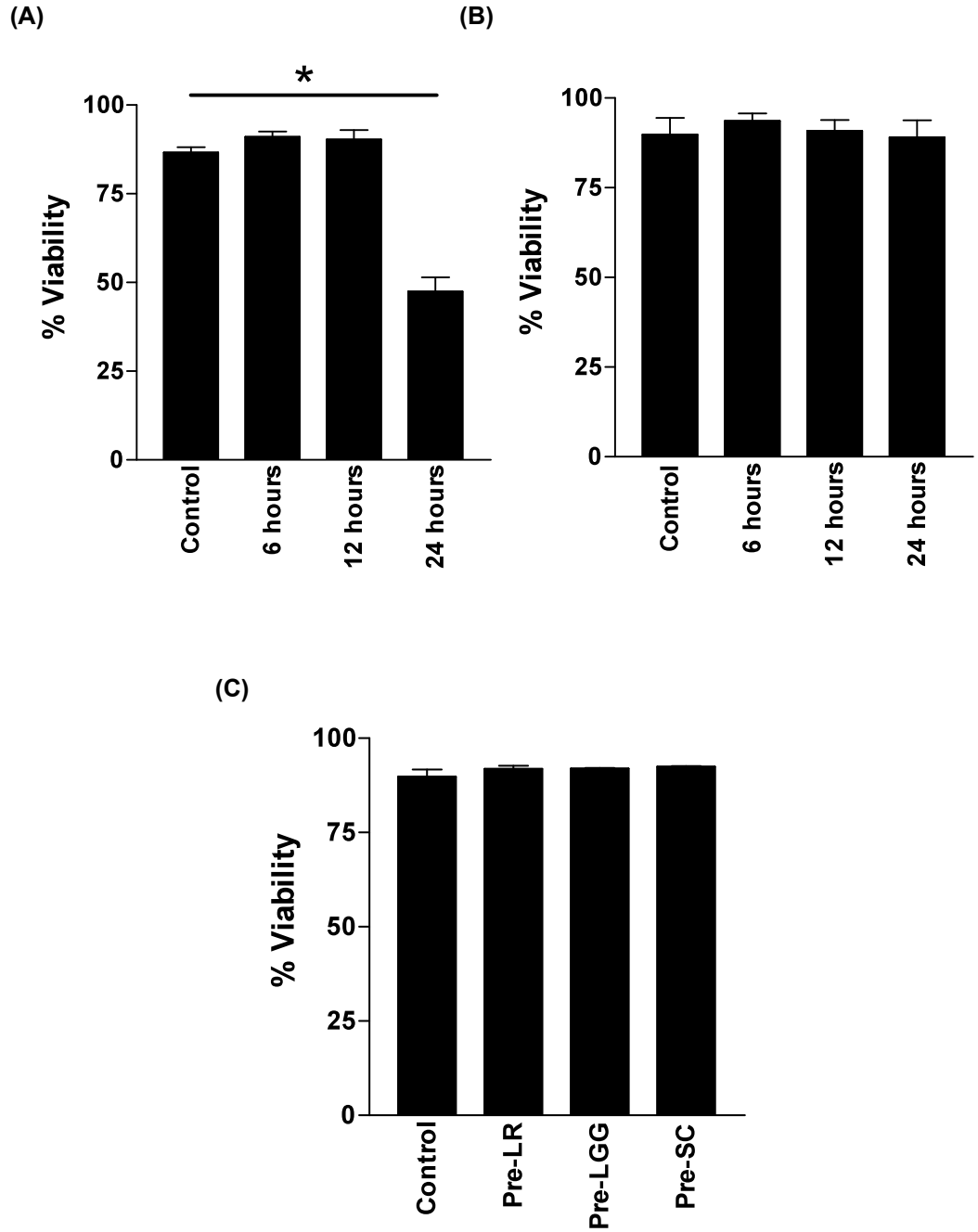


Figure 6.1. Exposure to *S. capitis*, *L. rhamnosus* GG or pre-exposure to probiotics or commensals prior to *S. aureus* infection did not cause cell death in NHEK after 12h exposure.

A) Time response assay for *S. capitis*. There was no significant change in the total viability of NHEK exposed to *S. capitis* for 12h (90.3 ± 2.7%) compared to control cells (86.6 ± 1.5%, P>0.05). NHEK exposed to *S. capitis* for 24h were significantly less viable (47.5 ± 4%, P=0.0004*) than controls. **B)** The percentage viability of NHEK was unaffected by exposure to *L. rhamnosus* GG for 12h (90.9 ± 3%) compared to control untreated cells (89.8 ± 4.7%, P>0.05). **C)** The viability of NHEK pre-exposed to *L. reuteri* for 12h (Pre-LR) (91.9 ± 0.7%) was similar to the viability of untreated control NHEK (89.8 ± 1.9%). There was no significant difference in the viability of NHEK pre-exposed to *L. rhamnosus* GG (Pre-LGG) (91.99 ± 0.2%) or pre-exposed to *S. capitis* (Pre-SC) (92.5 ± 0.2%) compared to control cells. Results are expressed as mean ± SEM (n=3).

6.1 Toll-like receptor-2 expression by NHEK

TLR-2 is thought to play an important role in *S. aureus* skin infections, as TLR-2 deficient mice are highly susceptible to *S. aureus* infection (Takeuchi et al., 2000). Therefore, experiments were performed to determine whether TLR-2 expression by NHEK changes in response to the different bacteria used in these experiments. However, previous investigations have measured only mRNA levels of TLRs. In the current chapter, TLR-2 protein expression was also measured using flow cytometry because it allows an analysis of changes in TLR-2 expression in individual cells, unlike more traditional methods such as western blotting. Additionally, flow cytometry has the capacity to gate out dead cells and define cells by their size, allowing the exclusion of aggregates and debris.

It was initially decided to confirm that both qRT-PCR and flow cytometry methods could accurately detect changes in TLR-2 in NHEK. NHEK exposure to 10μg/ml lipoteichoic acid (LTA) has previously been shown to induce a significant increase in expression of TLR-2 mRNA and protein expression in NHEK using qRT-PCR and western blotting techniques (Lew et al., 2009). Therefore, experiments were performed to determine whether this result could be repeated using RT-PCR and flow cytometry to detect TLR-2 mRNA and protein respectively. For flow cytometry, total (intracellular and cell surface) expression was measured because the expression of TLR-2 is known to be predominantly intracellular (Baker et al., 2003, Begon et al., 2007). Quantitative RT-PCR and flow cytometry confirmed that treatment of NHEK with LTA resulted in increased expression of TLR-2 mRNA and protein ($P=0.033$ and $P=0.015$ respectively) (Figure 6.2a and b).

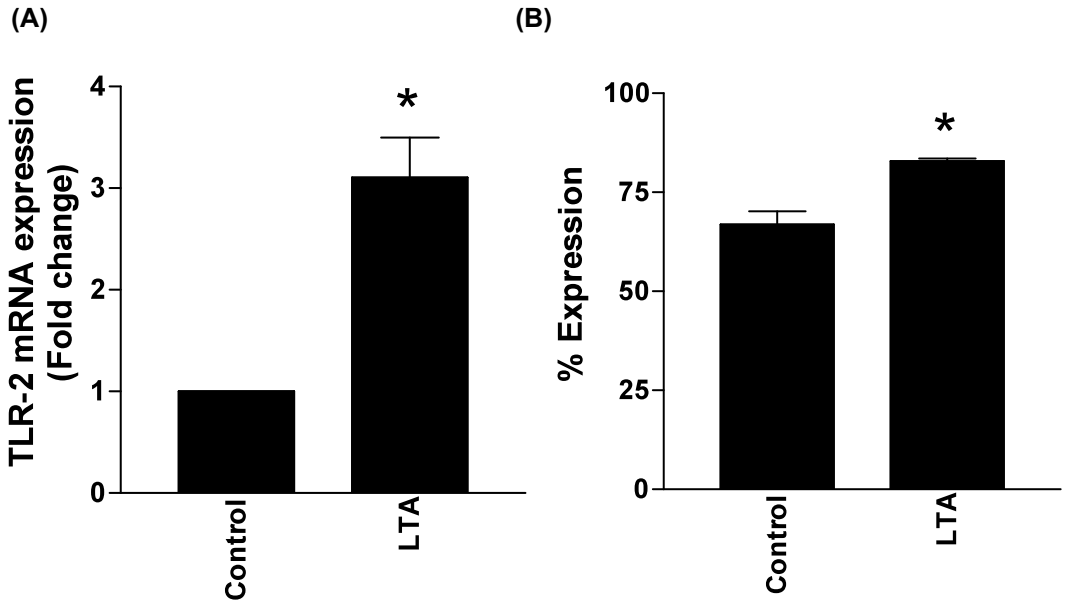


Figure 6.2. Expression of TLR-2 in NHEK in response to lipoteichoic acid (LTA).
A) TLR-2 mRNA expression in NHEK was analysed by quantitative RT-PCR. Results were normalised using HMBS and SDHA as endogenous controls and are shown as fold changes relative to untreated controls. Compared to control keratinocytes, treatment with 10µg/ml LTA for 24h induced a significant increase in the mRNA expression of TLR-2 (3.1 ± 0.4, P=0.033*) (n=3). **B)** Flow cytometry analysis of TLR-2 expression in NHEK demonstrated that significantly more LTA treated cells expressed TLR-2 protein (82.8 ± 0.8%) compared to untreated NHEK (66.9 ± 3.8%, P=0.015*) (n=4). Results are expressed as the mean ± SEM.

Next, the effect of *S. aureus*, *S. capitis*, *L. reuteri*, and *L. rhamnosus* GG on TLR-2 expression in NHEK was examined. NHEK were exposed to one strain of bacteria for 12h or were pre-exposed to the probiotic or *S. capitis* for 1h prior to a 12h infection with *S. aureus*. Treatment of NHEK with *S. aureus*, *S. capitis* or either of the probiotics did not induce any significant change in TLR-2 mRNA expression. However, NHEK pre-exposed to *S. capitis* prior to *S. aureus* infection had significantly increased TLR-2 mRNA production (22 ± 8.4 fold increase, P=0.009, Figure 6.3a). In agreement, flow cytometry also demonstrated a significant increase in the expression of TLR-2 protein in NHEK pre-exposed to *S. capitis* (P<0.0001, Figure 6.3b). Conversely, separate experiments demonstrated that treatment of NHEK with *S. aureus* alone for 12h reduced the protein expression of TLR-2 significantly (P=0.018, Figure 6.3c) while treatment with the probiotics or *S. capitis* had no significant effect.

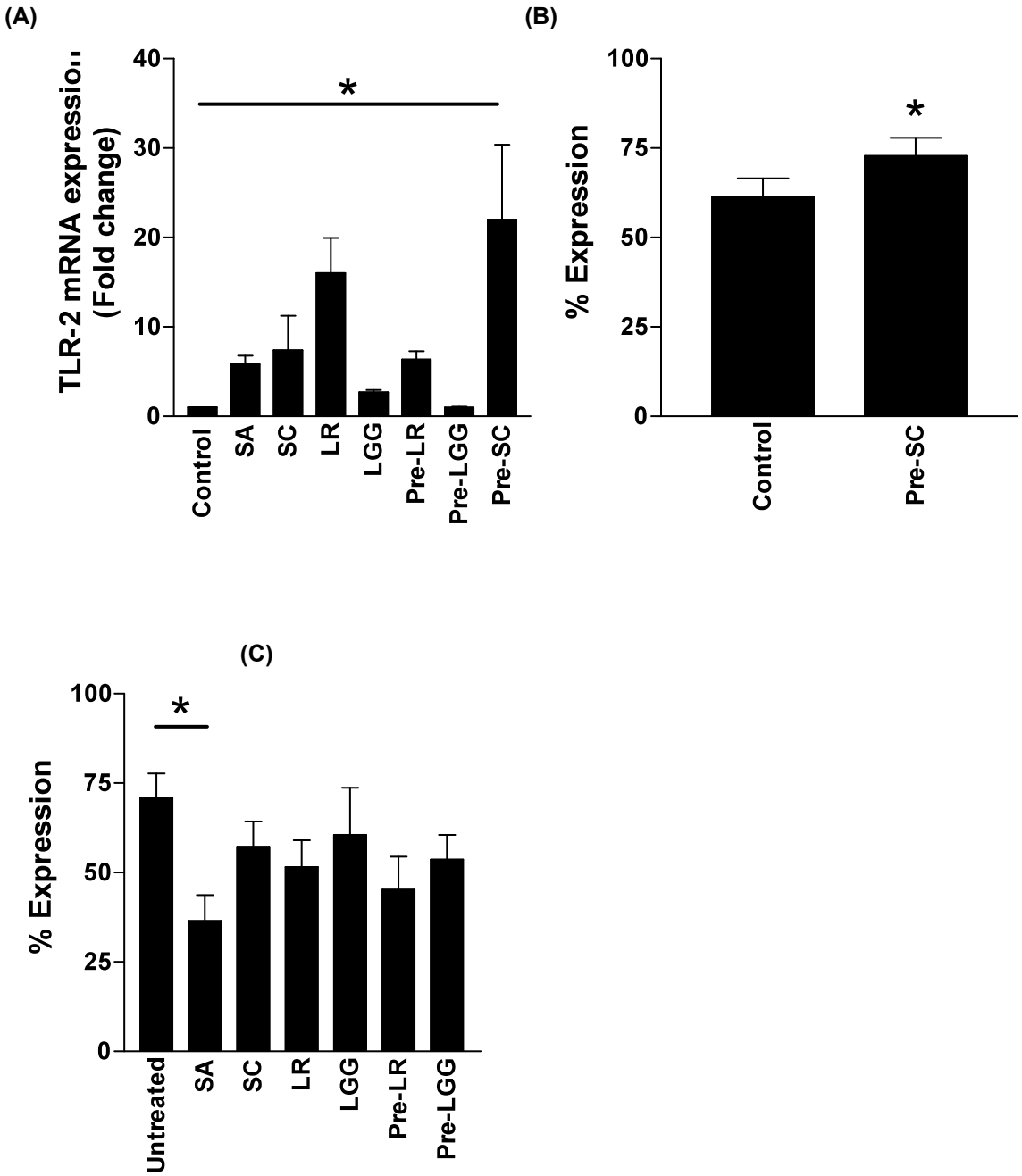


Figure 6.3. TLR-2 mRNA and peptide expression in NHEK exposed to bacteria.
A) TLR-2 mRNA expression in NHEK was analysed by quantitative RT-PCR. Results were normalised using HMBS and SDHA as endogenous controls and are shown as fold changes relative to untreated controls. There was no significant change in the mRNA expression in cells infected with *S. aureus* (SA) (10^6), *S. capitis* (SC) (10^4), *L. reuteri* (LR) (10^8), *L. rhamnosus* GG (LGG) (10^8), pre-exposed to *L. reuteri* (Pre-LR), or *L rhamnosus* prior to *S. aureus* infection (Pre-LGG), compared to the control. However, NHEK pre-exposed to *S. capitis* prior to *S. aureus* infection (Pre-SC) had a significantly increased mRNA expression (22 ± 8.4 fold increase, P=0.009*). **B)** Flow cytometry analysis of TLR-2. NHEK pre-exposed to *S. capitis* prior to *S. aureus* (Pre-SC) infection had a significantly higher percentage of cells expressing TLR-2 (72.8 ± 5.1%) compared to untreated controls (61.3 ± 5.3%, P<0.001*). **C)** Flow cytometry analysis of TLR-2. Treatment of NHEK with *S. aureus* resulted in a significant reduction in the number of NHEK expressing TLR-2 (36.4 ± 7.3%) compared to control untreated cells (70.9 ± 6.8 %, P=0.018*). Results are expressed as the mean ± SEM (n=3).

