DEVELOPMENT OF *LACTOBACILLUS PLANTARUM* ANTIBACTERIAL PROTEINS AS BACTERIOCIDES AGAINST *STAPHYLOCOCCUS AUREUS*

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DEVELOPMENT OF *LACTOBACILLUS PLANTARUM* ANTIBACTERIAL PROTEINS AS BACTERIOCIDES AGAINST *STAPHYLOCOCCUS AUREUS*

by

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LIST OF ABBREVIATIONS

±	Plus or minus
°C	Degree Celsius
%	Percentage
$2^{-\Delta\Delta C_T}$	A relative calibrator used in the analysis of real-time quantitative PCR (qPCR) data by the comparative C_T method
11-MUA	11-Mercaptoundecanoic acid
ACE	Angiotensin-I converting enzyme
AD	Atopic dermatitis
AHAs	A-hydroxy acids
ALP	Antileucoprotease
AMPs	Antimicrobial peptides
AO	Acridine orange
atl	Autolysin gene
ATP	Adenosine triphosphate
AU	Arbitury unit
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CFS	Cell-free supernatant
CFU	Colony forming unit
C_T	Threshold cycle
DC	Dendritic cell
DiOC5; DiOC5(3)	3,3-dipentyloxacarbocyanide

DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EB	Ethidium bromide
ELISA	Enzyme linked immunosorbent assay
EPS	Extracellular polymeric substances
FAME	Fatty acid methyl esterase
FBS	Fetal bovine serum
FESEM	Field emission scanning electron microscope
Fraction A	A protein fraction of *Lactobacillus plantarum* USM8613 with transglycosylase activity
Fraction B	A protein fraction of *Lactobacillus plantarum* USM8613 with glyceraldehyde-3-phosphate dehydrogenase activity. An extracellular enzyme (MW 37 kDa) that inhibits the growth of *S. aureus*
Fraction A+B	A combined fraction of *Lactobacillus plantarum* USM8613 with both transglycosylase activity and glyceraldehyde-3-phosphate dehydrogenase activities
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAPDH F	Glyceraldehyde 3-phosphate dehydrogenase forward primer
GAPDH R	Glyceraldehyde 3-phosphate dehydrogenase reverse primer
GCMS	Gas chromatography mass spectrometry

gyrB	DNA gyrase, subunit B
h	Hour
HaCaT	Immortalised human keratinocyte cell line
hBD	Human beta-defensin
hBD-2	Human beta-defensin 2
hBD-2 F	hBD-2 Forward primer
hBD-2 R	hBD-2 Reverse primer
hBD-3	Human beta-defensin 3
hBD-3 F	hBD-3 Forward primer
hBD-3 R	hBD-3 Reverse primer
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IC_{50}	Antimicrobial titer that gives 50 % inhibition
IFN-γ	Interferon-gamma
IL	Interleukin
IL-1α	Interleukin 1 alpha
IL-1α F	IL-1α Forward primer
IL-1α R	IL-1α Reverse primer
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-6 F	IL-6 Forward primer
IL-6 R	IL-6 Reverse primer
IL-8	Interleukin 8
in vitro	Performed in the test-tube
in vivo	Performed in live animal/human

kDa	kiloDalton
LAB	Lactic acid bacteria
L. plantarum	*Lactobacillus plantarum*
L. plantarum USM8613	*Lactobacillus plantarum* USM8613
LPS	Lipopolysaccharide
LysM	Lysine motif
MDA	Malonyldialdehyde
mg/ml	Miligrams per millilitre
mgrA	Global regulator gene
MIC	Minimum inhibitory concentration
MM	Molecular mass in kDa
MMPs	Matrix metalloproteinases
MOWSE	Molecular weight search engine
mRNA	Messenger ribonucleic acid
MRS	De Man-Rogosa-Sharpe medium
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSA	Mannitol salt agar
MS/MS	Tandem mass spectrometry
MTT	3-(4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight in g/mol
NAG	*N*-acetylglucosamine
NAM	*N*-acetylmuramic acid
NF-κB	Nuclear factor κB
n	Number or sample number

nm	Nanometres
NOD	Nucleotide oligomerisation domain
OD	Optical density
(P<0.05)	Probability less than 0.05
PAMPs	Pathogen-associated molecule patterns
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PGN	Peptidoglycan
qPCR	Quantitative PCR
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse-transcription polymerase chain reaction
S. aureus	*Staphylococcus aureus*
SC	Stratum corneum
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
sigB	Stress regulator gene
SPR	Surface plasmon resonance
SPSS	Statistical Package for the Social Science, a software package used for statistical analysis
TBA	Thiobarbituric acid
TEM	Transmission electron microscope
Th	T-helper cell
TLRs	Toll like receptors
TLR-2	Toll-like receptor-2

TLR-2 F	Toll-like receptor-2 forward primer
TLR-2 R	Toll-like receptor-2 reverse primer
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	Tumor necrosis factor alpha
TNF-α F	Tumor necrosis factor alpha forward primer
TNF-α R	Tumor necrosis factor alpha reverse primer
Total RNA	Total ribonucleic acid
TSA/B	Trypticase soy agar/broth
µl	Microlitre
UV	Ultra-violet
VP	Variable pressure

PENGGUNAAN PROTEIN ANTI-BAKTERIA DARIPADA *LACTOBACILLUS PLANTARUM* SEBAGAI BAKTERIOCIDESTERHADAP *STAPHYLOCOCCUS AUREUS*

