PRODUCTION OF BACTERIOCIN SF BY *LACTOBACILLUS GASSERI* SF FOR USE IN DERMAL BACTERIAL INFECTION AND WOUND HEALING

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by

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Tan Pei Lei Date:

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LIST OF ABBREVIATIONS AND SYMBOLS

AD	=	atopic dermatitis
ALP	=	alkaline phosphatase
AMPs	=	antimicrobial peptides
AU/mL	=	arbitary unit per mililitre
β (1-4)	=	beta (1-4)
BHI	=	brain heart infusion
CD	=	cluster of differentiation
CSLM	=	confocal scanning laser microscopy
CFU	=	colony forming unit
(CFU/mL, $\log_{10}$ CFU/mL)		
DMEM	=	Dulbecco's modified Eagle's medium
DC	=	dendritic cell
DNA	=	deoxyribonucleic acid
ECM	=	extracellular matrix
eDNA	=	extracellular deoxyribonucleic acid
ELISA	=	enzyme-linked immunosorbent assay
EMBL	=	European molecular biology laboratory
EPS	=	extracellular polymeric substances
FGFR2	=	fibroblast growth factor 2
FITC	=	fluorescein isothiocyanate
g	=	gram
GAS	=	group A *Streptococcus*
GC	=	guanine-cytosine
h	=	hour
H_2O_2	=	hydrogen peroxide
HA	=	hyaluronic acid
hBD	=	human beta defensin
(hBD-1, hBD-2, hBD-3)		
HPLC	=	high performance liquid chromatography
IBS	=	irritable bowel syndrome
IFN	=	interferon alpha
(IFN-α, IFN- γ)		

KGFR	=	keratinocyte growth factor receptor
Ig	=	immunoglobulin
IL (IL-1, IL-4,IL-8)	=	interleukin
LAB	=	lactic acid bacteria
LPS	=	lipopolysaccaride
LTA	=	lipoteichoic acid
M	=	Molar
MIC (MIC_{50}, MIC_{90})	=	minimum inhibitory concentration
min	=	minute
mL	=	millilitre
mL/min	=	milliliter per minute
mm	=	milimeter
mol/L	=	mole per litre
mRNA	=	messenger ribonucleic acid
MRS	=	de Mann Rogosa Sharpe
MTT	=	3-(4,5-dimethylthiazol-2-y)-2,5-diphenyl tetrazolium bromide
MRSA	=	methicillin resistant *Staphyloccus aureus*
NF-κB	=	nuclear factor kappa-light-chain-enhancer of activated B cells
ng/mL	=	nanogram per mililitre
$(NH_4)_2SO_4$	=	ammonium sulfate
nm	=	nanometer
OD	=	optical density
PAMP	=	pathogen-associated molecule pattern
PBS	=	phosphate buffered saline
PDLLA	=	poly(D,L-lactide)
PEO	=	poly(ethylene oxide)
Pglyrp	=	peptidoglycan recognition protein
PGN	=	peptidoglycan
PRMs	=	pattern recognition molecules

QS	=	quorum sensing
RNA	=	ribonucleic acid
RSM	=	reconstituted skimmed milk
RT-PCR	=	reverse transcription-polymerase chain reaction
SC	=	stratum corneum
SCORAD	=	Severity Scoring of Atopic Dermatitis
SDS-PAGE	=	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	=	scanning electron microscope
SMase	=	sphingomyelinase
TEM	=	transmission electron microscope
TGF-β	=	transforming growth factor beta
TLR	=	toll like receptor
TNF-α	=	tumor necrosis factor alpha
µL	=	microliter
µm	=	micrometer
USM	=	Universiti Sains Malaysia
v/v	=	volume per volume
VEGF	=	vascular endothelial growth factor
w/v	=	weight per volume
%	=	percent
°C	=	degree Celcius

PENGHASILAN BAKTERIOSIN SF OLEH *LACTOBACILLUS GASSERI* SF UNTUK APLIKASI KULIT YANG DIJANGKITI BAKTERIA DAN PENYEMBUHAN LUKA