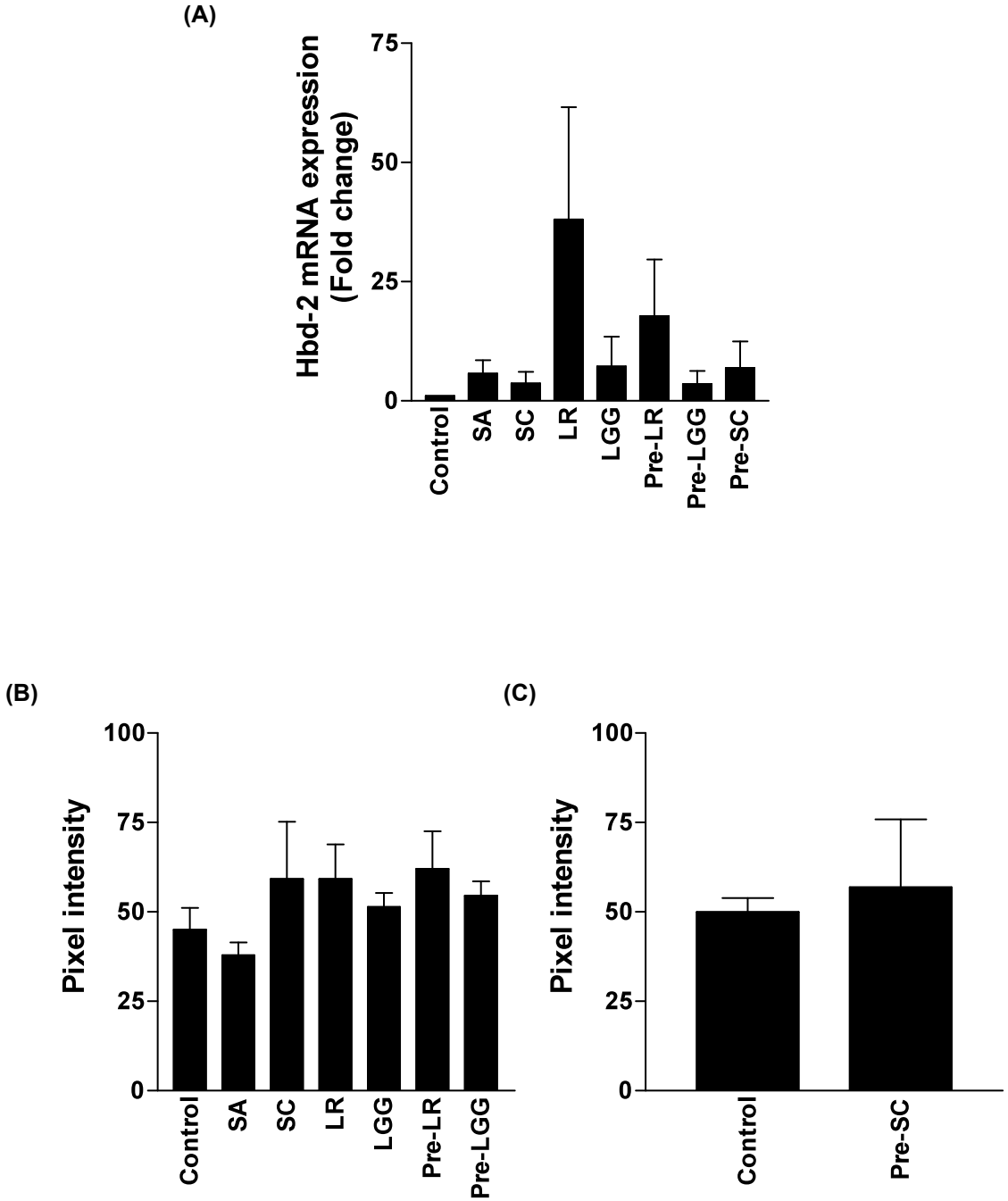
6.2 Antimicrobial peptide (AMP) production by NHEK

Previous studies have demonstrated that a number of antimicrobial peptides (AMPs) are produced by keratinocytes in response to bacteria. These include human beta defensins -2 and -3 (Hbd-2 and Hbd-3), cathelicidin, RNase-7 and psoriasin. Each AMP is considered active against specific microorganisms. For example, Hbd-3, cathelicidin and RNase-7 contribute to *S. aureus* killing (Harder et al., 2001, Braff et al., 2005c, Dorschner et al., 2001, Harder and Schröder, 2002) while psoriasin is particularly active against *E. coli* (Gläser et al., 2005). Hbd-2 is effective at killing *E. coli* and *P. aeruginosa* and bacteriostatic for *S. aureus* (Harder et al., 2004). In the gut, probiotics can induce production of Hbd-2, enhancing the mucosal barrier against pathogen infection (Wehkamp et al., 2004). In addition, *S. epidermidis* can amplify the production of AMPs in skin (Wanke et al., 2011). However, no work exists on the ability of probiotics to induce AMP production in keratinocytes. Therefore, the production of AMPs by NHEK in response to probiotic bacteria was compared to the response of NHEK to a skin pathogen (*S. aureus*) and a skin commensal (*S. capitis*).

6.2.1 Human beta defensin-2 (Hbd-2)

Human beta defensin-2 (Hbd-2) is found within the lamellar bodies of keratinocytes in the *stratum spinosum* of the epidermis (Oren et al., 2003). Hbd-2 is constitutively produced by NHEK, though its expression can be induced by exposure to proinflammatory cytokines (e.g. TNF-α) and heat-killed bacteria (Harder et al., 1997).

Hbd-2 production was analysed using immunocytochemistry and quantitative reverse transcription PCR (qRT-PCR). Quantitative RT-PCR demonstrated that there was no significant change in the expression of Hbd-2 mRNA in NHEK exposed to bacteria for 12h compared to controls ($P>0.05$) (Figure 6.4a). Immunocytochemistry was performed as described in Section 2.4.1, and the pixel intensity of AMP expression adjacent to cell nuclei measured using Image-J. Immunocytochemistry results confirmed that there was no significant difference in the expression of Hbd-2 peptide pixel intensity with exposure of NHEK to any of the bacteria used after 12h ($P>0.05$) (Figure 6.4b, c and d).


(A)
Hbd-2 mRNA expression
(Fold change)
75
50
25
0
Control
SA
SC
LR
LGG
Pre-LR
Pre-LGG
Pre-SC
(B)
Pixel intensity
100
75
50
25
0
Control
SA
SC
LR
LGG
Pre-LR
Pre-LGG
(C)
Pixel intensity
100
75
50
25
0
Control
Pre-SC

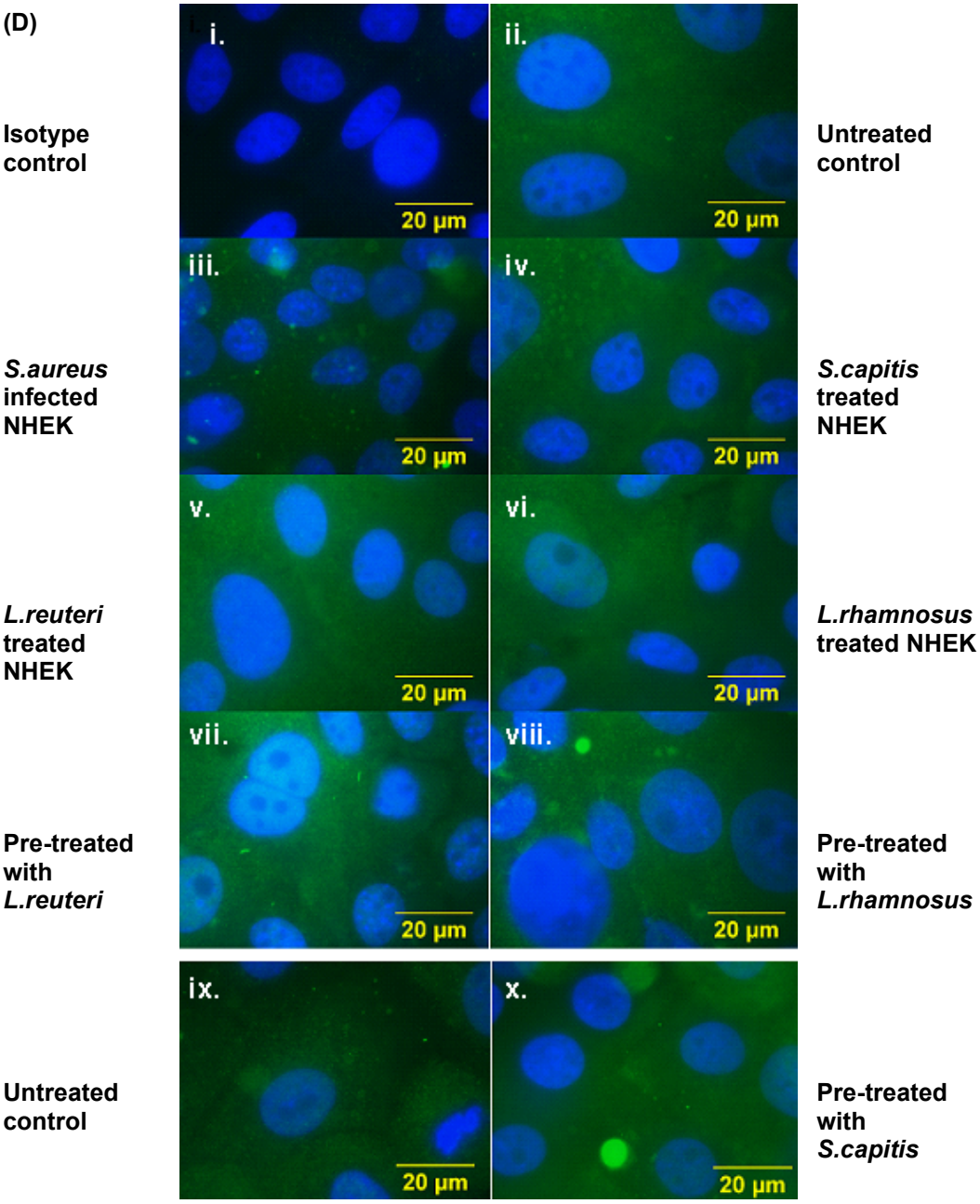
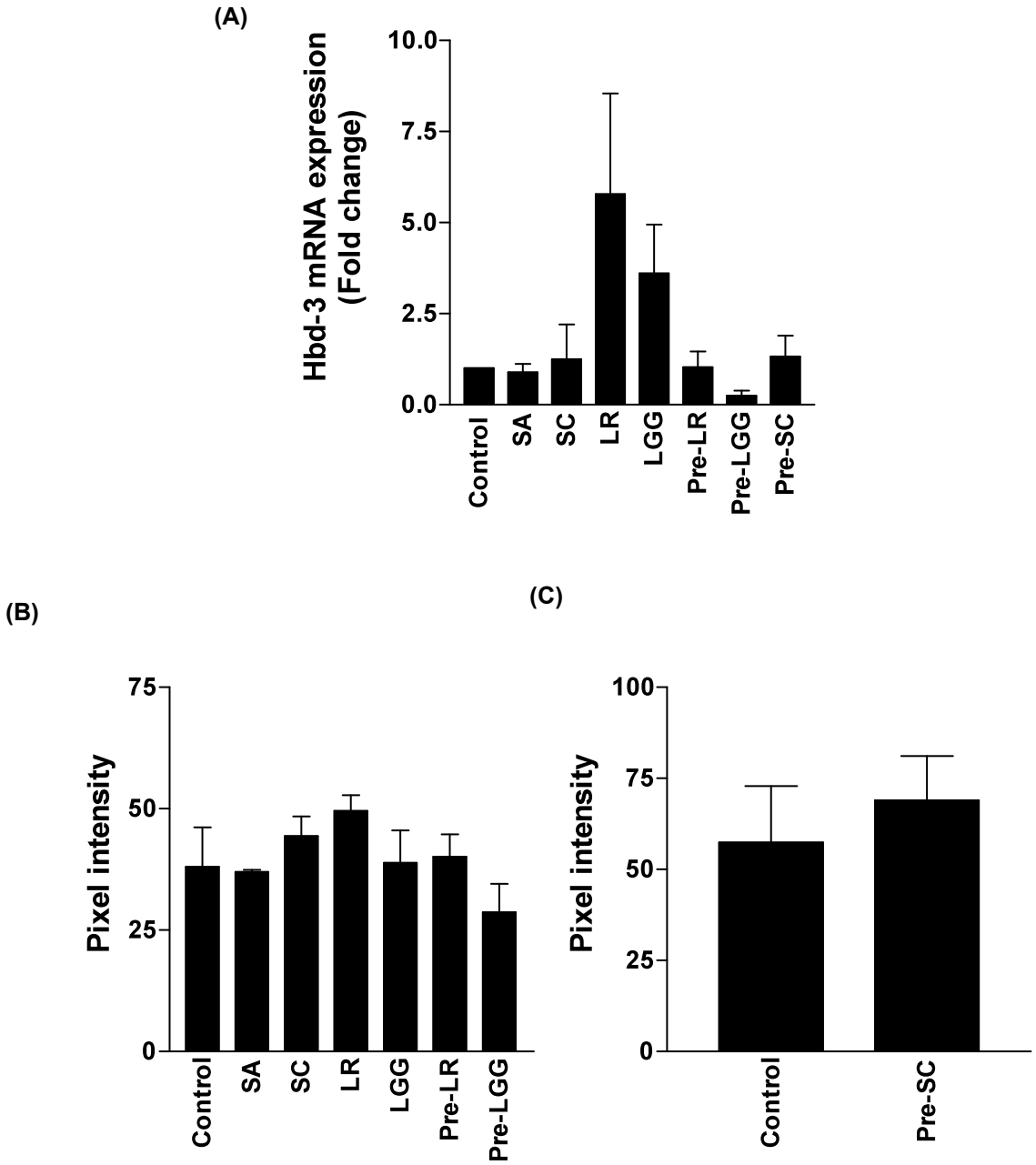


Figure 6.4. Hbd-2 expression did not change significantly with exposure to bacteria for 12h. **A)** Hbd-2 mRNA expression (n=3). Results were normalised using HMBS and SDHA as endogenous controls and are shown as fold changes relative to untreated controls. There was no significant change in Hbd-2 mRNA expression in NHEK exposed to bacteria compared to controls (P>0.05). **B)** Immunocytochemistry results. Untreated NHEK expressed Hbd-2 at a pixel intensity of 45 ± 6.1, while *S. aureus* (SA) treated NHEK expressed Hbd-2 at a pixel intensity of 37.8 ± 3.6. *S. capitis* (SC) treated NHEK expressed Hbd-2 at a pixel intensity of 59.2 ± 16. *L. reuteri* (LR) treated NHEK expressed Hbd-2 at a pixel intensity of 59.2 ± 9.7 while *L. rhamnosus* GG treated NHEK expressed Hbd-2 at a pixel intensity of 51.4 ± 3.9. NHEK pre-exposed to *L. reuteri* for 1h prior to *S. aureus* infection (Pre-LR) expressed Hbd-2 at a pixel intensity of 62.1 ± 10.5 while NHEK pre-exposed to *L. rhamnosus* GG (Pre-LGG) expressed Hbd-2 at a pixel intensity of 54.6 ± 4. (n=4). **C)** There was no significant difference in Hbd-2 expression in untreated NHEK (49.9 ±3.9) and NHEK pre-exposed to *S. capitis* prior to *S. aureus* infection (Pre-SC) (56.87±18.96). **D)** Representative images of NHEK from one individual experiment. Blue = nuclei, Green = AMP. i) isotype control, ii) untreated control, iii) *S. aureus* infected NHEK, iv) *S. capitis* infected NHEK v) NHEK exposed to *L. reuteri*, vi) NHEK exposed to *L. rhamnosus* GG, vii) NHEK pre-exposed to *L. reuteri* prior to *S. aureus* infection, viii) NHEK pre-exposed to *L. rhamnosus* GG prior to *S. aureus* infection, ix) untreated control, x) NHEK pre-exposed to *S. capitis* prior to *S. aureus* infection. Results are expressed as the mean ± SEM.