ABSTRAK

Empat puluh tiga strain bakteria asid laktik telah diasingkan dan dikenalpasti daripada sayur-sayuran segar, buah-buahan segar dan produk penapaian. Supernatan bebas sel (CFS) *Lactobacillus plantarum* USM8613 (*L. plantarum* USM8613) yang telah dineutralkan yang menunjukkan kesan rencatan lebih kuat (P<0.05) terhadap *Staphylococcus aureus* (*S. aureus*) berbanding semua strain yang dikaji telah dipilih untuk analisis seterusnya. CFS *L. plantarum* USM8613 telah diasingkan kepada fraksi protein, polisakarida dan lemak, dengan semua fraksi merencat *S. aureus* secara lebih ketara (P<0.05), dengan kesan yang lebih menonjol daripada fraksi protein mentah. Kajian permukaan plasmon resonans menunjukkan fraksi protein mentah mempunyai kecenderungan ikatan yang kuat terhadap *S. aureus* dan morfologi membran kedutan dan kasar diperhatikan dalam *S. aureus* yang dirawat dengan fraksi protein mentah melalui imbasan mikroskop elektron. Fraksi protein mentah telah ditulenkan lagi untuk kehomogenan dengan kaedah penulenan tiga langkah. Dua protein antimikrob anggapan yang ditetapkan sebagai Fraksi A dan Fraksi B masing-masing telah ditemui dan dikenalpasti sebagai enzim transglikosilase ekstrasel dan gliseraldehid-3-fosfat dehidrogenase. Ketiga-tiga fraksi protein (A, B dan A+B) daripada *L. plantarum* USM8613 menunjukkan kesan bakterisidal terhadap *S. aureus*, dengan Fraksi A mempunyai aktiviti anti-stafilokokal yang lebih kuat. Kedua-dua fraksi A dan B mempunyai mekanisme anti-stafilokokal yang berbeza. Fraksi A memusnahkan peptidoglikan dinding sel *S. aureus*. Sementara itu, Fraksi B menembusi sel *S. aureus* dan kemudiannya

menyebabkan autolysis *S. aureus* melalui induksi ekspresi lebihan regulator autolisis, gen *sigB, mgrA* dan *atl*. Akibatnya, Fraksi A dan Fraksi B menyebabkan penelapan membran dalam *S. aureus*. Fraksi A dan Fraksi A+B melesapkan potensi membran, meningkatkan pengoksidaan membran lipid, mengubah sifat berubah-ubah membran dan meningkatkan kebocoran kandungan intrasel dalam *S. aureus*. Ini menunjukkan Fraksi A mempunyai kesan gangguan membran sel secara langsung dan lebih kuat terhadap *S. aureus* dan seterusnya meningkatkan tindakan Fraksi B. Ketiga-tiga fraksi protein (A, B dan A+B) adalah tidak sitotoksik kepada sel HaCaT pada semua kepekatan yang dikaji (100-12800 AU/ml). Ketiga-tiga fraksi protein antimikrob melindungi (P<0.05) sel HaCaT yang dijangkiti oleh *S. aureus* daripada serangan *S. aureus* berterusan dan meningkatkan pembiakan sel HaCaT. Ketiga-tiga fraksi protein antimikrob mempunyai kesan anti-inflamasi setelah penghapusan bakteria. Di antara kesan anti-inflamasi ini ialah kekurangan secara ketara (P<0.05), ekspresi dan penghasilan reseptor-2 seakan tol (toll-like receptor-2, TLR-2), β-defensin (hBDs) dan sitokin pro-inflamasi (IL-1α, IL-1β, IL-6, TNF-α dan IL-8). Secara kolektifnya, hasil kajian ini menunjukkan keberkesanan dan potensi terapeutik protein antimikrob daripada *L. plantarum* USM8613 untuk memerangi *S. aureus*, seterusnya dapat digunakan sebagai agen anti-stafilokokal alternatif dalam industri dermatologi untuk rawatan jangkitan kulit stafilokokal.

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ABSTRACT

Forty-three strains of lactic acid bacteria (LAB) were isolated and identified from fresh vegetables, fresh fruits and fermented products. Neutralised cell-free supernatant (CFS) of *Lactobacillus plantarum* USM8613 (*L. plantarum* USM8613) exerted the strongest inhibitory effect ($P<0.05$) against *Staphylococcus aureus* (*S. aureus*) compared to all LAB strains studied. Thus, it was selected for subsequent analyses. CFS of *L. plantarum* USM8613 was fractionated into protein, polysaccharide, and lipid fractions. All three fractions significantly inhibited *S. aureus* ($P<0.05$), but the most profound inhibitory effect was from the crude protein fraction. Surface plasmon resonance study demonstrated strong binding affinity of the crude protein fraction to *S. aureus* and rough and wrinkled membrane morphology was observed in *S. aureus*, treated with crude protein fraction via scanning electron microscopy. The crude protein fraction was further purified to homogeneity by a three-step purification method. Two putative antimicrobial proteins, designated as Fraction A and Fraction B, were discovered and identified as extracellular transglycosylase and glyceraldehyde-3-phosphate dehydrogenase respectively. Individual fractions A and B, and combined fraction A+B from *L. plantarum* USM8613 exerted a bactericidal effect against *S. aureus*, with a stronger anti-staphylococcal activity from Fraction A, suggesting Fraction A and Fraction B have different anti-staphylococcal mechanisms. Fraction A degraded the cell wall peptidoglycan of *S. aureus*. Meanwhile, Fraction B penetrated *S. aureus* cells and subsequently caused *S. aureus* autolysis via induction of overexpression of autolysis regulators−*sigB*, *mgrA* and *atl* genes. Consequently, both Fraction A and Fraction B

caused membrane permeabilisation in *S. aureus*. Fraction A and Fraction A+B prevalently dissipated the membrane potential, induced membrane lipid peroxidation, altered membrane fluidity, and enhanced leakage of intracellular contents of *S. aureus*, suggesting Fraction A exhibited a direct and stronger cell membrane disruptive effect against *S. aureus*, thereby enhancing the action of Fraction B. Fraction A, Fraction B, and Fraction A+B did not exhibit cytotoxicity effects on HaCaT cells at all concentrations studied (100-12800 AU/ml). These antimicrobial proteins significantly ($P<0.05$) protected *S. aureus*-infected HaCaT cells from continued *S. aureus* invasion and enhanced HaCaT cell proliferation. These antimicrobial proteins exerted anti-inflammatory effect upon bacterial clearance, where the expression and production of toll-like receptor-2 (TLR-2), β-defensins (HBDs), and various pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, TNF-α, and IL-8) were significantly reduced ($P<0.05$). Collectively, results obtained illustrated that the therapeutic potential of the antimicrobial proteins from *L. plantarum* USM8613 to combat *S. aureus* and could be applied as alternative anti-staphylococcal agents in the dermatological industry to treat staphylococcal skin infections.