ABSTRAK

Enam belas strain *Lactobacillus* dan *Bifidobacterium* telah disaring berdasarkan pertumbuhan di dalam susu skim. *B. longum* 8643, *L. plantarum* 8943, *L. casei* 1268, *L. fermentum* 8312, *L. fermentum* 8848, dan *L. gasseri* SF menunjukkan kemandirian yang lebih tinggi (P< 0.05), dan ekstrak extrasel daripada enam strain ini juga mengandungi bioaktif pada konsentrasi yang mampu meningkatkan kesihatan kulit. Di samping itu, protein mentah yang diperolehi daripada ekstrak extrasel juga menunjukkan aktiviti penghambatan yang lebih tinggi (P< 0.05) terhadap pertumbuhan patogen kulit, dan mungkin disebabkan oleh sebatian antimikrob protein. Unsur seperti bakteriosin yang dihasilkan oleh *L. gasseri* SF menunjukkan aktiviti penghambatan yang lebih tinggi terhadap *Enterococcus faecalis* FM 2138, dan memenuhi ciri-ciri bakteriosin kelas II, justeru dinamakan sebagai bakteriosin SF. Bakteriosin SF juga didapati stabil haba dengan jisim molekul ketara sebanyak 3.5 kDa. Bakteriosin SF pada kepekatan 10240 AU/mL juga mengurangkan cas negatif pada permukaan sel *E. faecalis* FM 2138 dengan signifikan (P <0.05), dan seterusnya menyebabkan depolarisasi membran dan pembentukan liang seni, seperti yang ditunjukkan dalam mikrograf elektron. Bakteriosin SF juga menurunkan tahap ungkapan mRNA dalam gen yang berkaitan dengan *fsr* korum penderiaan dan pembentukan biofilem dalam *E. faecalis* FM 2138. Potensi bakteriosin SF dalam penyembuhan luka juga telah dipamerkan *in vitro*, di mana bakteriosin SF pada kepekatan 5120 AU/mL meningkatkan proliferasi dan migrasi sel HaCaT dengan

signifikan (P< 0.05). Bakteriosin SF juga telah meningkatkan tahap ungkapan mRNA untuk faktor pertumbuhan (FGFR-IIIb) dan sitokin (TGF-β1 dan IL-8) yang memainkan peranan penting dalam proses penyembuhan luka. Selanjutnya, arnab yang dirawat dengan rumusan topikal yang mengandungi bakteriosin pada kepekatan 5120 AU/mL tidak mempamerkan sebarang tanda-tanda kerengsaan kulit dan perubahan histologi. Rumusan topical bakteriosin SF ini juga meningkatkan kadar pengecutan luka, kandungan hydroksiprolin, dan mengurangkan pertumbuhan *E. faecalis* FM 2138 dengan signifikan (P < 0.05) pada bahagian luka kulit tikus. Ungkapan mRNA untuk CX3CR1, IL- 8, dan TGF- β1, dan tahap ungkapan protein untuk IL- 8, TGF- β1, dan IFN- α juga telah dipertingkatkan semasa rawatan topical bakteriosin SF, menunjukkan bahawa bakteriosin SF boleh menggalakkan penyembuhan luka dengan mempengaruhi efektor imun yang terlibat dalam penyembuhan luka. Secara keseluruhan, keputusan dalam kajian ini mencadangkan bahawa bakteriosin SF pada kepekatan 5120 AU/mL adalah selamat, berkesan untuk menghambat pertumbuhan *E. faecalis* FM 2138 dan menggalakkan penyembuhan luka. Justeru, bakteriosin SF berpotensi digunakan dalam bidang dermatologi sebagai bahan bioaktif yang berkesan untuk mengatasi jangkitan *E. faecalis* dan/atau rawatan penjagaan luka.