6.2.2 Human beta defensin-3 (Hbd-3)

Human beta defensin-3 (Hbd-3) is constitutively expressed by keratinocytes *in vitro.* Hbd-3 expression can be induced by exposure to proinflammatory cytokines and heat-killed bacteria (Harder et al., 2001). Hbd-3 exerts potent antimicrobial activity particularly against *S. aureus* (Harder et al., 2001, Schibli et al., 2002).

Quantitative RT-PCR revealed there was no significant change in the expression of Hbd-3 mRNA on exposure of NHEK to bacteria (Figure 6.5a). This was confirmed using immunocytochemistry (P>0.05, Figure 6.5b, c and d).



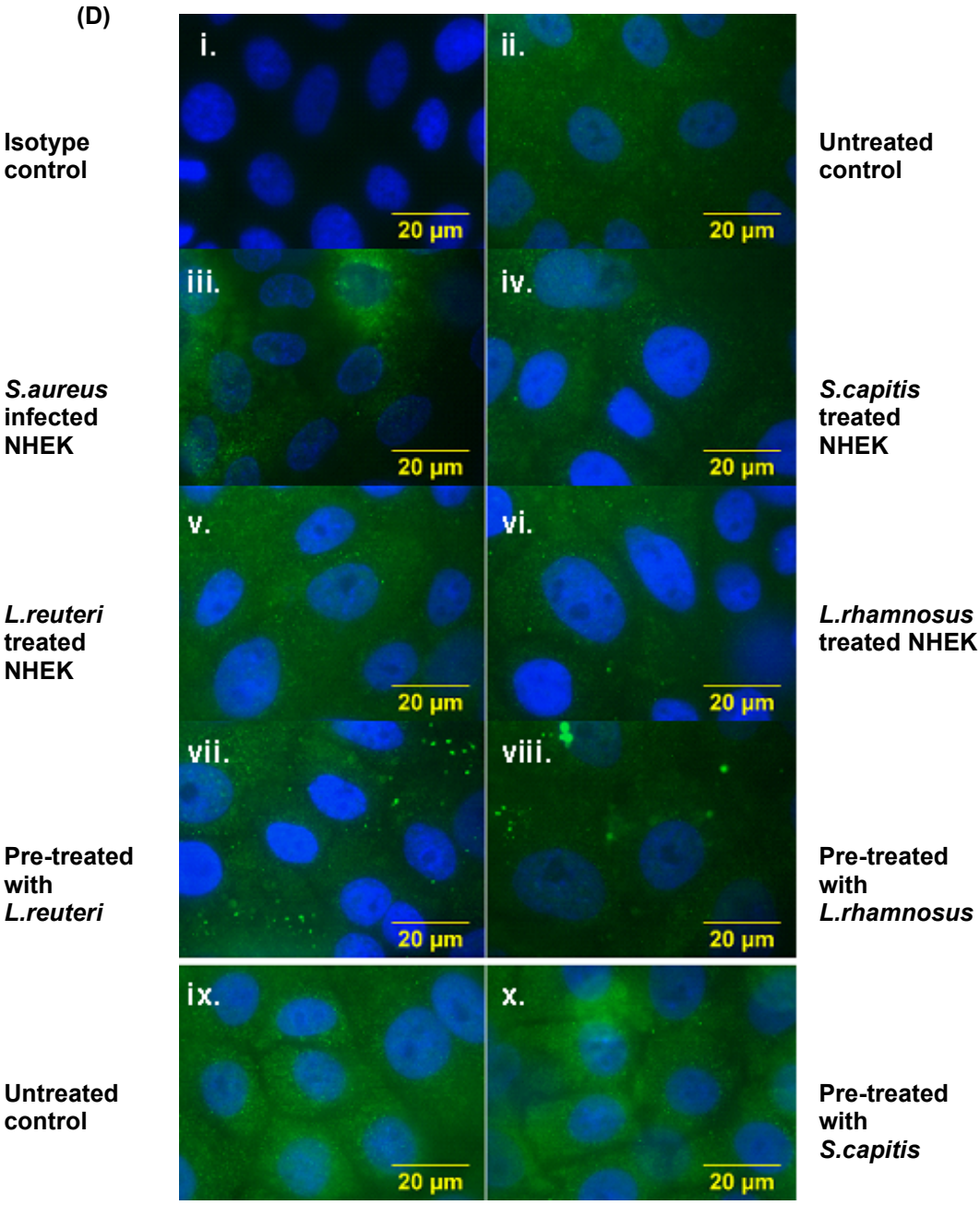
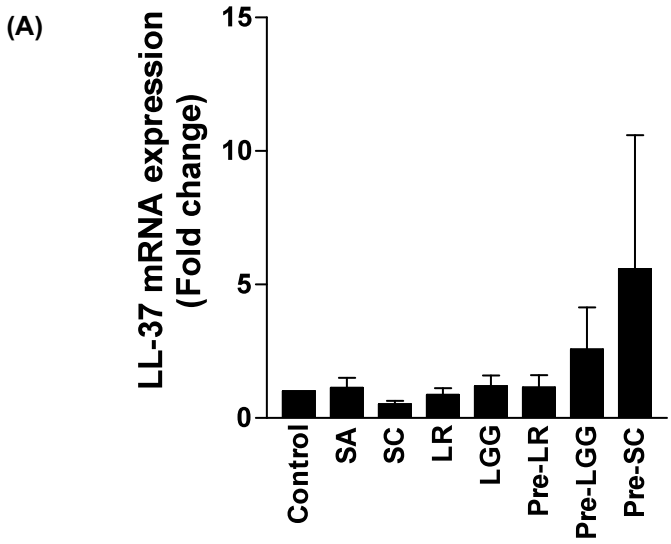
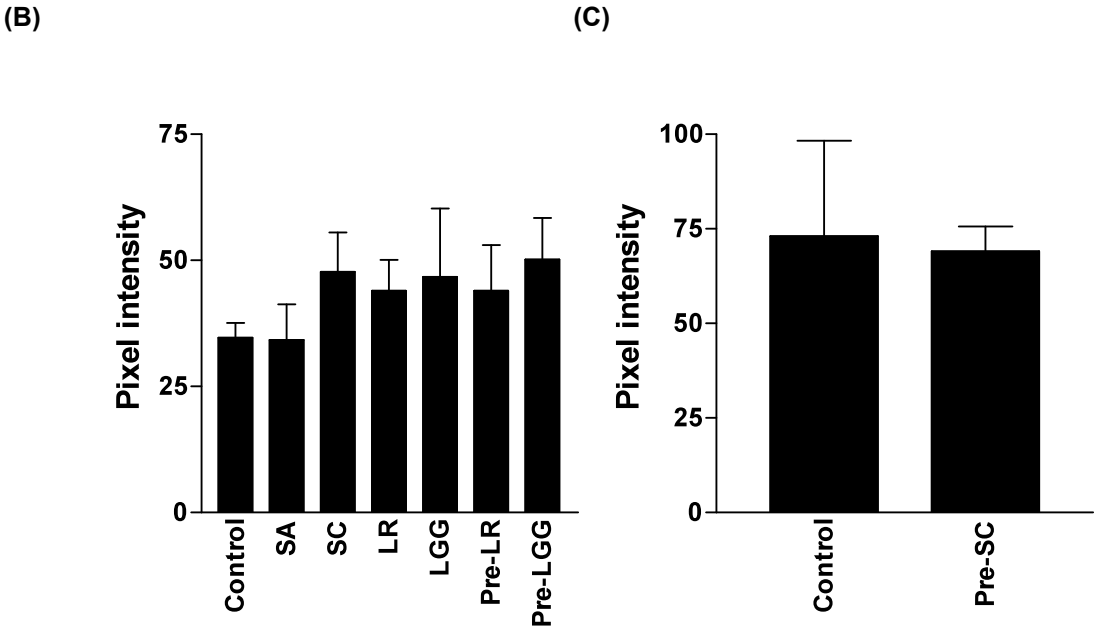


Figure 6.5. Hbd-3 expression did not significantly change after 12h exposure to bacteria. **A)** Hbd-3 mRNA expression. Results were normalised using HMBS and SDHA as endogenous controls and are shown as fold changes relative to untreated controls. There was no significant change in the expression of Hbd-3 mRNA in NHEK exposed to bacteria compared to controls ($P>0.05$). **B)** Immunocytochemistry results. Untreated NHEK expressed Hbd-3 at a pixel intensity of 38 ± 8.2. *S. aureus* (SA) infected NHEK expressed Hbd-3 at a pixel intensity of 36.9 ± 0.4 while cells exposed to *S. capitis* (SC) expressed Hbd-3 at a pixel intensity of 44.3 ± 4.1. NHEK exposed to probiotics *L. reuteri* (LR) and *L. rhamnosus* GG (LGG) expressed Hbd-3 at pixel intensities of 49.5 ± 3.2 and 38.9 ± 6.7 respectively. NHEK pre-exposed to *L. reuteri* prior to *S. aureus* infection (Pre-LR) expressed Hbd-3 at a pixel intensity of 40.1 ± 4.6 while cells pre-exposed to *L. rhamnosus* GG prior to *S. aureus* infection (Pre-LGG) expressed Hbd-3 at a pixel intensity of 28.7 ± 5.9. **C)** There was no significant difference in the pixel intensity of untreated NHEK (57.38 ± 13) compared to NHEK pre-exposed to *S. capitis* prior to *S. aureus* infection (Pre-SC) (68.95 ± 12.2). **D)** Representative images from one experiment. Blue = nuclei, Green = AMP. i) isotype control, ii) untreated control, iii) *S. aureus* infected NHEK, iv) *S. capitis* infected NHEK v) NHEK exposed to *L. reuteri*, vi) NHEK exposed to *L. rhamnosus* GG, vii) NHEK pre-exposed to *L. reuteri* prior to *S. aureus* infection, viii) NHEK pre-exposed to *L. rhamnosus* GG prior to *S. aureus* infection, ix) untreated control, x) NHEK pre-exposed to *S. capitis* prior to *S. aureus* infection. Results are expressed as the mean ± SEM (n=3).

6.2.3 Cathelicidin (LL-37)

Cathelicidin is active against the skin pathogens *S. aureus* and *S. pyogenes* (Braff et al., 2005c). Cathelicidin is stored in an inactive form (hCAP-18) and activated by cleavage of the C-terminal group to the active form, LL-37 (Sorensen et al., 2001) which is stored in the lamellar granules, ready for release (Braff et al., 2005a). The expression of LL-37 in response to exposure to *S. aureus*, *S. capitis*, and lactobacilli was analysed using immunocytochemistry and qRT-PCR. There was no significant change in the expression of the mRNA or peptide on exposure of NHEK to bacteria for 12h (P>0.05) (Figure 6.6a, b, c and d).