CHAPTER 1

INTRODUCTION

1.1 Background

Lactic acid bacteria (LAB) are gram-positive, catalase-negative, immobile, non-sporulating, aerotolerant cocci or rods that produce lactic acid as their main metabolic end product during carbohydrate fermentation (Khalid, 2011). LAB are mainly divided into four genera: *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*. They are normally used in dairy products, meat, vegetables, cereals, and wine fermentation. LAB are generally regarded as safe under the US Food and Administration (FDA) guidelines and in recent years they have been renowned for their health promoting effects and some were claimed with probiotic properties (Patrick, 2012). LAB, particularly members of the genus *Lactobacillus*, have traditionally been documented to confer beneficial effects on gut health including modulation of unbalanced indigenous microbiota, reduction of gastro-intestinal discomfort, and prevention and treatment for diarrhea and irritable bowel syndrome (Collado *et al.*, 2009). Recently, LAB have drawn attention for their capabilities to exert therapeutic functions beyond the gut, for instance the skin.

The human skin is the largest organ of human body that functions as an important barrier preventing the escape of moisture and protecting human body from invasion and growth of infectious bacteria (Segre, 2006). It is an intricate habitat for enormous variability of microbial communities. The skin is colonised by a diverse population of microbes, many of which are commensal or symbiotic, during birth and in subsequent post-natal exposure. The skin microbiota is mainly comprised of *Staphylococcus sp., Micrococcus sp., Corynebacterium sp., and Propionibacterium*

sp. (Chiller *et al.*, 2001). They are beneficial for a healthy person, which supplement the barrier function of the skin by inhibiting the growth of pathogenic species and maintain skin balance. However, some of the skin microbiota may become pathogenic to an impaired skin barrier or in an immuno-compromised person. *Staphylococcus aureus*, which is an opportunistic pathogen that resides and colonises on human skin and mucous membrane, plays an undeniable role in human skin infections.

S. aureus is a common commensal of humans and its primary site of colonisation is anterior nares and the skin (Plata *et al.*, 2009). Colonisation predisposes an individual to *S. aureus* infections as it provides a reservoir from which bacteria can be introduced when host defenses are breached (Kluytmans *et al.*, 1997). *S. aureus* causes a wide array of staphylococcal infections ranging from minor skin infections such as impetigo, folliculitis, furuncle, and abscesses to invasive and life-threatening diseases including septic arthritis, osteomyelitis, pneumonia, meningitis, septicaemia and endocarditis (Lowy, 1998; Foster, 2005; Iwatsuki *et al.*, 2006). In recent years, *S. aureus* has received great attention due to its intrinsic virulence and the emergence of the antibiotic resistant variants that are increasingly resistant to a vast number of antimicrobial agents. Several newer agents against the antibiotic-resistant virulent strains have recently been discovered or under clinical development, yet resistance to these new classes of antibiotics has already been reported (Ruiz *et al.*, 2002; Aksoy and Unal, 2008). Inevitably, this has left fewer effective bactericidal antibiotics to fight against this often life-threatening causative agent and therefore a paradigm shift in the treatment of staphylococcal skin infection is necessary to prevent antibiotics becoming obsolete. Decolonisation of *S.*

aureus and treatment of its skin infections via non-antibiotic measures ought to be considered.

The increasing interest in treating bacterial skin infection in a natural way has intensified the use of LAB as a feasible biotherapeutic alternative. LAB have been proposed to augment the skin barrier function to inhibit skin pathogens, prevent or treat bacterial skin infections, and promote skin health by either or both competitive exclusion and production of antimicrobial substances (Gan *et al.*, 2002; Gueniche and Castiel, 2009; Charlier *et al.*, 2009). For instance, Prince *et al.* (2012) have demonstrated that *Lactobacillus reuteri* inhibited *S. aureus* adherence and protected epidermal keratinocytes from *S. aureus*-induced cell death by competitive exclusion. Whilst either live bacteria or lysate of *L. rhamnosus* GG have been reported to inhibit the growth of *S. aureus* and reduce bacterial adhesion on epidermal keratinocytes. However, the safety of using live bacteria, especially in situations where the skin barrier is breached remains an important concern. In fact, the application of viable bacteria to wounds can lead to the risk of bacteraemia. Study has suggested that LAB metabolites such as the bacteriocin, nisin F can potentially treat subcutaneous skin infections caused by *S. aureus* (De Kwaadsteniet *et al.*, 2010). For this reason, the inhibitory substances produced by LAB may be the preferred choice.

Considering the increasing levels of antibiotic resistance in *S. aureus* remains as a serious problem to public health and it is essential to seek for a better alternative, we hypothesised that LAB could be an interesting biotherapeutic agent. The inhibitory substances produced by LAB can potentially inhibit *S. aureus* and/or treat staphylococcal skin infections. Moreover, the anti-staphylococcal activity and the mechanisms of the potential inhibitory substances produced by LAB remains to be

elucidated. In addition, the efficacy and immuno-modulating effects of the inhibitory substances on human skin are scarcely reported. Thus, in depth investigation is needed to acquire a better understanding on how LAB inhibitory substances interfere with the skin pathogenic bacteria, *S. aureus* and promote skin health.

1.2 Aim and Objectives for Research

The main aim of this study is to evaluate the effects of inhibitory substances from LAB against the skin pathogen, *S. aureus*.

Specific and measurable objectives were:

1. To isolate, identify and select a potential strain of LAB that produces inhibitory metabolites against *S. aureus*

2. To fractionate, characterise and evaluate the potential LAB inhibitory metabolites against *S. aureus*

3. To purify and characterise the putative anti-staphylococcal compounds from the fractionated cell-free supernatant of LAB

4. To elucidate the mechanisms of action of the purified putative anti-staphylococcal compounds of LAB against *S. aureus*

5. To evaluate the protective effect, efficacy and immuno-modulating effect of the purified putative anti-staphylococcal compounds of LAB on human keratinocytes

CHAPTER 2

LITERATURE REVIEW

2.1 LAB

LAB are a group of non-motile and non-spore forming Gram-positive bacteria. They ferment carbohydrate and produce lactic acid as the major end-product (Wong *et al.*, 2014; Nair and Surendran, 2005). Members of the genera *Lactobacillus, Lactococcus, Enterococcus, Leuconostoc, Pediococcus* and *Streptococcus* are commonly recognised as lactic acid producing bacteria (Jay, 2000; Holzapfel *et al.*, 2001). LAB are nutritionally fastidious in nature as they require rich media to grow. Hence, LAB are widely distributed in niches with rich nutrient supplies such as humans, animals, dairy products, meats, plants, vegetables, fruits, beverages, fermented products, and sewage (König and Fröhlich, 2009).