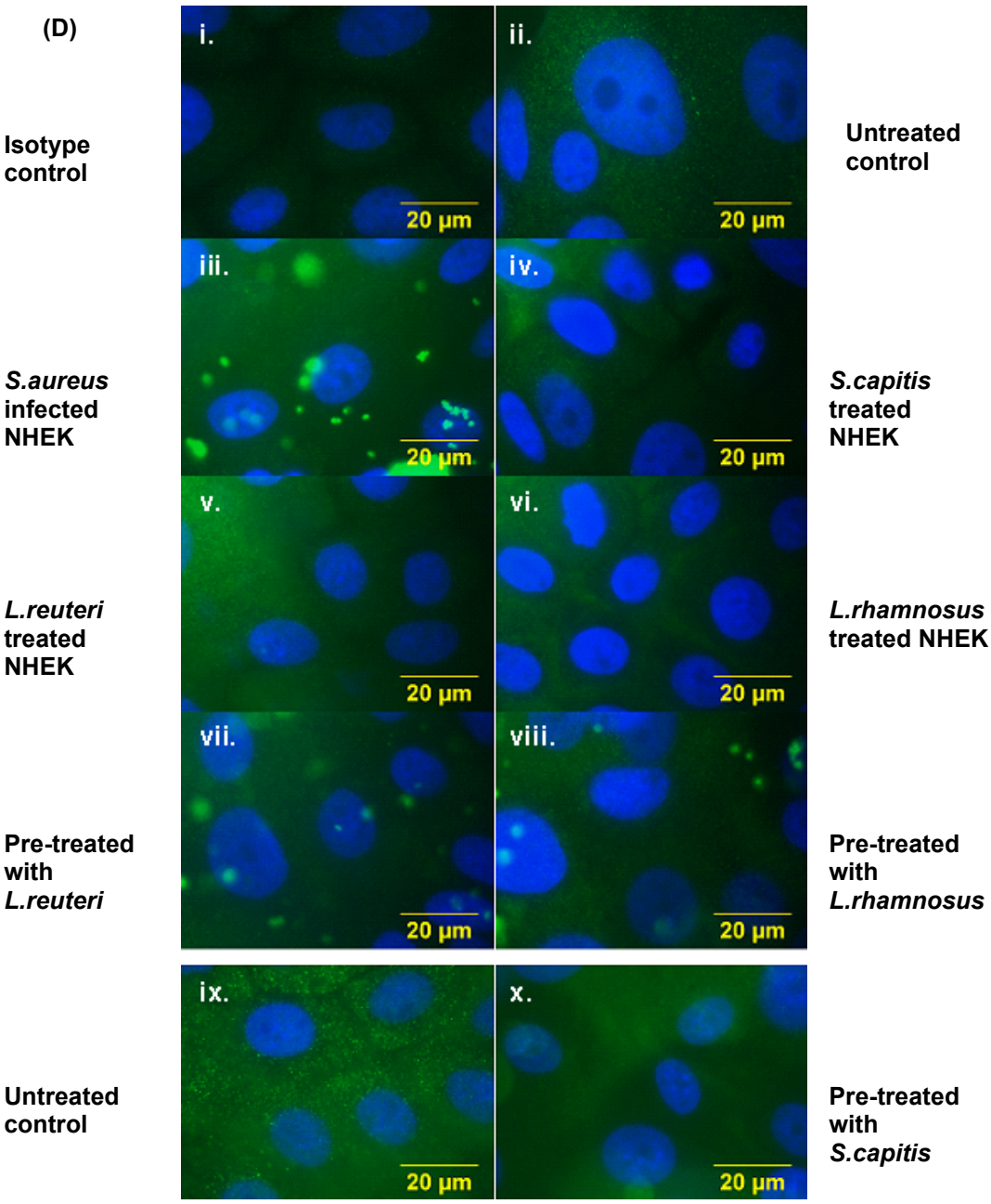
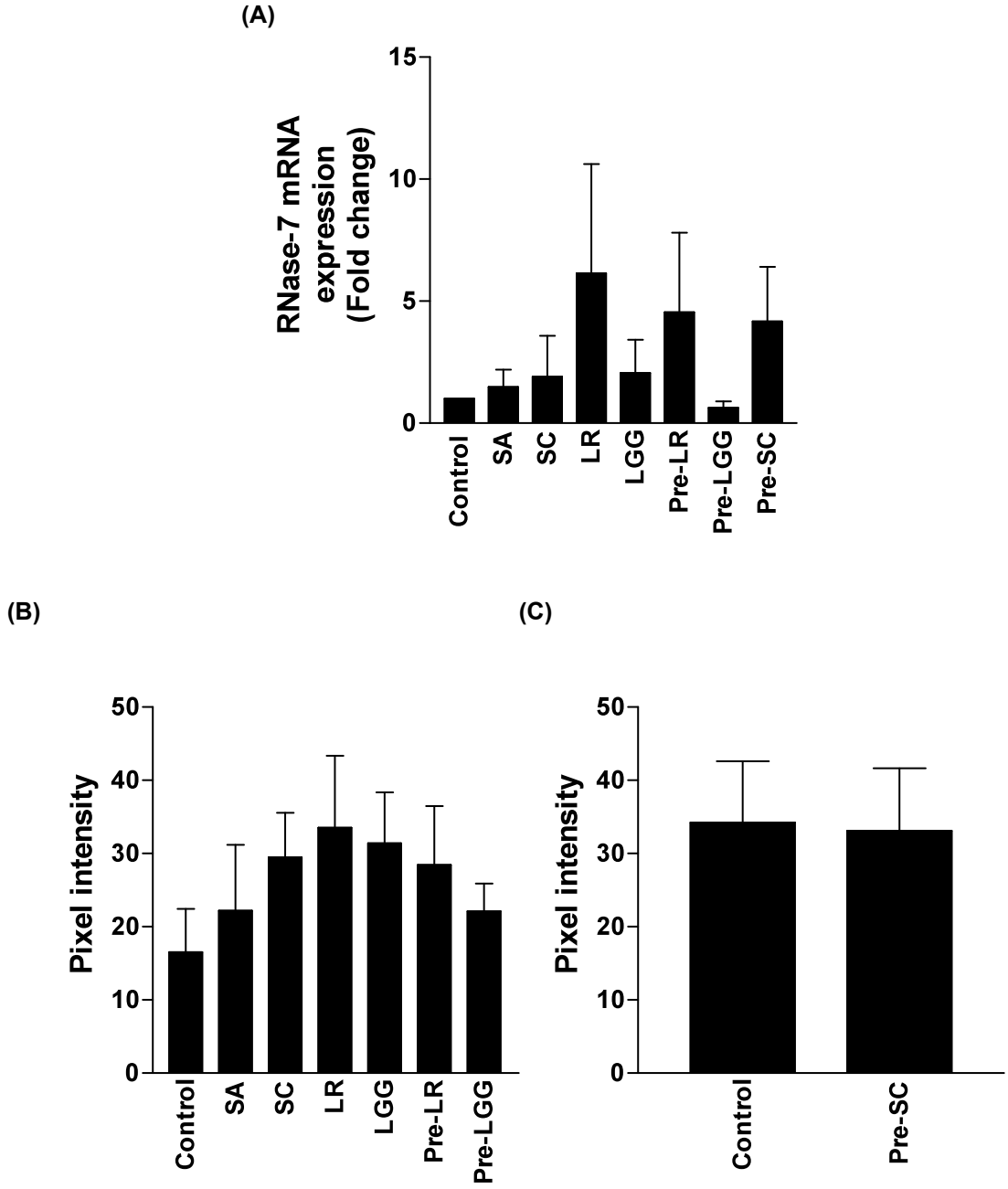


Figure 6.6. LL-37 expression in NHEK did not change with exposure to bacteria for 12h. **A)** LL-37 mRNA expression (n=3). Results were normalised using HMBS and SDHA as endogenous controls and are shown as fold changes relative to untreated controls. There was no significant change in the expression of LL-37 mRNA in NHEK exposed to bacteria compared to controls (P>0.05). **B)** Immunocytochemistry results (n=4). Untreated NHEK had a pixel intensity of 34.7 ± 3. *S. aureus* (SA) infected NHEK expressed LL-37 at a pixel intensity of 34.2 ± 7 while cells exposed to *S. capitis* (SC) expressed LL-37 at a pixel intensity of 47.7 ± 7.8. NHEK exposed to probiotics *L. reuteri* (LR) and *L. rhamnosus* GG (LGG) expressed LL-37 at pixel intensities of 44 ± 6.1 and 46.7 ± 13.6 respectively. NHEK pre-exposed to *L. reuteri* prior to *S. aureus* infection (Pre-LR) expressed LL-37 at a pixel intensity of 44 ± 9.1 while cells pre-exposed to *L. rhamnosus* GG prior to *S. aureus* infection (Pre-LGG) expressed LL-37 at a pixel intensity of 50.2 ± 8.3. **C)** There was no significant difference in the pixel intensity of LL-37 in untreated control NHEK (73.07 ± 25.19) compared to NHEK pre-exposed to *S. capitis* prior to *S. aureus* infection (Pre-SC) (69.06 ± 6.6). **D)** Representative images from one experiment. Blue = nuclei, Green = AMP i) isotype control, ii) untreated control, iii) *S. aureus* infected NHEK, iv) *S. capitis* infected NHEK v) NHEK exposed to *L. reuteri*, vi) NHEK exposed to *L. rhamnosus* GG, vii) NHEK pre-exposed to *L. reuteri* prior to *S. aureus* infection, viii) NHEK pre-exposed to *L. rhamnosus* GG prior to *S. aureus* infection, ix) untreated control, x) NHEK pre-exposed to *S. capitis* prior to *S. aureus* infection. Results are expressed as the mean ± SEM.

6.2.4 RNase-7

RNase-7 is found in all layers of the skin but predominantly in the granular keratinocytes where it is secreted into the *stratum corneum* (Köten et al., 2009). RNase-7 is particularly active against *S. aureus* (Harder and Schröder, 2002) and can be induced in NHEK in response to *S. aureus* (Wanke et al., 2011). However, exposure of NHEK to bacteria did not induce any significant change in expression levels of the mRNA or peptide expression in NHEK (P>0.05) (Figure 6.7a, b, c, and d).



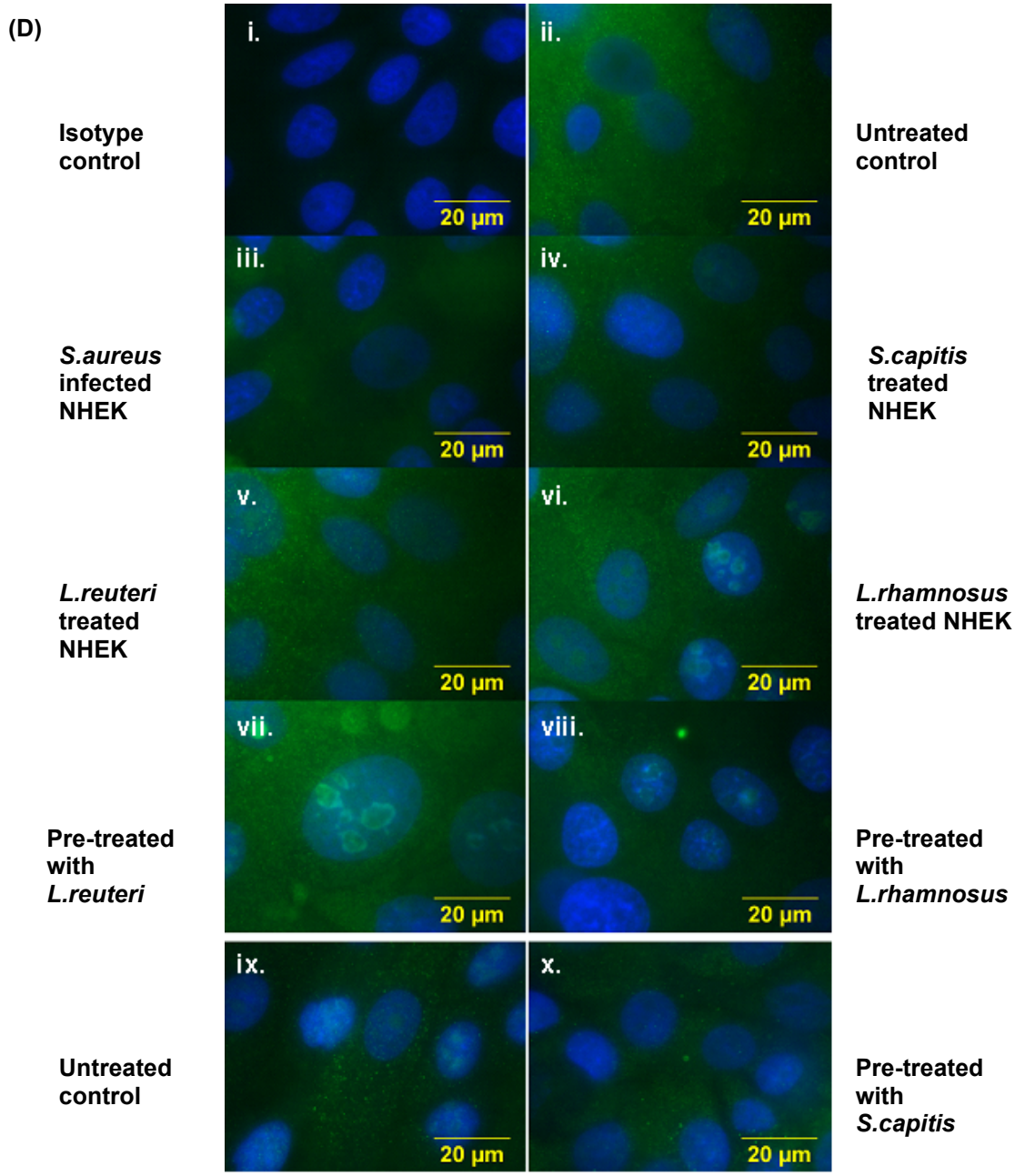
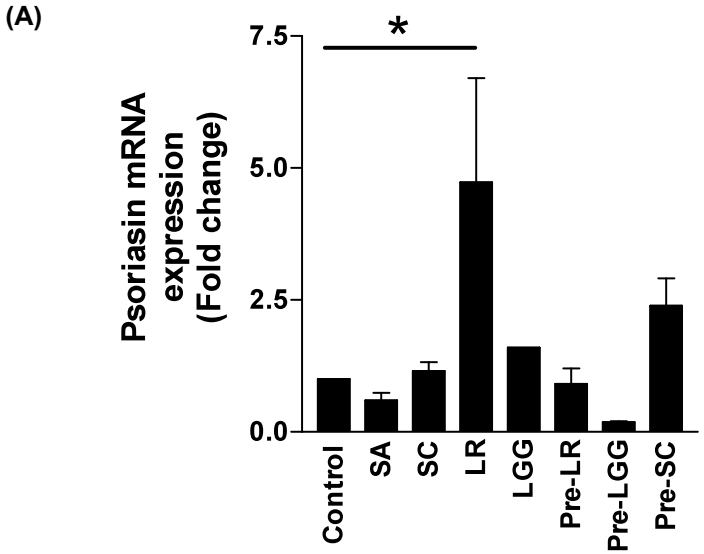
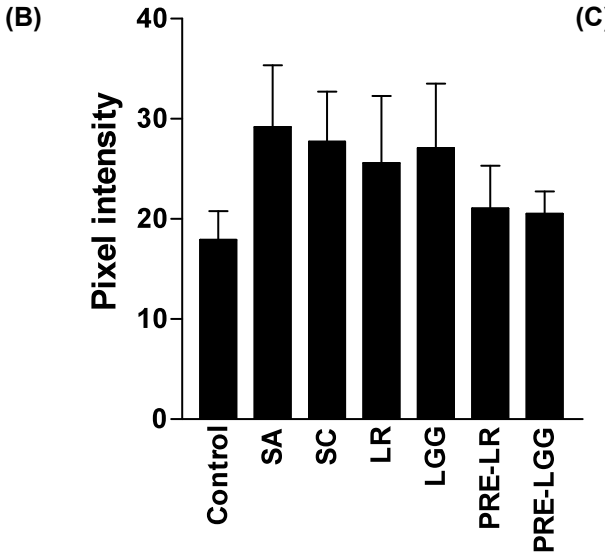


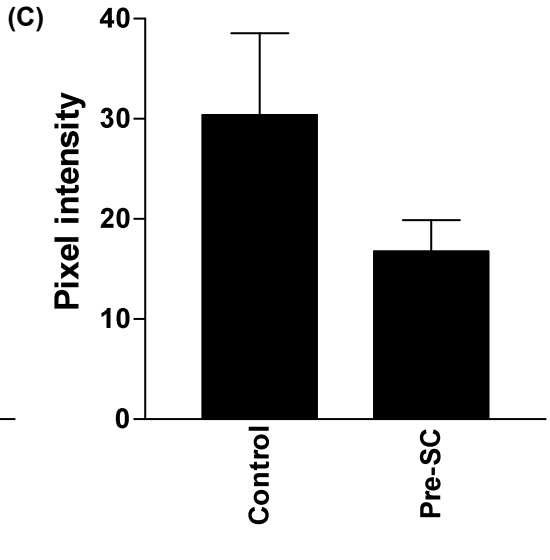
Figure 6.7. RNase-7 expression in NHEK did not change in response to 12h exposure to bacteria.
A) RNase-7 mRNA expression (n=3). Results were normalised using HMBS and SDHA as endogenous controls and are shown as fold changes relative to untreated controls. There was no significant change in the expression of RNase-7 mRNA in NHEK exposed to bacteria compared to controls (P>0.05). **B)** Immunocytochemistry results (n=4). Untreated NHEK expressed RNase-7 at a pixel intensity of 16.5 ± 5.9, while *S. aureus* (SA) treated NHEK expressed RNase-7 at a level of 22.2 ± 9. *S. capitis* (SC) treated NHEK expressed RNase-7 at a level of 29.5 ± 6.2. *L. reuteri* (LR) expressed RNase-7 at a level of 33.5 ± 9.8 while *L. rhamnosus* GG expressed RNase-7 at a level of 31.4 ± 7. NHEK pre-exposed to *L. reuteri* for 1h prior to *S. aureus* infection (Pre-LR) expressed RNase-7 at a level of 28.5 ± 8 while NHEK pre-exposed to *L. rhamnosus* GG (Pre-LGG) expressed the peptide at a level of 22.1 ± 3.8. **C)** There was no significant difference in the pixel intensity of RNase-7 in untreated NHEK (34.2 ± 8.4) compared to NHEK pre-exposed to *S. capitis* prior to *S. aureus* infection (Pre-SC) (33.1 ± 8.5). **D)** Images taken from one experiment. Blue = nuclei, Green = AMP i) isotype control, ii) untreated control, iii) *S. aureus* infected NHEK, iv) *S. capitis* infected NHEK v) NHEK exposed to *L. reuteri*, vi) NHEK exposed to *L. rhamnosus* GG, vii) NHEK pre-exposed to *L. reuteri* prior to *S. aureus* infection, viii) NHEK pre-exposed to *L. rhamnosus* GG prior to *S. aureus* infection, ix) untreated control, x) NHEK pre-exposed to *S. capitis* prior to *S. aureus* infection. Results are expressed as the mean ± SEM.