Fresh fruits and vegetables are essential components of the human diet and natural habitats for various beneficial LAB. For instance, *L. plantarum* has been successfully isolated from olives, pineapple, papaya, and grapefruit juice and found to exert antimicrobial activity against several spoilage bacteria, including *Staphylococcus aureus* (Kato *et al.*, 1994; Todorov and Dicks, 2005; Todorov *et al.*, 2011; Wong *et al.*, 2014). Moreover, various LAB with probiotic characteristics have also been isolated from fermented products. The presence of LAB in fermented products also improves the safety, nutritional values, and sensory properties of the foods (Lucke, 2000; Papamanoli *et al.*, 2003). Examples include *L. sakei, L. curvatus*, and *L. plantarum* strains which have been successfully isolated from naturally fermented dry sausages and found to exert antimicrobial activity against common

food spoilage bacteria, *Listeria monocytes* and *Staphylococcus aureus* (Papamanoli *et al.*, 2003).

LAB have been well-documented for their important technological properties in food production which increase the nutritional values, aroma, texture and shelf-life of the foods (Lebeer *et al.*, 2008). The preservative effect of LAB is mainly due to the production of antimicrobial substances such as organic acids, hydrogen peroxide, diacetyl, bacteriocins, and bacteriolytic enzymes (Klaenhammer 1988; Stiles and Hastings, 1991). In addition, LAB are also incorporated into food and beverages products as dietary adjuncts to promote gastrointestinal health and improve gut immune functions (Marini and Krugman, 2012). Numerous studies have revealed the potential use of LAB to offer benefits beyond the gut. This includes improving lactose intolerance, preventing gut inflammation, enhancing natural immunity, and reducing serum cholesterol and colon cancer (Liu *et al.*, 2007).

2.1.1 *Lactobacillus*

The genus *Lactobacillus* is a group of Gram-positive, rod-shaped, catalase-negative, non-motile, and non-sporulating microorganisms with genomic guanine-cytosine content that varies from 32 to 51 % (Otieno, 2011). The genus *Lactobacillus* is a very diverse genus with 185 recognised species and 28 subspecies identified to date (Euzeby, 2013).

Lactobacilli have different fermentation characteristics and produce lactic acid as the major metabolic acid. They can be divided into three classes, namely obligate homofermentative, facultative heterofermentative, and obligate heterofermentative (Tham *et al.*, 2011). Various studies have reported that

Lactobacillus species such as *L. gasseri*, *L. reuteri*, and *L. rhamnosus* are the most dominant bacteria in the gastrointestinal tract and oral cavity (Reuter, 2001; Saito, 2004). Moreover, *Lactobacillus* species are also widely distributed in ubiquitous environments rich-in carbohydrates such as fruits, vegetables, plants, beverages, dairy products, fermented foods, and sewage (Giraffa *et al.*, 2010). Clinical evidences have demonstrated the potential use of lactobacilli in foods and beverages for human consumption due to their ability to improve food quality and promote human health (Reid *et al.*, 2003).

2.1.2 Conventional Health Benefits

LAB have been long used in food fermentation since the discovery of their preservative and beneficial effects on gastrointestinal health. It is crucial to maintain gastrointestinal health as the gastrointestinal tract contains approximately 70 % of the immune cells of the entire immune system (Vighi *et al.*, 2008). LAB, commonly found in healthy intestinal microflora, interact with both the innate and adaptive immune systems to exert health promoting effects on the host (Purchiaroni *et al.*, 2013).

LAB are well-known for their antimicrobial effects. LAB have been shown to produce surface active components that inhibit the adhesion of other pathogenic bacteria while facilitate them to adhere to the small intestine (Pereira *et al.*, 2003). LAB can also exert antimicrobial effects via the production of antimicrobial metabolites such as organic acids, hydrogen peroxide, and bacteriocins. The production of lactic acid, for example, lowers the environmental pH and thus further inhibits the growth of pathogens (Fayol-Messaoudi *et al.*, 2005). The antimicrobial

effect of hydrogen peroxide is due to its strong oxidising nature. Studies have shown that hydrogen peroxide produced by *L. gasseri* and *L. johnsonii* NCC33 inhibited the growth of both Gram-positive *S. aureus* and Gram-negative *Salmonella* sp. (Pridmore *et al.,* 2006; Otero and Nader-Macias, 2006). Bacteriocins are one of the major antimicrobial metabolites from LAB. One study has demonstrated that plantaricin ZJ008 by *L. plantarum* ZJ008 formed pores and caused leakage of K+ out of the cells of various *Staphylococcus* spp., including the methicillin-resistant strains (Zhu *et al.*, 2014).

Besides secreting antimicrobial metabolites, LAB can also stimulate the host immune response against pathogen invasion. The outer membrane of LAB, consisting mostly of peptidoglycan and lipoteichoic acid, enhances the host innate immunity response. Both peptidoglycan and lipoteichoic acid are detected by host toll-like receptor-2 (TLR-2) and peptidoglycan recognition protein, subsequently initiating innate immune response in which pro-inflammatory cytokines and secretory immunoglobulin A (sIgA) are produced (McDonald *et al.*, 2005; Warchakoon *et al.*, 2009; Brandt *et al.*, 2013). The cytokines employ chemotactic mechanisms upon encounter with pathogens while sIgA prevents the binding and penetration of foreign invaders to the epithelia cells (Erickson and Hubbard, 2000). The interaction between LAB peptidoglycan and peptidoglycan recognition proteins act as antibacterial molecules which activates either of the two-component systems, CssR-CssS or CpzA-CpxR. This activation results in events responsible for cell death such as membrane depolarisation, oxidative stress, and inhibition of RNA, DNA, and cell wall synthesis (McDonald *et al.*, 2005; Park *et al.*, 2011). Claes *et al.* (2012) have reported that lipoteichoic acid isolated from *L. rhamnosus* GG induced

intestinal IL-8 production and NF-kB activation via TLR-2 or TLR-6 interaction, thereby enhanced the pro-inflammatory activities in HEK293T cells.