6.2.5 Psoriasin (S100A7)

Psoriasin is found in normal skin in the cytoplasm of suprabasal keratinocytes, and is increased in psoriatic skin (Madsen et al., 1991, Gläser et al., 2005). Psoriasin is particularly effective against *E. coli* (Gläser et al., 2005) though at higher concentrations it has also been shown to kill *S. aureus* (Gläser et al., 2008). Quantitative RT-PCR demonstrated that NHEK exposed to *L. reuteri* had significantly higher levels of psoriasin mRNA than controls (4.7 ± 2 fold increase, P=0.047) (Figure 6.8a). However, immunocytochemistry demonstrated that there was no change in protein expression in NHEK exposed to bacteria (P>0.05) (Figure 6.8b, c and d).







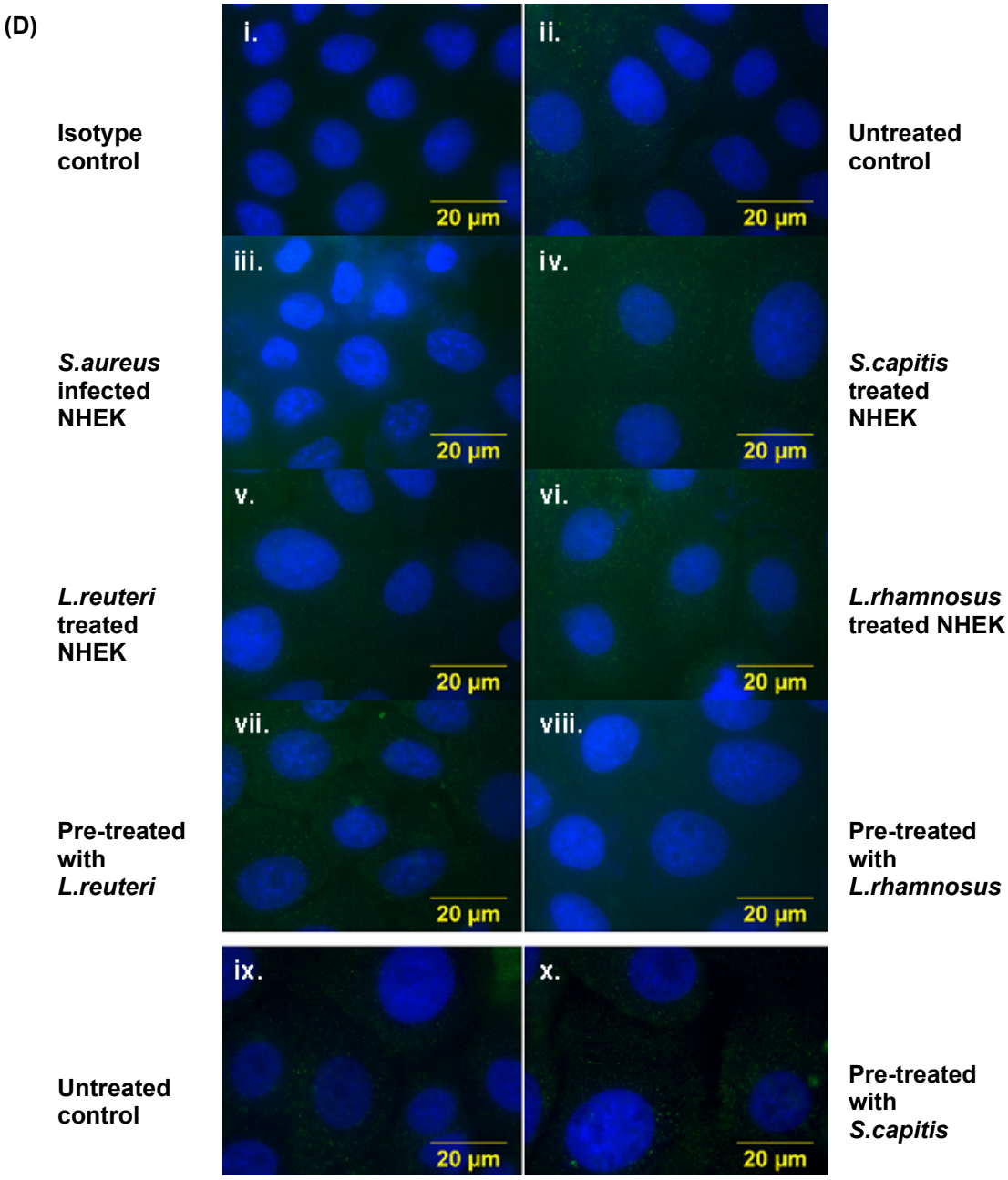


Figure 6.8. Psoriasin expression by NHEK in response to 12h exposure to bacteria.
A) Psoriasin mRNA expression (n=3). Results were normalised using HMBS and SDHA as endogenous controls and are shown as fold changes relative to untreated controls. NHEK exposed to *L. reuteri* expressed significantly more psoriasin mRNA (4.7 ± 2 fold increase) compared to untreated controls (P=0.047*). **B)** Immunocytochemistry results (n=4). Untreated NHEK expressed psoriasin at a pixel intensity of 17.9 ± 2.9. *S. aureus* (SA) infected NHEK had a pixel intensity of 29.2 ± 6.2 while cells exposed to *S. capitis* (SC) had a pixel intensity of 27.7 ± 5. NHEK exposed to *L. reuteri* (LR) and *L. rhamnosus* GG (LGG) had pixel intensities of 25.6 ± 6.7 and 27.1 ± 6.5 respectively. NHEK pre-exposed to *L. reuteri* prior to *S. aureus* infection (Pre-LR) had a pixel intensity of 21 ± 4.3 while cells pre-exposed to *L. rhamnosus* GG prior to *S. aureus* infection (Pre-LGG) had a pixel intensity of 20.5 ± 2.2. **C)** There was no significant difference in the pixel intensity of psoriasin expression in untreated control NHEK (30.4 ± 8.2) compared to NHEK pre-exposed to *S. capitis* prior to *S. aureus* infection (Pre-SC) (16.7 ± 3.1). **D)** Images taken from one experiment. Blue = nuclei, Green = AMP. i) isotype control, ii) untreated control, iii) *S. aureus* infected NHEK, iv) *S. capitis* infected NHEK v) NHEK exposed to *L. reuteri*, vi) NHEK exposed to *L. rhamnosus* GG, vii) NHEK pre-exposed to *L. reuteri* prior to *S. aureus* infection, viii) NHEK pre-exposed to *L. rhamnosus* GG prior to *S. aureus* infection, ix) untreated control, x) NHEK pre-exposed to *S. capitis* prior to *S. aureus* infection. Results are expressed as the mean ± SEM (n=4).

6.3 IL-8 expression by NHEK

As cytokine production is a direct result of TLR signalling, it was decided to explore whether any of the bacteria utilised had any effect upon the production of cytokines by NHEK. NHEK are known to produce IL-8, a neutrophil chemoattractant *in vitro*. *L. reuteri* reportedly has immunostimulatory activity (Jones and Versalovic, 2009) while *L. rhamnosus* GG has been shown to have immunomodulatory properties upon cytokine production in the gut (Pessi et al., 2000). Therefore, a comparison of the effect of these two probiotics upon IL-8 production in NHEK was performed. IL-8 ELISAs revealed that NHEK pre-exposed to *L. reuteri* prior to *S. aureus* infection secreted significantly higher levels of IL-8 compared to untreated control cells (P=0.0001). Additionally, there was a non-significant trend toward increased IL-8 production in NHEK exposed to *L. reuteri* alone (Figure 6.9).

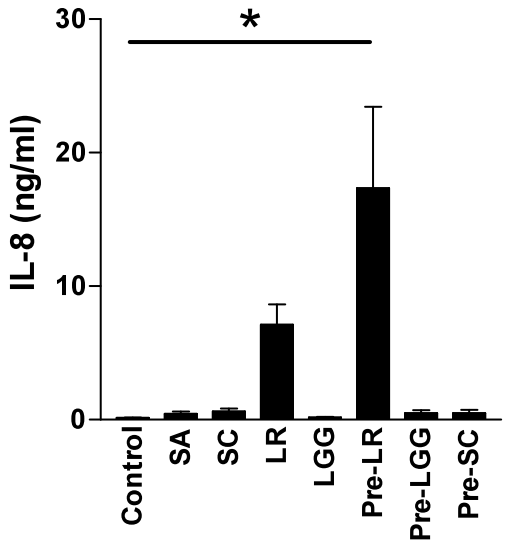


Figure 6.9. IL-8 levels in CFS of NHEK exposed to bacteria.
IL-8 ELISA of CFS from NHEK exposed to bacteria. Untreated control NHEK produced 0.2 ± 0.02 ng/ml IL-8. *S. aureus* treated NHEK (SA) produced 0.44 ± 0.17 ng/ml IL-8. NHEK exposed to *S. capitis* (SC) produced 0.6 ± 0.2 ng/ml IL-8. NHEK exposed to *L. reuteri* (LR) produced 7.1 ± 1.5 ng/ml IL-8. NHEK pre-exposed to *L. reuteri* prior to *S. aureus* infection (Pre-LR) had significantly higher levels of IL-8 (17.3 ± 6.1 ng/ml) compared to untreated controls (P=0.0001*). NHEK pre-exposed to *L. rhamnosus* GG (Pre-LGG) produced 0.5 ± 0.2 ng/ml while NHEK pre-exposed to *S. capitis* prior to *S. aureus* infection (Pre-SC) produced 0.5 ± 0.2 ng/ml. Results are expressed as the mean ± SEM (n=6).

6.4 Conclusions

6.4.1 Toll-like receptor-2 expression

TLR-2 is a pattern recognition receptor (PRR) known to recognise several Gram positive cell wall components including lipopeptides and lipoteichoic acid (Section 1.1.5.1). There has been some debate as to whether TLR-2 also recognises peptidoglycan (Kawai et al., 2002, Travassos et al., 2004, Dziarski and Gupta, 2005). TLR-2 plays an important role in recognition of *S. aureus* by the innate immune system, as TLR-2 deficient mice are more susceptible to infection by *S. aureus* (Takeuchi et al., 2000).

TLR-2 is expressed by keratinocytes in all layers of the epidermis, though principally in the basal layers of the epidermis *in vivo* (Baker et al., 2003, Begon et al., 2007). In contrast, expression of TLR-2 has been shown to increase with differentiation of keratinocytes in culture (Lew et al., 2009). Keratinocytes are known to express TLR-2 predominantly cytoplasmically, perhaps as a mechanism by which keratinocytes prevent over-stimulation by the commensal microflora (Begon et al., 2007). TLR-2 expression by NHEK can be induced by exposure of keratinocytes to the proinflammatory cytokines TNF-α and IFN-γ (Begon et al., 2007, Lew et al., 2009). Bacterial ligands, such as peptidoglycan (PGN) (Kobayashi et al., 2009) and lipoteichoic acid (LTA) (Lew et al., 2009) reportedly induce TLR-2 expression in keratinocytes. To date, little work has been performed using viable bacteria to determine their effect upon TLR-2 expression in NHEK. Therefore, experiments were performed to determine whether the expression of TLR-2 in NHEK changes in response to a skin pathogen, a skin commensal, and probiotic lactobacilli.

In the current study, exposure of NHEK to *L. reuteri* or *L. rhamnosus* GG alone did not significantly alter the expression of TLR-2 mRNA or protein by NHEK (Figure 6.3a and c). Similarly, *S. capitis* did not induce any increase in mRNA or protein levels of TLR-2 (Figure 6.3a and b). This agrees with the results from previous studies demonstrating that viable *S. epidermidis* only slightly increased TLR-2 mRNA expression (<5-fold) in nasal epithelia after 12h exposure (Quinn and Cole, 2007). Overall, this work suggests that NHEK do not respond to commensal staphylococci or probiotic bacteria by up-regulating TLR-2 expression.

Examination of the effect of the skin pathogen *S. aureus,* upon TLR-2 expression demonstrated that TLR-2 protein (but not mRNA) expression in NHEK was reduced following 12h infection with *S. aureus*. By contrast, *S. aureus* derived LTA did promote an increase in TLR-2 both at the message and protein levels (Figure 6.2).

Other studies have also demonstrated an increase in TLR-2 in response to *S. aureus* derived components (Lew et al., 2009), though few studies have utilised viable bacteria to examine TLR expression. The observation that viable *S. aureus* decreases TLR-2 protein expression could be due to cell death. However, this is unlikely given that time course assays demonstrated no significant cell death at 12h (Figure 4.2) and viability dye was used to exclude any dead cells from the analysis. There are a number of possible reasons for this, e.g. cross talk between different pattern recognition receptors could explain this result.

The use of individual ligands for the study of TLR signalling in keratinocytes has limited use because bacteria possess multiple ligands which may activate more than one PRR pathway. The interaction and possible feedback loops of such signalling are as yet unclear and may explain why different results are obtained using whole bacteria compared to single ligand experiments. Gram positive bacteria may stimulate multiple PRRs (e.g. TLR-2, TLR-9 and NOD-2) through lipopeptides, CpG DNA and peptidoglycan respectively. Therefore, it is possible that signalling interactions between different PRRs may result in suppression of inflammation (Kelly and Conway, 2005). For example, in the gut, NOD-2 signalling seemingly inhibits TLR-2 signalling (Watanabe et al., 2004) while stimulation of gut cells with TLR-2 ligands leads to reduced TLR-5 expression (van Aubel et al., 2007). In addition, it may be that prolonged exposure of NHEK to *S. aureus* for 12h results in a down-regulation of TLR-2 as a mechanism to prevent overstimulation. Bacterial lipoprotein (BLP) has been shown to induce tolerance in THP-1 monocytes *in vitro* through down-regulation of TLR-2 expression. This effect is similar to that observed with LPS tolerance, a well-known mechanism whereby pre-exposure to a small amount of LPS prevents overstimulation and excessive cytokine production in the face of a subsequent larger dose of LPS (Wang et al., 2002). A detailed investigation into the cellular signalling pathways occurring in NHEK response to *S. aureus* may give some further clarification into the mechanisms at work.

An alternative explanation for the reduction in TLR-2 expression by *S. aureus* is that pathogenic strains of *S. aureus* may suppress innate immune responses in order to promote colonisation. A strain of *S. aureus* able to colonise the nasal epithelium (carrier strain) has been compared with a lab strain for its ability to stimulate innate immune responses in nasal epithelia. Both strains of *S. aureus* were able to induce TLR-2, though the carrier strain was shown to have a delayed response. In addition, the carrier strain down-regulated defensin (AMP) production by nasal epithelia compared to the lab strain (Quinn and Cole, 2007).