LAB are able to produce β-galactosidase, phospho-β-galactosidase, and phospho-β-glucosidase enzymes that digest lactose in dairy products into glucose and galactose, through activation of the two lactose transportation systems, namely the lactose-permeate transportation and lactose-specific phosphoenolpyruvate-dependent phosphotransferase systems, and subsequently alleviate lactose intolerance symptoms (Honda *et al.*, 2007). Upon ingestion of sufficient amount of lactose, lactose maldigesters may experience various symptoms which include abdominal discomfort, bloating, diarrhoea, and flatulence (Vesa *et al.*, 2000). One study has shown that the consumption of *L. acidophilus*- and *L. casei*-fermented milk successfully reduced the development of gastrointestinal discomfort, and suppressed intestinal motility as well as hydrogen gas production in 18 lactose deficient patients (Gaón *et al.*, 1995).

LAB have also been demonstrated to ease antibiotic-associated diarrhoea and inflammatory diseases such as ulcerative colitis and Crohn's disease. This was achieved by regulating the intestinal microbiota and stabilising antibiotic- induced dysbiosis as demonstrated by *Lactobacillus* GG (Zhang *et al.*, 2005). Three possible mechanisms of LAB to inhibit growth of pro-inflammatory intestinal pathogens are through the production of inhibitory substances, adherence to mucosal layers, and iron-siderophores (Fung *et al.*, 2011).

Several studies have demonstrated the anti-carcinogenic effects of LAB. Liong (2008) suggested that the short-chain fatty acids from LAB lowered the colonic pH, and suppressed the growth of tumor-promoting and pro-carcinogenic

pathogenic microorganisms. In addition, LAB have been shown to possess anti-neoplastic activity for the prevention of colorectal cancer (Boyle *et al.*, 2006). The anti-carcinogenic activity of LAB was achieved via enhancement of intestinal detoxification and transit immune status, as well as suppression of *as-p21*oncoprotein expression (Singh *et al.*, 1997; Cabana *et al.*, 2007). Other studies suggested that the anti-carcinogenic effect of LAB was attributed to the binding of the cell wall skeleton of LAB and heterocyclic amines by intestinal probiotics to the mutagens (Zhang and Ohta, 1991; Orrhage *et al.*, 1994). In one such study, the administration of LAB alleviated the aberrant crypt foci counts in carcinogen-induced rats via the suppression of nitroreductase and β-glucoronidase activities (Verma and Shukla, 2013). Another study by Rafter *et al.* (2007) demonstrated that the secretion of IL-12 was significantly increased, the faecal flora was changed, and the production of genotoxins, colorectal proliferation and the capacity of faecal water to induce necrosis in colonic cells were decreased in 43 polypectomized patients after consuming symbiotic food containing *L. rhamnosus* LGG and *B. lactic* BB12.

LAB are also well-known for their serum cholesterol lowering ability. Shah (2007) reported that the administration of probiotic fermented milk (10^9 bacteria per mL) significantly reduced 50 % of the serum cholesterol level in hypercholesterolaemic human subjects. The hypocholesterolaemic effect of LAB was attributed to the ability of LAB to assimilate the serum cholesterol into the cell membrane (Liong and Shah, 2005a and 2005b). The serum cholesterol level was also reduced via the production of bile salt hydrolase (BSH) by LAB (Lye *et al.*, 2009). The hypercholesterolaemic effect of BSH was achieved via deconjugation of the bile salt, which limited re-absorption in the gut and facilitated excretion in faeces (Liong and Shah, 2005a and 2005b).

LAB have also been found to lower the blood pressure level. The production of bioactive angiotensin-I converting enzyme (ACE) inhibiting peptides by LAB have been shown to affect the rennin-angiotensin system. One study showed that the administration of *L. helveticus*-fermented milk significantly reduced the systolic and diastolic blood pressure by 4.1 mm Hg and 1.8 mm Hg respectively. The production of ACE-inhibitory peptides was also significantly increased in cheese upon addition of LAB during the fermentation process (Rhyänen *et al.*, 2001; Donkor *et al.*, 2007; Ong and Shah, 2008).

In addition, the gastrointestinal tract colonising-LAB can produce various nutrients for the host. Gomes and Malcata (1999) reported that LAB synthesised various vitamins such as folic acid, niacin, thiamine, riboflavin, pyridoxine, cyanocobalamin, and vitamin K where these vitamins were slowly absorbed by the host. However, the ability to synthesise vitamins and the concentration of vitamins produced was strain dependent (Biavati and Mattarelli, 2006). Several studies have reported the ability of *L. lactis* and *L. bulgaricus* to produce higher amount of folic acid, niacin, biotin, pantothenic acid, vitamin B6 and vitamin B12 as compared to their unfermented counterparts (unfermented control) (Hugenholtz and Kleerebezem, 1999; Kleerebezem and Hugenholtz, 2003).

2.1.3 LAB for Dermal Health

Increasing evidences indicate the possible use of LAB for treating extra-intestinal disorders by maintaining the intestinal microbiota balance, and thus ameliorating the immune system at local and systemic levels. The use of LAB to

exert health benefits beyond the gut through the gut-brain-skin axis hypothesis was proposed by Arck *et al.* (2010).

The potential roles of LAB to promote dermal health have been highlighted by numerous studies. LAB act as immune-modulators and improve skin health by regulating the production of cytokines and growth factors such as tumor-necrosis factor-alpha (TNF-α), interferon-gamma (IFN-Ɣ), transforming growth factor (TGF), and immunoglobulins (IgA and IgE). Guéniche *et al.* (2009) have reported that the ingestion of *L. johnsonii* NCC533 daily for 8 weeks significantly increased the production of cytokines and TGF-β, resulting in the preservation of cutaneous immune homeostasis in 57 volunteers upon exposure to ultraviolet ray of 2 x 1.5 minimal erythema dose. In addition, several studies have demonstrated alleviation of cow milk allergy and atopic dermatitis (AD) lesions via the consumption of *L. rhamnosus* GG. Upon administration, the level of IL-10 and IFN-Ɣ was significantly increased, resulting in preservation of cutaneous homeostasis (Pessi *et al.*, 2000; Pohjavouri *et al.*, 2004). Recently, the consumption of probiotics for 6 months was shown to reduce the risk of Ig-E- associated atopic eczema of the subjects (mothers at 35 weeks of gestation age and continued after the birth of infants up to the age of 6 months) via interaction with the neuropeptide S receptor 1 gene SNP hopo546333 (Kauppi *et al.*, 2014).