It is also possible that in the present study, the loss of TLR-2 may be due to pathogen derived factors that cleave the receptor e.g. proteases. However, further work would be needed to confirm this.

Pre-exposure of NHEK to *L. reuteri* or *L. rhamnosus* GG prior to infection with *S. aureus* was measured to determine whether probiotics can influence the expression of TLR-2 in response to pathogenic bacteria. Neither TLR-2 mRNA nor protein expression was significantly altered in NHEK pre-exposed to probiotics (Figure 6.3a and c). This suggests that both probiotics were able to prevent the down-regulation of TLR-2 observed with infection with *S. aureus*. Previous work demonstrated that *L. reuteri* could inhibit the adhesion to, and invasion of, keratinocytes by *S. aureus* (Figure 5.2 and Figure 5.3). Therefore, it is possible that this is a mechanism by which both probiotics could protect NHEK from interaction of *S. aureus* with both cell surface and intracellular TLR-2.

Interestingly, pre-exposure of NHEK to *S. capitis* prior to *S. aureus* infection resulted in a significant up-regulation of TLR-2 mRNA expression (P=0.009) and protein expression (P=0.015) (Figure 6.3a and b respectively). *S. epidermidis* conditioned medium reportedly amplifies NHEK innate immune responses to *S. aureus* when used sequentially to stimulate NHEK (Wanke et al., 2011). Therefore, this may be a mechanism by which skin commensals protect the skin from the harmful effects of skin pathogens like *S. aureus*.

Overall, *S. capitis* appeared to not only prevent *S. aureus* induced TLR-2 down-regulation in NHEK, but pre-treatment with *S. capitis* appeared to up-regulate TLR-2 expression. In comparison, *L. reuteri* and *L. rhamnosus* did not appear to up-regulate TLR-2 but did prevent *S. aureus* induced down-regulation of TLR-2 in NHEK. The results suggest that NHEK respond to pathogenic and commensal bacteria in different ways, and that probiotic bacteria appear to induce responses more akin to skin commensals.

6.4.2 Antimicrobial peptide (AMP) expression in NHEK

The activation of TLRs by bacterial ligands results in signalling cascades that culminate in the production of AMPs and cytokines. For example, a <10KDa fraction from *S. epidermidis* conditioned media can induce up-regulation of Hbd-2 and -3 in NHEK in a TLR-2 dependent manner (Lai et al., 2010). The importance of AMP expression for skin defence against bacterial infection can be observed in skin disorders where AMP expression is altered. This is illustrated in atopic skin where expression of Hbd-3 (known to be effective against *S. aureus*) is diminished and *S. aureus* infections are more common than in healthy individuals (Nomura et al., 2003). Likewise, individuals with staphylococcal skin infections exhibit lower levels of RNase-7 in unaffected skin compared to healthy matched control subjects (Zanger et al., 2009). Additionally, cathelicidin deficient mice are more susceptible to streptococcal infections (Nizet et al., 2001).

The mRNA and protein expression of the AMPs Hbd-2 (Figure 6.4), Hbd-3 (Figure 6.5), cathelicidin (Figure 6.6) and RNase-7 (Figure 6.7) in NHEK were not significantly altered in response to 12h exposure to *S. aureus*, *S. capitis* or the probiotics. Each of these AMPs is capable of bactericidal/static activity against *S. aureus*. In previous experiments *S. aureus* viability was not affected by co-culture with keratinocytes (Section 4.6), suggesting that AMPs produced by NHEK under these conditions did not have a significant bactericidal effect *in vitro*. A comparison of these data with that in the literature is difficult due to the different protocols, incubation times, bacterial concentrations and preparations used, as many studies have utilised heat-killed bacteria, or bacteria conditioned media to study AMP production in keratinocytes.

In previous studies, viable *S. aureus* induced the up-regulation of Hbd-2, Hbd-3 and RNase-7 mRNA expression in NHEK after 24h infection *in vitro*. A longer exposure was possible than was used in the present work because a strain of *S. aureus* which did not induce cell death was utilised. However, in agreement with the present study, no change in AMP expression was observed at 12h exposure (Wanke et al., 2011). This contrasts with other studies which have reported the up-regulation of Hbd-3 in NHEK after just 1.5h infection with viable *S. aureus* (Menzies and Kenoyer, 2005). Others have found significant up-regulation of Hbd-2 at 16h incubation with viable *S. aureus in vitro* (Dinulos et al., 2003). *In vivo* studies have demonstrated Hbd-2 and Hbd-3 are induced in *S. aureus* skin infections compared to healthy controls (Zanger et al., 2010). The differing results found in these studies are likely due to differences in the strains of *S. aureus* used, exposure times, and concentrations used.

Viable *S. epidermidis* is purported to induce Hbd-2 significantly after 24h while Hbd-3 and RNase-7 expression are only slightly induced (Wanke et al., 2011). Others have found significant up-regulation of Hbd-3 in response to viable *S. epidermidis* after 24h (Lai et al., 2010) and Hbd-2 after 16h exposure (Chung and Dale, 2004, Dinulos et al., 2003). However, in the current study, *S. capitis* had no effect upon AMP expression. This may be due to the shorter exposure time used, so an investigation into the effect of *S. capitis* over time upon AMP expression may be required. However, in the present study, *S. capitis* killed significant numbers of NHEK after 24h exposure.

Wanke *et al* (2011) found that pre-exposure of NHEK to the conditioned media of *S. epidermidis* prior to incubation with *S. aureus* conditioned media resulted in a significant increase in the expression of Hbd-3 and RNase-7 mRNA and protein levels in comparison to cells exposed to *S. aureus* alone, suggesting that skin commensals can amplify the innate immune response to pathogenic bacteria (Wanke et al., 2011). This result was not observed in the current study when NHEK were pre-exposed to *S. capitis* prior to *S. aureus* infection (Figure 6.5 and Figure 6.7). However, Wanke *et al* exposed NHEK to bacterial conditioned media for longer periods of time, making a comparison difficult.

Keratinocyte AMP induction by probiotic bacteria has not yet been measured, though probiotics can stimulate Hbd-2 expression in intestinal cells. For example, a study in 2009 found that stimulation of gut epithelial cells (Caco-2) with *L. plantarum* for 12h induced a significant increase in Hbd-2 protein and mRNA levels (Paolillo et al., 2009). Others have found that Hbd-2 mRNA levels were up-regulated by 4.5h exposure to heat-killed probiotic bacteria including *E. coli* Nissle 1917 and *L. fermentum* but not pathogenic *E. coli* strains (Wehkamp et al., 2004). Another study demonstrated that lysozyme generated cell wall fragments of *L. rhamnosus* GG, when fed to rats, resulted in up-regulation of cathelicidin related antimicrobial peptide (CRAMP) mRNA expression and protein in peritoneal macrophages (Bu et al., 2006). These studies contrast with the present study where no up-regulation of Hbd-2 or LL-37 was found in response to probiotic bacteria. As TLR-2 and AMP expression was unchanged in response to probiotic bacteria, this may be a mechanism by which keratinocytes prevent unnecessary inflammation in the presence of commensal or non-pathogenic bacteria. Some AMPs play roles in cytokine signalling in addition to their antimicrobial activities. For example, LL-37 has been shown to induce proinflammatory cytokines including IL-8, TNF-α and IL-6 in keratinocytes (Braff et al., 2005b).

In contrast to the results observed for Hbd-2, Hbd-3, LL-37 and RNase-7, psoriasin mRNA expression was significantly increased in NHEK exposed to *L. reuteri* (Figure 6.8a). However, immunocytochemistry revealed no significant change in the expression of psoriasin protein with exposure to bacteria compared to controls (Figure 6.8b, c and d). This demonstrates the importance of measuring both mRNA and protein levels, as while changes may occur in the mRNA expression of AMPs, the protein expression may not necessarily be affected.

Psoriasin expression can be induced by exposure of NHEK to *E. coli*. This involves TLR-5 through activation by flagellin on the surface of *E. coli* (Abtin et al., 2008). There are reports of *S. aureus* induction of psoriasin in lung cells and corneal cells (Andresen et al., 2011, Garreis et al., 2011), but induction of psoriasin by other bacteria in keratinocytes has not been noted previously. In these experiments, no induction was observed upon exposure to *S. aureus*.

The ability to induce up-regulation of AMPs is often strain dependent. A study in 2007 demonstrated that a nasal carrier isolate of *S. aureus* could suppress induction of Hbd-2 and Hbd-3, while a lab strain could induce both AMPs significantly. Additionally, the relative AMP levels changed over time, peaking at 4h and 20h, suggesting that further examination of the AMP levels over time is necessary to give a more accurate picture of the effect of bacteria upon AMP induction (Quinn and Cole, 2007).

6.4.3 IL-8 Production by NHEK

Inflammation in response to bacterial challenge is regulated in the skin through the production of a variety of cytokines and chemokines. The keratinocytes of the epidermis are able to produce a variety of cytokines and chemokines (Section 1.1.5.3) including the chemokine, IL-8. IL-8 is a chemoattractant for neutrophils and T-cells (thus providing a link with the adaptive immune response) (Larsen et al., 1989). In NHEK, IL-8 production is low in unstimulated cells, but can be induced by exposure to proinflammatory cytokines such as TNF-α (Li et al., 1996). IL-8 neutrophil recruitment is particularly important in the limitation of *S. aureus* infections, as observed in neutropenic mice where intradermally injected *S. aureus* skin infections progressed to bacteraemia compared to control mice where *S. aureus* infections resolved after a period (Mölne et al., 2000).

In the current study, IL-8 secretion by NHEK in response to *S. aureus*, *S. capitis*, *L. reuteri* and *L. rhamnosus* GG was measured using ELISAs. *S. aureus*, *S. capitis* and *L. rhamnosus* GG exposure did not result in any significant production of IL-8 compared to untreated cells. However, *L. reuteri* exposure resulted in a non-significant trend toward increased IL-8 production. NHEK pre-exposed to *L. reuteri* prior to *S. aureus* infection produced significantly more IL-8 than untreated NHEK (P=0.0001) (Figure 6.9). This suggests that topically applied *L. reuteri* could act to prime the skin to respond to *S. aureus* infection in a prompt manner.

L. reuteri ATCC 55730 is reportedly an immunostimulatory probiotic. *L. reuteri* DSM 17398 (an equivalent daughter strain of *L. reuteri* ATCC 55730 (Rosander et al., 2008)) induces IL-8 production in cultured intestinal epithelial cells (Liu et al., 2010a). Jones and Versalovic (2009) demonstrated that *L. reuteri* ATCC 55730 cell-free supernatant (CFS) from planktonic cultures, when added to monocytoid THP-1 cells could induce TNF-α production (Jones and Versalovic, 2009). As NHEK can be induced to produce IL-8 by TNF-α (Li et al., 1996, Griffiths et al., 1991), this could be a mechanism by which IL-8 production is increased in response to *L. reuteri* in the current study. However, in other experiments, no TNF-α induction was observed (data not shown).

In contrast to *L. reuteri*, *L. rhamnosus* GG (LGG) has immunomodulatory properties, able to reduce inflammation in both *in vitro* and *in vivo* models. Both viable and dead *L. rhamnosus* GG inhibit TNF-α induced IL-8 production (Zhang et al., 2005) and flagellin induced IL-8 production (Lopez et al., 2008) by Caco-2 enterocytes *in vitro,* though higher concentrations (>10^{10} CFU) of the organism appear to induce IL-8 production in Caco-2 cells (Zhang et al., 2005). *In vivo* studies have demonstrated that oral administration of LGG to atopic dermatitis patients resulted in an up-regulation of IL-10 in sera, a cytokine known to down-regulate production of TNF-α (Pessi et al., 2000). As *S. aureus* did not appear to induce IL-8 (Figure 6.9), it is not surprising that there was no change in the production of IL-8 by NHEK pre-exposed to *L. rhamnosus* GG prior to *S. aureus* infection. However, it would be interesting to discover whether *L. rhamnosus* GG could be used to treat inflammatory skin disorders (e.g. psoriasis), through down-regulation of proinflammatory cytokines involved in the pathophysiology of the disorder.

S. aureus did not induce production of IL-8 by NHEK (Figure 6.9). This is in contrast to other studies demonstrating that both viable and heat-killed *S. aureus* induce IL-8 secretion by keratinocytes (Mempel et al., 2003, Secor et al., 2011, Olaru and Jensen, 2010). Midorikawa *et al* (2003) demonstrated that exposure of NHEK to heat-killed *S. aureus* for 4h resulted in increased IL-8 mRNA expression. However, this was found to drop again after 8h (Midorikawa et al., 2003). IL-8 stimulation by *S. aureus* appears to be dependent upon TLR-2 signalling (Mempel et al., 2003, Olaru and Jensen, 2010). Therefore, as TLR-2 expression was down-regulated in response to *S. aureus* (Figure 6.3), this may explain why IL-8 was not induced by *S. aureus*. *S. aureus* possesses other mechanisms to avoid neutrophil clearance, such as the extracellular adherence protein (Eap) which disrupts leucocyte receptor mediated adhesion and neutrophil chemotaxis (Chavakis et al., 2002). Therefore, this may be another mechanism by which *S. aureus* defends itself against detection.

A previous study utilising viable *S. epidermidis* demonstrated that it did not induce IL-8 production by NHEK, due to cell cytotoxicity caused by the bacteria. However, heat-killed *S. epidermidis* induced IL-8 production (Sasaki et al., 2003). *S. epidermidis* filtrates also induce IL-8 production in neutrophils, thought to be due to the action of phenol soluble modulins (a known TLR-2 agonist) (Cheung et al., 2010). Other coagulase-negative staphylococcal species have elsewhere also been shown to induce IL-8 production by HaCaT keratinocytes (Layer et al., 2007). These studies are in contrast to the results found in the present study, where *S. capitis* did not induce production of IL-8 by NHEK. However, as IL-8 production by NHEK may differ with respect to time (Midorikawa et al., 2003), an investigation into the effect of bacteria over time on IL-8 production by NHEK would be appropriate.

Overall, while knowledge of the innate immune response of keratinocytes to probiotics in comparison to *S. aureus* and *S. epidermidis* is incomplete, what is apparent is that both *L. reuteri* and *L. rhamnosus* do not appear to be “seen” by NHEK in the same manner as the skin pathogen *S. aureus*. The down-regulation of TLR-2 by *S. aureus* leads to a hypothesis that infection with *S. aureus* in some way acts to dampen the innate immune response. This down-regulation was abrogated by both probiotic lactobacilli and commensal *S. capitis*. Additionally, *L. reuteri* appeared to stimulate an innate immune response through IL-8 production, suggesting a topical application (e.g. in skin creams and hand washes) could act to prime the skin to respond to *S. aureus* skin infection in an efficient manner through neutrophil recruitment.

Conclusions and future work

The overall aim of this doctoral project was to explore the possibility that probiotic bacteria of enteric origin contribute towards the protection of the epidermal barrier. Specifically, the potential of probiotics to protect skin against *S. aureus* infections was examined. Probiotics are known to protect the gut epithelium through a number of mechanisms including i) direct interactions with pathogens ii) competitive exclusion for binding sites on epithelial cells and iii) immunomodulation.