Besides promoting dermal health through the gut-skin axis, LAB have been employed in topical applications that exert dermal-promoting effects directly on the skin. One animal study has reported that wound closure and healing were significantly accelerated in the ischaemic and infected wounds of New Zealand white rabbits upon treatment with an adhesive gas permeable patch containing nitric oxide gas-producing probiotics (Figure 2.1; Jones *et al.*, 2012).

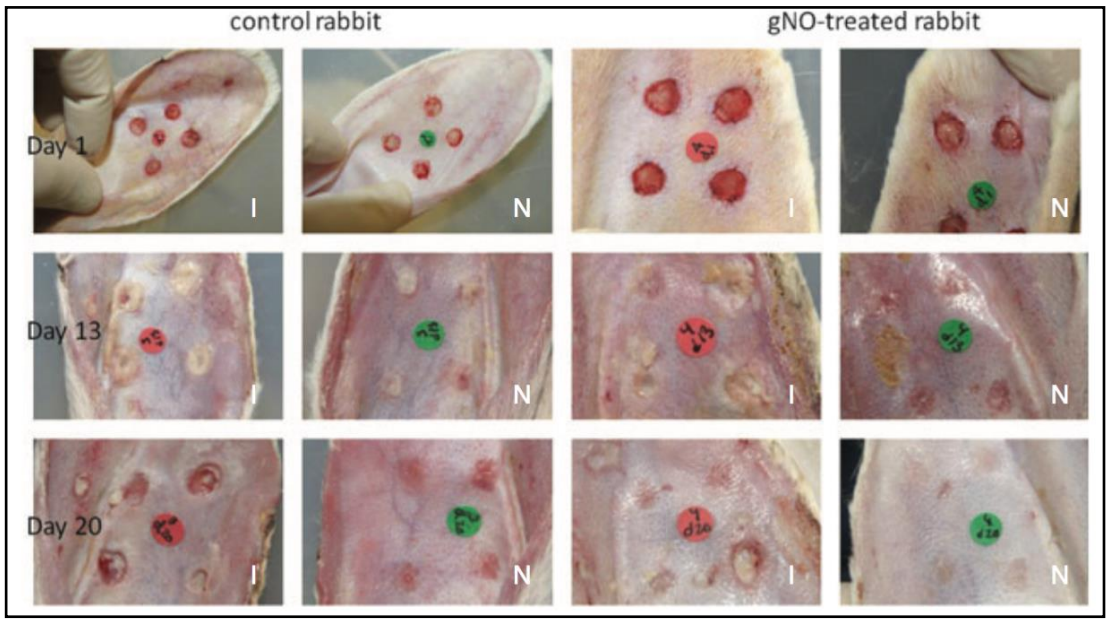

Figure 2.1 Photographs of infected full-thickness dermal wounds on ears that are either ischaemic "I" or non-ischaemic "N" and treated with nitric oxide gas-producing probiotic patchers or treated with vehicle control patches at days 1, 13 and 20 post-surgery. Wound healing was monitored daily and photographic records were kept for computer-aided morphometric analysis. Reprinted from Jones *et al.* (2012); with permission from John Wiley and Sons (License number: 3791820268326).

Dermal health can be improved not only by using whole LAB cells but also by using bioactive metabolites from LAB. Lysate from *Lactobacillus* and *Bifidobacterium* modulated the protein components such as claudin 3 of keratinocytes and increased the tight-junction (Sultana *et al.*, 2013), while *L. helveticus*-fermented milk promoted keratinocyte cell differentiation via enhancement of keratin-10 mRNA expression (Baba *et al.*, 2006). Another study has also suggested that bioactive metabolites produced by LAB such as bacteriocins and lipoteichoic acid (LTA) could kill skin pathogens and promote the host skin defence system (Tan *et al.*, 2014).

2.1.4 LAB-Derived Bioactive Metabolites for Dermal Health

2.1.4(a) Lactic Acid

LAB ferment carbohydrates via the Embden-Meyerhof-Panas pathway and produce lactic acid as the major metabolic end product. LAB also use the 6-P-gluconate/phosphoketolase pathway for carbohydrate fermentation, and produce lactic acid, acetic acid/ethanol and carbon dioxide as the end products (König and Fröhlich, 2009). There are two optical isomer forms of lactic acid, namely the L-(+)- and D-(-)-lactic acid.

Besides its antimicrobial ability, lactic acid has also demonstrated profound effects on epidermal and dermal layers by stimulating the secretion of cytokines. Topical application of 5 % lactic acid lotion over a year in 22 acne patients illustrated a significant reduction in inflammatory lesion counts and comedones (Garg *et al.*, 2002). Another study by Rendl *et al.* (2001) demonstrated that the secretion of vascular endothelial growth factor (VEGF) was significantly increased after the topical application of 1.5-3.0 % of lactic acid over the skin; subsequently wound repair was improved via stimulation of endothelial cells proliferation and migration and the expression of angiogenesis-related genes. In addition, lactic acid also enhanced the production of IL-17a that subsequently increased the re-epithelisation of skin wound healing, regardless of IL-23 dependent or independent pathway (Tesmer *et al.*, 2008; Yabu *et al.*, 2010). Lactic acid has also been used as a chemical peeling agent and exfoliator for different skin conditions. Another study has demonstrated a significant reduction of lengtigines and mottled hyper-pigmentation in the left forearm of a 62-year-old subject after the topical treatment of 25 % lactic acid twice daily for 6 months, as compared to the right forearm

(placebo; Green *et al.*, 2009). Sachdeva (2010) reported that treatment with 95 % (pH 2.0) lactic acid on seven patients of age 20-30 with superficial acne scaring for three months significantly improved the texture, pigmentation, and appearance of the treated skin with lightening of scars (Figure 2.2).