7.0 Conclusions

This thesis has examined whether probiotic bacteria can inhibit the growth of *S. aureus* (Chapter 3), whether probiotics can protect keratinocytes from the cytotoxic effects of *S. aureus* (Chapter 4), and the mechanisms behind protective effects (Chapter 5). Finally, a comparison of the innate immune response of keratinocytes exposed to probiotics, *S. aureus* and a skin commensal has been performed (Chapter 6).

An exploration of the use of three probiotics (*L. reuteri* ATCC 55730, *L. rhamnosus* AC413, and *L. salivarius* UCC118) in keratinocyte cell culture suggests that probiotics are well tolerated by keratinocytes *in vitro*. Furthermore, lactobacilli could be used to protect keratinocytes against the cytotoxic effects of *S. aureus* because *L. reuteri* has a significant protective effect on normal human epidermal keratinocytes (NHEK) in both undifferentiated and differentiated NHEK. Importantly, this depends on the timing of application because *L. reuteri* only protects NHEK if it is applied before, or at the same time as *S. aureus*. Lysates of *L. reuteri* also provide protection against *S. aureus* infection. However, heat-killed *L. reuteri* do not, suggesting a heat-labile component is involved. Furthermore, the protective effect of probiotics appears to be species specific because *L. salivarius* does not provide any significant protection against *S. aureus*.

The protective effect observed for *L. reuteri* could be the result of several different mechanisms. For example, lactobacilli produce antimicrobial substances such as organic acids and bacteriocins. *L. reuteri* inhibited the growth of *S. aureus* in well diffusion assays through the production of organic acids. However, this property is growth medium-dependent, and in cell culture assays, the pH of keratinocyte culture medium is unchanged in the presence of lactobacilli.

Competition assays likewise demonstrated that the growth rate and productivity of *S. aureus* is unchanged by the presence of *L. reuteri*. Therefore, it is unlikely that the protective effect is due to *L. reuteri* inhibition of *S. aureus* growth.

At least part of the mechanism behind the protective effect is the ability of *L. reuteri* to prevent *S. aureus* binding to keratinocytes and subsequent invasion. This conclusion is supported by several lines of evidence. Firstly, when *S. aureus* adhesion is inhibited using an integrin-blocking antibody, the ability to induce cell death in keratinocytes is inhibited. Secondly, *L. salivarius*, (which does not confer protection) does not inhibit the binding of *S. aureus* to keratinocytes. Finally, lysates of *L. reuteri* (which are protective) inhibit the adhesion of *S. aureus*, while heat-killed *L. reuteri* (which is not protective) does not. Therefore, it is likely that the mechanism by which *L. reuteri* adheres to keratinocytes involves a heat-labile component.

The ability of *L. reuteri* to inhibit *S. aureus* adhesion to keratinocytes is also dependent upon the timing of application. Taken together, these data suggest that the protective effect of *L. reuteri* is at least partly due to a mechanism involving competitive exclusion of *S. aureus* from binding sites on keratinocytes. This suggests that the prophylactic use of probiotics and/or their lysates in soaps and creams for the skin could aid in the prevention of staphylococcal skin colonisation and subsequent infection.

Subsequent comparison of the innate immune responses of NHEK to probiotics, *S. aureus* and *S. capitis* (a skin commensal) were performed to determine whether probiotic lactobacilli modulate the innate immune responses of keratinocytes, and if probiotics are well tolerated by keratinocytes. TLR-2 expression by NHEK was initially examined since it is the primary pattern recognition receptor involved in the detection of Gram positive bacteria by keratinocytes. TLR-2 protein (but not mRNA) expression in NHEK significantly decreases in cells infected with *S. aureus*. Therefore, the use of single TLR ligands (as commonly used in other studies) may not be relevant to the *in vivo* process, since bacteria such as *S. aureus* may possess multiple pattern recognition receptor (PRR) ligands (e.g. LTA and PGN). Cross talk between PRRs could lead to modulation of receptor expression. However, it is also possible that *S. aureus* possesses a mechanism by which it can reduce the expression of TLR-2 by keratinocytes (e.g. proteases).

In contrast, TLR-2 mRNA and protein expression is significantly increased in NHEK pre-exposed to the skin commensal, *S. capitis*, prior to *S. aureus* infection. This suggests that skin commensals play a role in promoting an innate immune response toward pathogenic bacteria. While probiotics do not induce an increase in TLR-2 expression, pre-exposure to lactobacilli abrogates the effects of *S. aureus* upon TLR-2 expression, suggesting that probiotics influence TLR-2 expression in a similar manner to commensal bacteria in the skin.

An analysis of the expression of antimicrobial peptides (AMPs) by NHEK in response to bacteria demonstrated no significant changes. Hbd-2, Hbd-3, LL-37 and RNase-7 mRNA and protein does not change in response to exposure to any of the bacteria for 12h. Psoriasin mRNA is up-regulated in response to *L. reuteri*. However, this result was not supported by protein analysis, demonstrating the importance of examining both mRNA and protein levels to determine the true expression of genes involved in the innate immune response.

Examination of the effect of bacteria upon IL-8 secretion by NHEK revealed that IL-8 production is increased upon exposure to the immunostimulatory probiotic, *L. reuteri* ATCC 55730. IL-8 production in response to *L. reuteri* may be advantageous in priming the skin innate immune system and clearance of *S. aureus* skin infections. Further work must be undertaken to confirm whether *L. reuteri* has an immunostimulatory effect *in vivo*.

Analysis of the innate immune responses at a fixed time point is not sufficient to provide an accurate picture of the response of NHEK to different bacteria. Further work is required to explore how expression of TLRs, AMPs and cytokines change over time. Additionally, comparison of this work with that of others is complicated by the ability of different strains of bacteria to behave differently. However, while limited conclusions can be made, NHEK appear to respond to *L. reuteri* and *L. rhamnosus* GG in a manner more akin to that of the skin commensal *S. capitis*. Further work should include analysis of the signalling pathways involved in PRR signalling to determine if probiotic bacteria utilise different signalling pathways to skin pathogens and/or commensals.

7.1 Future work

Work presented in this thesis suggests that the protective effect of *L. reuteri* was at least partly due to competitive exclusion of *S. aureus* binding to NHEK. However, probiotic bacteria possess other mechanisms by which they may prevent bacterial pathogenesis. Some probiotics can inhibit the expression of virulence associated genes by *S. aureus*. For example, *L. reuteri* RC-14 has been shown to inhibit the production of TSST-1 by *S. aureus* (Li et al., 2011). As *S. aureus* pathogenesis of keratinocytes is known to involve expression of the virulence factor α-toxin (Walev et al., 1993, Menzies and Kourteva, 2000), further work could involve an analysis of the effect of *L. reuteri* upon gene expression in *S. aureus,* and vice versa.

Probiotics may also influence adhesive factors in keratinocytes. For example, *L. rhamnosus* GG and *L. plantarum* 299v up-regulate mRNA expression of genes involved in mucin production in intestinal epithelial cells, thereby inhibiting the adhesion of enteropathogenic *Escherichia coli* (Mack et al., 1999). Finally, probiotic fermented whey products purportedly increase mRNA expression of differentiation markers in NHEK (Baba et al., 2006) suggesting that “postbiotics” or metabolites of probiotics may regulate epidermal proliferation and differentiation.

This study focussed on the use of probiotic bacteria to inhibit *S. aureus* pathogenesis using primary human epidermal keratinocytes, and found that *L. reuteri* could inhibit *S. aureus* adhesion to differentiating NHEK. In skin, this model may represent the *stratum granulosum* layer of keratinocytes directly beneath the outermost *stratum corneum*, and as such, an open wound. In intact skin, bacteria first come into contact with the *stratum corneum*, so the ability of probiotic bacteria to prevent colonisation of the *stratum corneum* is of interest. Adhesion of *S. aureus* to corneocytes involves elements of the cornified envelope (e.g. involucrin and keratin-10) (Clarke et al., 2009, O'Brien et al., 2002). Therefore, further work is needed to determine whether *L. reuteri* can afford similar protection in a more complete skin model.

A cell culture model of the epidermis restricted to the use of primary keratinocytes provides a valid but limited view of the skin as a whole. Future work exploring the potential role of probiotics as a preventative therapy against *S. aureus* infections would need to utilise more complete models of the skin. Many questions remain to be determined, such as the viability of probiotic bacteria on whole skin, and the ability to colonise the skin under normal conditions.

Preliminary work has commenced using *ex-vivo* skin biopsies maintained in organ culture to determine whether topically applied *L. reuteri* can colonise whole skin explants and what effect *L. reuteri* has upon apoptosis and proliferation in the epidermis using TUNEL assays (results not shown). Initial results suggest that *L. reuteri* remains viable on the skin for approximately 24h, but thereafter it is not detected using culture techniques. However, this work is unfinished and requires further investigation.

This study was limited to the evaluation of topical probiotics to prevent or treat skin infection with *S. aureus*. However, many other skin pathogens cause significant morbidity and deserve investigation also. Group A streptococci (e.g. *S. pyogenes*) can similarly cause skin infections ranging from impetigo to necrotising fasciitis (Tognetti et al., 2012). *P. aeruginosa* is a Gram negative organism which causes severe infections in burn victims lacking an effective epidermal barrier. Treatment of *P. aeruginosa* infections is particularly problematic thanks to the inherent antibiotic resistance of the organism coupled with its ability to form resistant biofilms (Agger and Mardan, 1995). *Propionibacterium acnes* is associated with the development of *acne vulgaris,* though treatment with antibiotics alone does not seem sufficient to treat this condition, suggesting that further treatment with immunomodulating agents may be required (Bek-Thomsen et al., 2008).

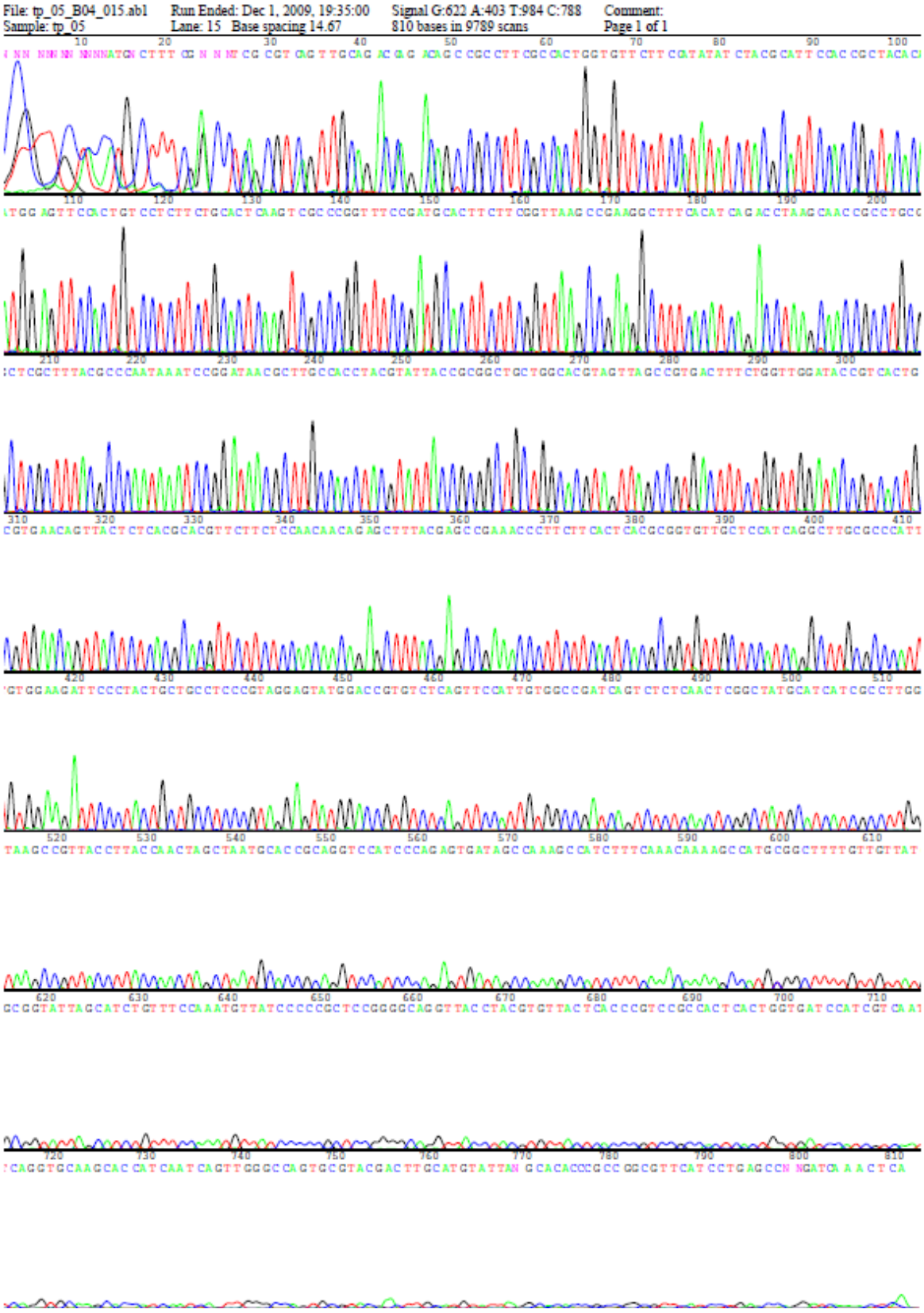
Many other facets of topical probiotic therapeutics for skin are currently being investigated and remain to be determined fully. These include i) cutaneous wound healing (Valdéz et al., 2005, Peral et al., 2009a, Peral et al., 2009b) ii) treatment of skin disorders e.g. atopic dermatitis (Di Marzio et al., 2003, Guéniche et al., 2006b) iii) anti-ageing benefits (Di Marzio et al., 2008) and iv) improved barrier function (Guéniche et al., 2010, Di Marzio et al., 2008).

To date, relatively little is understood of the role of the commensal microbiota on the skin, as the true diversity in species is only being acknowledged now through the use of molecular techniques. How probiotic bacteria will affect the composition of the normal skin microbiota, (including fungi and protists) and the resultant positive or negative effects of this need to be determined.