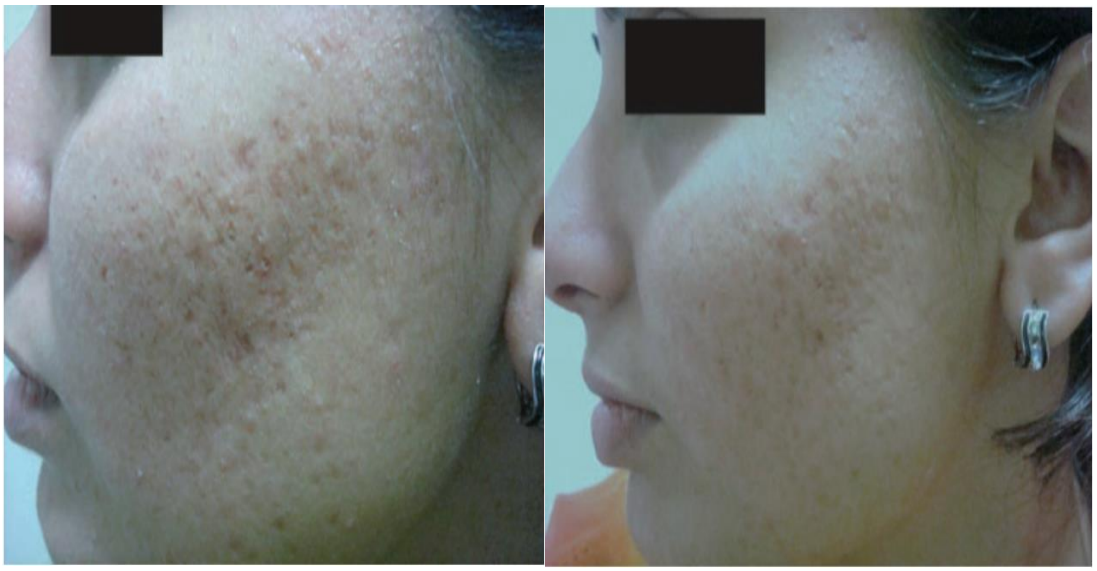

Figure 2.2 A 23-year-old female, Fitzpatrick skin type IV, (a) with comedonal acne and superficial acne scarring on the left side of the face, and (b) after four chemical peels with lactic acid showing good improvement, 3 months after treatment. Reprinted from Sachdeva (2010); with permission from John Wiley and Sons (License number: 3792311215214).

2.1.4(b) Acetic Acid

In addition to lactic acid, acetic acid produced by heterofermentative LAB such as *L. buchneri* is also known to improve dermal health. Numerous studies have suggested the potential use of acetic acid as topical antibacterial agents, especially in superficial wounds. The bactericidal effect of acetic acid was due to the chemical action of acetic acid itself which lowered the surrounding pH to a range that was unsuitable for the growth of pathogens (Nagoba *et al.*, 2008). Ryssel *et al.* (2009) demonstrated that 3 % acetic acid actively inhibited the growth of both Gram-

positive and Gram-negative pathogenic bacteria commonly found in burn units. In this study, acetic acid was capable of inhibiting the growth of *P. aeruginosa* upon 5 min treatment while the growth of Gram-positive *S. aureus* and *S. epidermidis* was completely inhibited upon 30 min treatment. Acetic acid was also able to inhibit the growth of *Escherichia coli, Enterococcus feacalis* and methicillin-resistant *Staphylococcus aureus* upon 60 min treatment. Another report has shown that the mean number of *S. aureus* and Gram-negative rods per ulcer were significantly reduced in 45 venous leg ulcer patients upon treatment with gauze dressing containing 0.25 % acetic acid (Hansson and Faergemann, 1995). Topical application of 3-5 % acetic acid daily for 12 days on seven hospitalised patients with diabetic foot ulcers successfully eliminated *P. aeruginosa* from the wounds, and a second application healed the wounds without grafting (Nagoba *et al.*, 2008).

2.1.4(c) Bacteriocins

Bacteriocins are small, ribosomal synthesised antimicrobial peptides (AMPs) that exhibit either broad or narrow spectrum of antimicrobial activity. LAB have been well- documented for bacteriocin production (O'Sullivan *et al.*, 2002; Reid *et al.*, 2003). One study has been reported that a bacteriocin (3.4 kDa) produced by *Lactococcus* sp. HY 449 inhibited the growth of numerous skin inflammatory bacteria such as *Pseudomonas aeruginosa, Staphylococcus aureus, Propionibacterium acnes,* and *Streptococcus pyrogenes* (Oh *et al.*, 2006). In addition, the bacteriocin from *Lactococcus lactis* KU24 exhibited significant inhibitory effect against methicillin-resistant *S. aureus*, indicating the potential application of

bacteriocin as an alternative antimicrobial agent against the growing number of antibiotic-resistant pathogens (Cotter *et al.*, 2013; Lee *et al.* 2013).

In addition to direct antimicrobial activity against skin pathogens, bacteriocins have also been shown to modulate the host skin immune system. Marzani *et al.* (2012) reported that plantaricin A from *L. plantarum* promoted the antioxidant defences, barrier functions, and antimicrobial activity of the skin by enhancing the mRNA expression of filaggrin, involucrin, β-defensin 2, and TNF-α. In addition, plantaricin A was also reported to accelerate the wound healing process by increasing the expression of TGF-β1, VEGF-A, and IL-8, resulting in proliferation and migration of human keratinocytes (Pinto *et al.*, 2011). Bacteriocins have also been incorporated into nanofibre scaffolds for dermal applications. Heunis *et al.* (2013) demonstrated that the number of viable *S. aureus* and the excision wound closure were significantly reduced on adult male BALB/c mice infected with *S. aureus* (Figure 2.3).

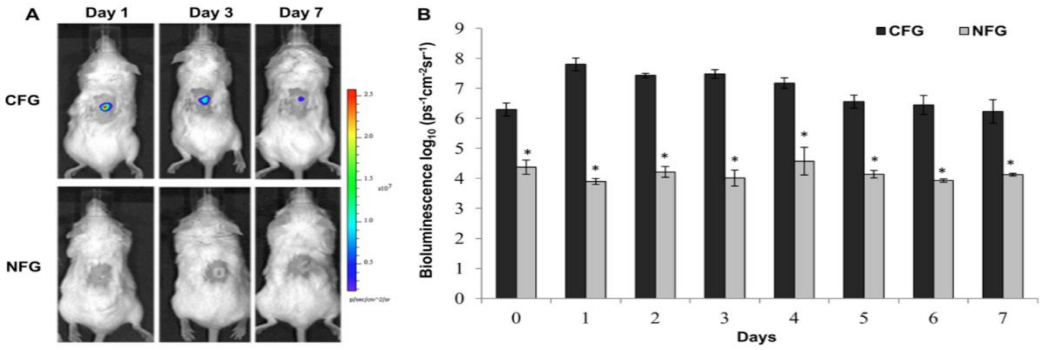

Figure 2.3 Efficacy of nisin-eluting electrospun nanofibre blend of Poly(ethylene oxide) (PEO) and Poly(D,L-lactide) (PDLLA) of ratios (50:50) wound dressings to reduce *Staphylococcus aureus* Xen 36 bioluminescence *in vivo* in a full-thickness excisional skin wound model in mice. Bioluminescent images (A) and bioluminescent measurements (B) of mice infected with 10 µl of 10^8 CFU/ml *S. aureus* Xen 36 and treated with nisin-containing PEO 50 –PDLLA 50 nanofiber wound dressings (NFG) and control PEO 50 –PDLLA 50 nanofiber wound dressings (CFG). *, $P<0.0001$ compared to CFG. Error bars represent standard deviations. Reprinted from Heunis *et al.* (2013) with permission from American Society for Microbiology (ASM).

2.1.4(d) Other Bioactive Metabolites

LAB are also capable of producing diacetyl, an organic compound with buttery flavour, as a metabolic by-product from citrate metabolism (Tan *et al.*, 2014). Diacetyl exerts a broad antimicrobial spectrum against both Gram-positive and Gram-negative skin pathogens (Jay, 1982). The exact mode of antimicrobial action of diacetyl is scarcely reported and still remains unclear. Holzapfel *et al.* (2003) suggested that the binding of diacetyl to guanidino sites of arginine in bacterial enzymes and inactivation of the protein via blockage or modification of the catalytic regions were the main attribute to the antimicrobial action of diacetyl. In one study, the growth of *E. coli* and *S. aureus* was significantly inhibited by 2.43 and 4.23 times respectively in the broth culture medium supplemented with 300 ppm of diacetyl, as compared to the untreated cells (Lanciotti *et al.*, 2003). Numerous *in vitro* studies have demonstrated the dermal potential of diacetyl; however, there are limited reports on the topical treatment of diacetyl for skin infections caused by skin pathogens in animal and clinical studies.

Besides diacetyl, LAB are also capable of producing hydrogen peroxide (H_2O_2). H_2O_2, a strong oxidiser generated by *L. gasseri* and *L. johnsonnii* NCC 533, significantly inhibited the growth of *S. aureus* and *Salmonella* sp. under aerobic conditions (Otero and Nader-Macias, 2006; Pridmore *et al.*, 2008). In addition to antimicrobial activity, numerous evidences have also reported the wound healing effects of H_2O_2. Sen *et al.* (2002) demonstrated that H_2O_2 at 250 µM facilitated angiogenesis by activating the transcriptional factor Sp1 and inducing the mRNA expression of VEGF in human keratinocytes. In a clinical study involving 60 patients with mild to moderate acne vulgaris, the inflammatory and non-inflammatory lesions were significantly reduced in subjects with topical application of stabilised H_2O_2

cream, resulting in better local tolerability profiles (erythema, dryness and burning sensation), as compared to the benzoyl peroxide gel (Milani *et al.*, 2003). Another study in zebrafish reported that H_2O_2 at a concentration of more than 166 mM resulted in detrimental effects such as delayed wound healing due to an increase in oxidative lipid damages and decrease in connective tissue formation (Niethammer *et al.*, 2009). Hence, it was suggested that the concentration of H_2O_2 lower than 166 mM could be safely applied to facilitate the wound healing process by enhancing the angiogenesis activity (Niethammer *et al.*, 2009).

LAB such as *L. rhamnosus* and *L. gasseri* also capable of producing hyaluronic acid (HA), which is an essential component of the extracellular matrix (ECM) of skin required for maintaining the normal skin structure of stratum corneum (SC), conserving epidermal barrier functions, and influencing cell proliferation, differentiation and tissue repair (Gold, 2007; Kogan *et al.*, 2007). It is a linear, anionic, and non-sulphated glycosaminoglycan polysaccharide comprised of D-glucuronic acid and *N*-acetylglucosamine monomer units. The high hydrophilic property of HA was shown to improve skin hydration and elasticity (Gold, 2007). In addition, HA was also shown to reduce wrinkle depth in 76 female subjects aged 30 to 60 years old after treatment with 0.1 % HA cream-based formulations (MW of 50, 130, 300, 800 and 2000 kDa, respectively) for 60 days (Pavivic *et al.*, 2011). HA was also shown to promote wound healing via enhancement of collagen deposition, cell proliferation, migration, angiogenesis, and pro-inflammatory activity (Weindl *et al.*, 2004). A clinical study involving 89 patients with one or several leg ulcers of venous or mixed venous origin treated with cotton gauze pad impregnated with 0.05 % HA demonstrated enhanced wound closure, healed ulcers rate, and reduced visual analogue scale, as compared to the placebo group (Humbert *et al.*, 2013).

2.2 Skin Defence System

The human skin serves as a protective barrier against environmental challenges and microbial pathogen invasion. The tough outer SC layer consists of overlapping, thin, and completely flattened keratinised cells connected by intercellular lipids that restrict the invasion of pathogenic microorganisms and chemicals into the body (Zaidi and Lanigan, 2010). This protective effect is enhanced by the naturally dry keratinised cell layers which are unfavourable for the growth of microorganisms. In addition, the SC layer is equipped with desquamation ability to help remove pathogenic microorganisms and chemicals from the skin surface (Chiller *et al.*, 2001; Zaidi and Laginan, 2010). The human skin immune system comes into play once the first line of defence is invaded.

The human skin defence mechanisms consist of the first line innate immunity, which involves the initial rapid pathogens clearance, and the second line adaptive immunity, which generates highly specific cytokines and antibodies, as well as immunological memory (Kang *et al.*, 2006). Both innate and adaptive immune systems have a coordinated effort in contributing to an effective immune response, even though both immune systems serve distinct functions.

2.2.1 Innate Immune System

Keratinocytes, Langerhans cells, neutrophils, and macrophages, as well as the production of pre-formed non-specific and broadly specific effector molecules are the major immune cells that are involved in the innate immune system (Oppemheim *et al.*, 2003). Upon invasion of pathogenic microorganisms, toll-like receptors (TLRs) and other PAMPs receptors recognise the pattern-associated molecule patterns