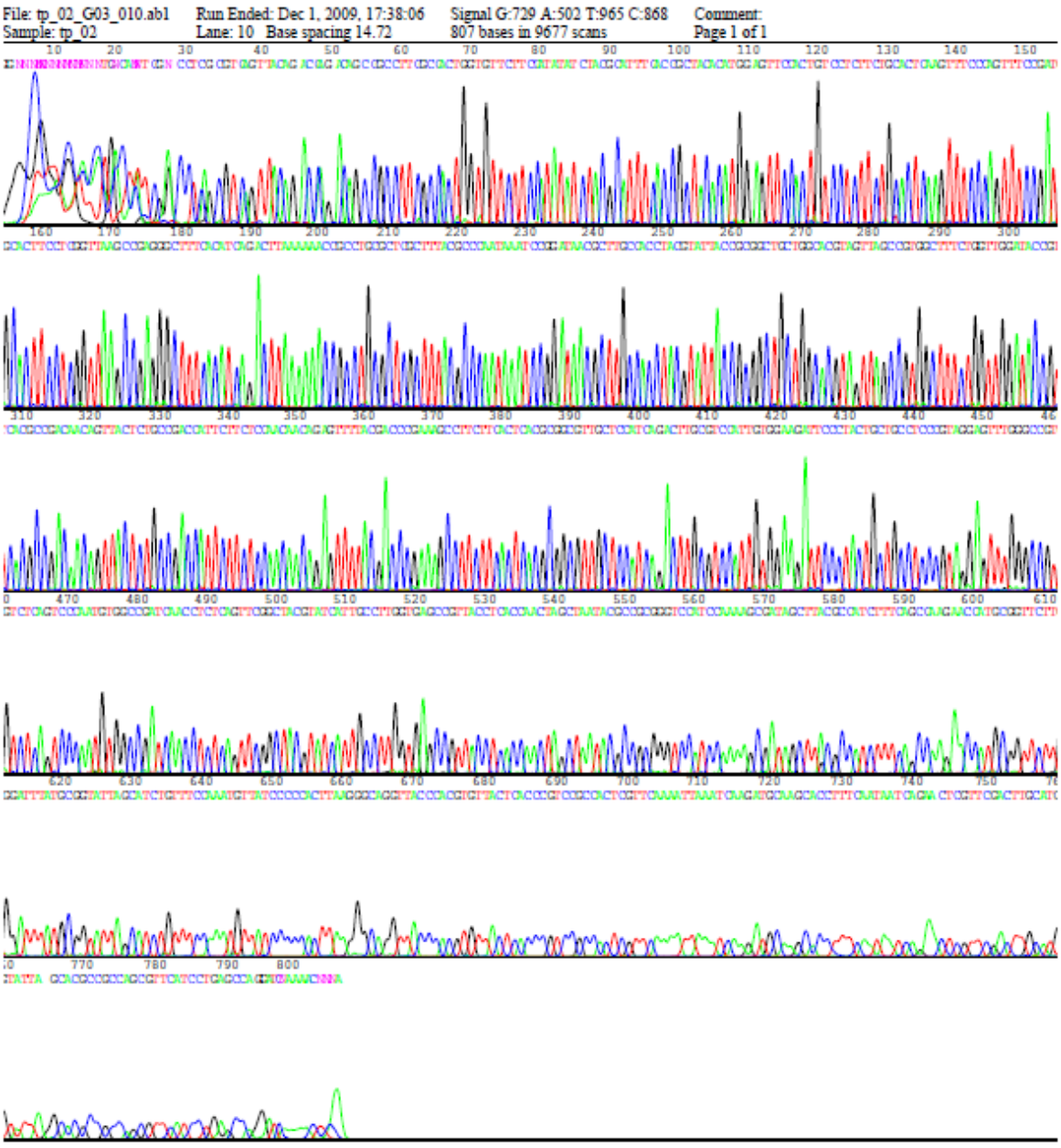
Overall, this study evaluated whether an enteric probiotic could potentially be used as a topical therapy to prevent *S. aureus* infections in skin. Using an *in vitro* model of epidermal keratinocytes, it was found that *L. reuteri* could protect keratinocytes from the cytotoxic effects of *S. aureus*, at least partly due to the competitive exclusion of *S. aureus* binding to NHEK. NHEK responded to *L. reuteri* in a manner similar to the skin commensal *S. capitis*, suggesting that *L. reuteri* is well tolerated by NHEK *in vitro*. Furthermore, addition of *L. reuteri* to NHEK led to elevated secretion of IL-8, an important chemokine involved in the recruitment of neutrophils to the site of infection. This implies that topical application of *L. reuteri* could act to prime the skin innate immune response against *S. aureus* infection through both neutrophil recruitment and inhibition of *S. aureus* colonisation of the skin. Importantly, it was found that *L. reuteri* could not protect keratinocytes against existing *S. aureus* infections. Therefore, it is proposed that *L. reuteri* be used as a prophylactic therapy against *S. aureus* infections, perhaps in barrier creams and soaps. Further work must be done to determine if *L. reuteri* can protect the skin against *S. aureus* infection *in vivo*.

Appendix A

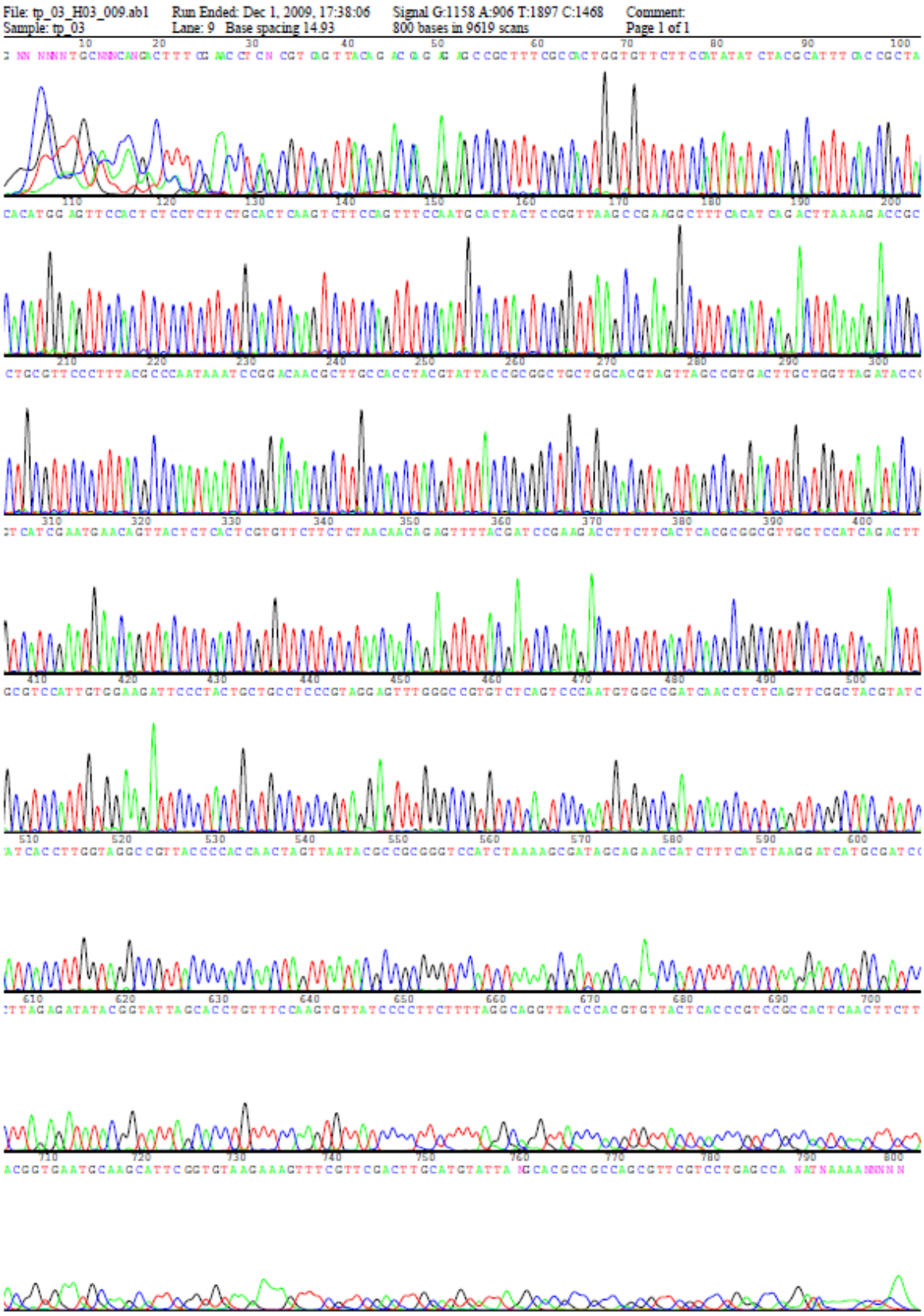
Lactobacillus reuteri ATCC 55730



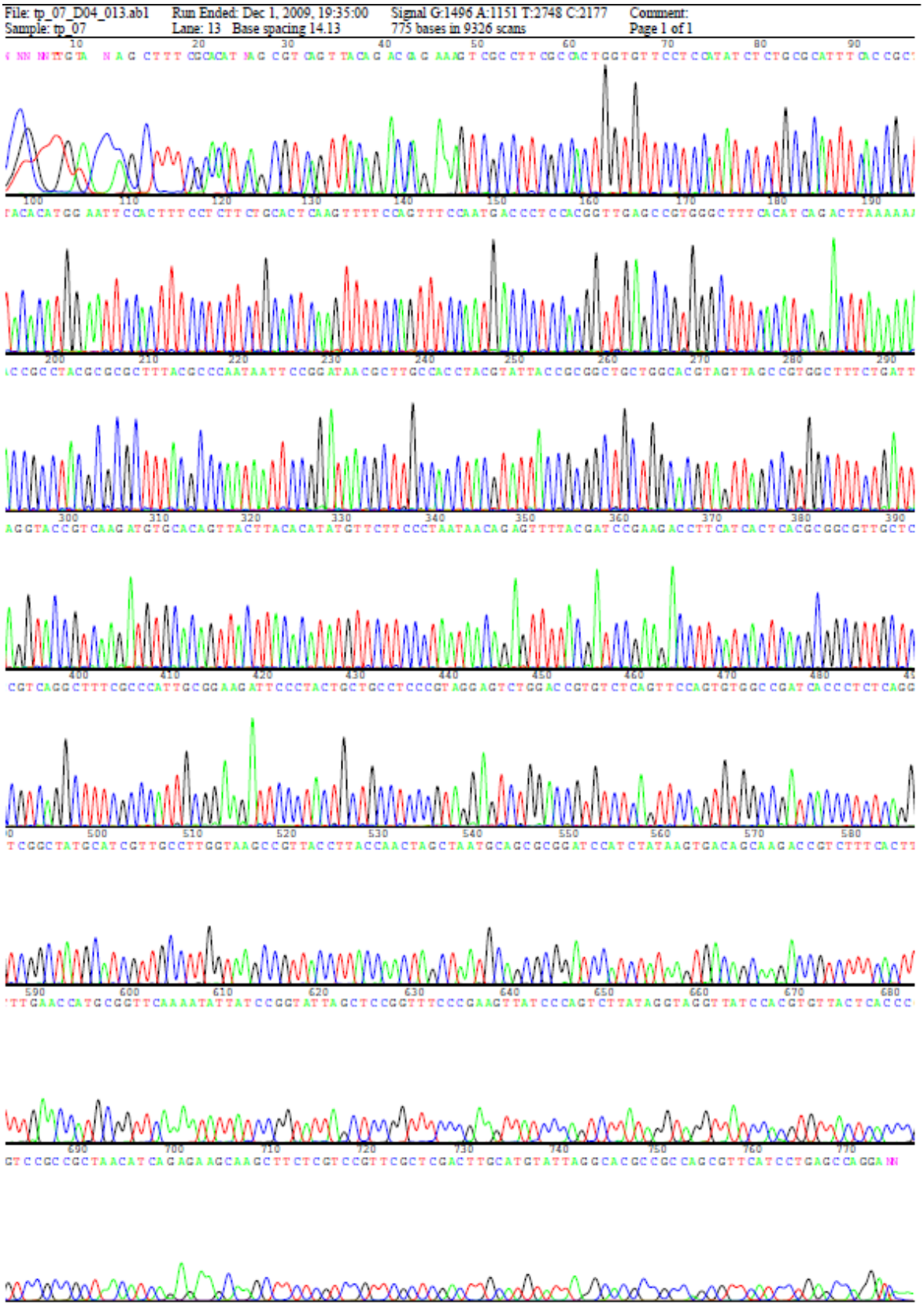
Lactobacillus rhamnosus AC413



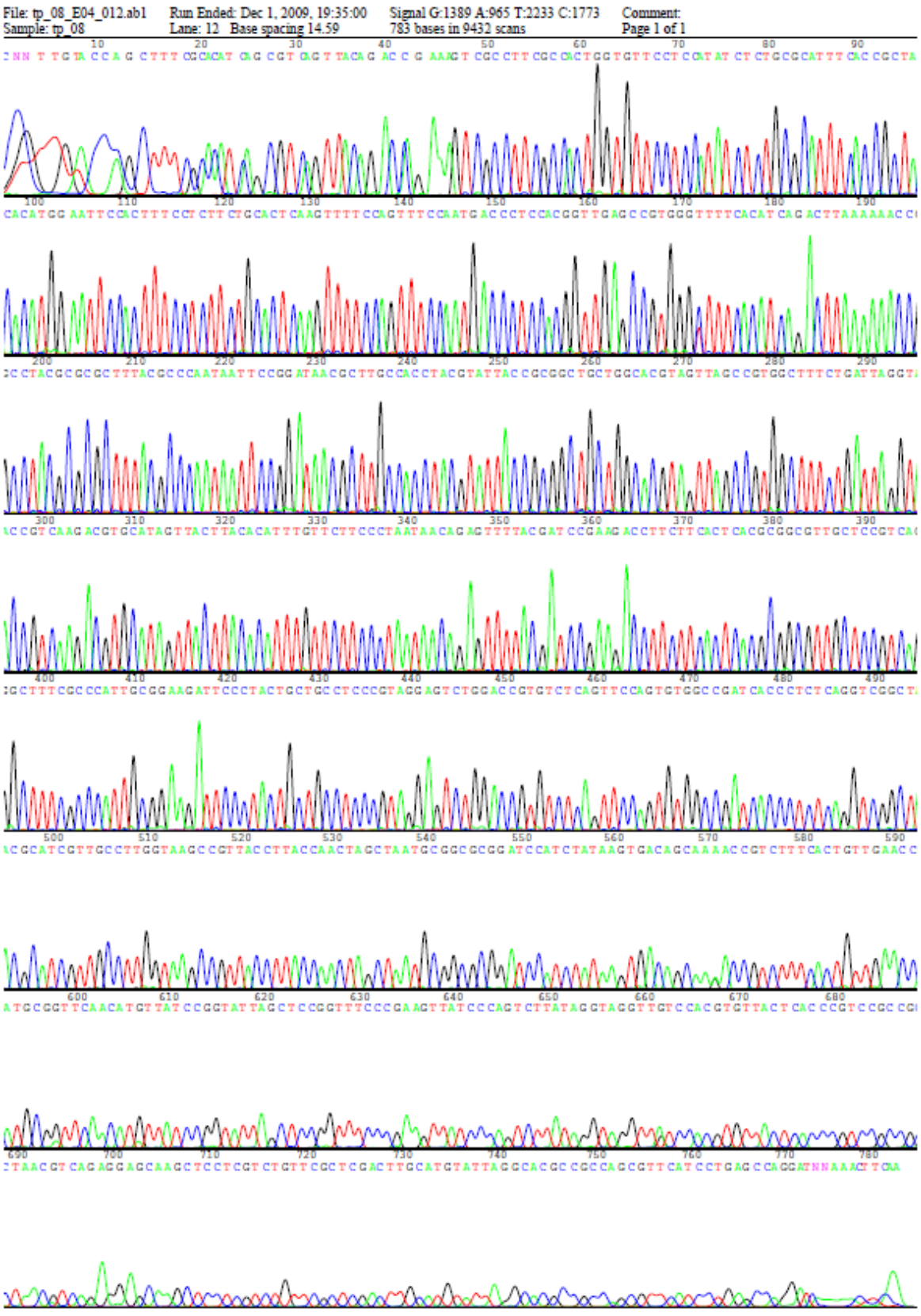
Lactobacillus salivarius UCC118



Staphylococcus aureus



Staphylococcus capitis



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