PRODUCTION OF BACTERIOCIN SF BY *LACTOBACILLUS GASSERI* SF FOR USE IN DERMAL BACTERIAL INFECTION AND WOUND HEALING

ABSTRACT

Sixteen strains of *Lactobacillus* and *Bifidobacterium* were screened based on their growth in reconstituted skimmed milk. However, six strains (*B. longum* 8643, *L. plantarum* 8943, *L. casei* 1268, *L. fermentum* 8312, *L. fermentum* 8848, and *L. gasseri* SF) exhibited significantly higher viability ($P < 0.05$). Extracellular extracts of these strains contained bioactives at concentrations capable of promoting dermal health. Meanwhile, crude protein fractions fractionated from extracellular extracts of all six strains exhibited significantly higher antagonistic activity on skin pathogens, probably due to the production of putative bacteriocins. Putative bacteriocin produced from *L. gasseri* SF exhibited significantly higher ($P < 0.05$) antagonistic activity on *Enterococcus faecalis* FM 2138, and fitted the characteristics of class II bacteriocin and was thus renamed as bacteriocin SF. Bacteriocin SF was found to be heat-stable, with an apparent molecular mass of 3.5 kDa. Bacteriocin SF at a concentration of 10240 AU/mL significantly reduced ($P < 0.05$) the negative charge on the cellular surface of *E. faecalis* FM 2138, subsequently leading to membrane depolarization and pore formation, as visible in electron micrographs. Bacteriocin SF also down-regulated mRNA expression levels of *fsr* quorum sensing- and biofilm associated genes of *E. faecalis* FM 2138. Wound healing potential of bacteriocin SF was also demonstrated *in vitro*, where bacteriocin SF at a concentration of 5120 AU/mL significantly increased ($P < 0.05$) HaCaT cells proliferation and migration. Bacteriocin SF also increased the mRNA expression of growth factor (FGFR2-IIIb), and protein expression level of cytokines (TGF-β1 and IL-8) in HaCaT cells that play an

important role in wound healing. Furthermore, rabbits treated with a topical formulation containing 5120 AU/mL of bacteriocin SF exhibited no signs of skin irritation or abnormal histological changes. This topical bacteriocin SF formulation also significantly increased ($P< 0.05$) the wound contraction rate, hydroxyproline content, and reduced the viability of *E. faecalis* FM 2138 in the wound sites of mice. mRNA expression of CX3CR1, IL-8, and TGF-β1, and protein expression levels of IL-8, TGF-β1, IFN-α in mice were also elevated during topical bacteriocin SF treatment, indicating that bacteriocin SF may promote wound healing by regulating the immune effectors that are involved in wound healing. Collectively, results in this study suggest that bacteriocin SF at a concentration of 5120 AU/mL is safe, effectively inhibits the growth of *E. faecalis* FM 2138 and promotes wound healing. Therefore, bacteriocin SF could be potentially applied in the field of dermatology as a bioactive ingredient against *E. faecalis* infections and/or wound care treatment.

CHAPTER 1

INTRODUCTION

1.1 Background

Lactobacilli and bifidobacteria are the most common genera of bacteria with claimed probiotic properties. Probiotics are defined as "live microorganisms which when administered in adequate amounts, confer health benefits on the host" (Guarner *et al*., 2005). The health benefits of these bacteria in the gut have been well documented, including therapeutic restoration of altered gut microbiota; and prevention and treatment for diarrhea (Fung *et al*., 2011). Although major studies have traditionally focused on the potential beneficial effects of probiotics on gut health, there has been a shift in recent years toward discovering the therapeutic possibilities of probiotics beyond the gut, such as the skin.

Skin is the largest organ of human body. For an adult, the total surface area and weight of skin area are about 1.75 m^2 and 5 kg, respectively (Percival *et al*., 2012). The skin functions as a vital physical barrier that protects the human body's underlying tissues from the external environment influences, such as ultraviolet and desiccation. The skin also communicates with the external environment to support a normal flora; and regulate water content, calcium homeostasis, and temperature in human body. In addition, the skin harbours an enormously complex immune system that is poised to react to toxins, infections, and injuries (Ilkovitch, 2011). However, under certain circumstances such as injury, infectious microorganisms can breach into the skin and produce an infection which is detrimental to wound healing.

Lactobacilli and bifidobacteria have been advocated for the treatment and prevention of a wide range of skin infections and skin diseases. Peral *et al*. (2010) have demonstrated that ingestion of viable *Lactobacillus plantarum* significantly reduced

wound bacterial number and promoted wound healing in diabetic and non-diabetic patients with chronic infected leg; while Yesilova *et al*. (2012) reported that eight week oral administration of a probiotic mix (*L. acidophilus*, *L. casei*, *L. salivarius*, and *Bifidobacterium bifidum*) significantly reduced serum cytokines IL-5, IL-6, IFN-γ and total serum IgE levels in children with moderate to severe atopic dermatitis (AD), a long term skin disorder that involves scaly and itchy rashes. Clinical studies also showed that consumption of viable *L. rhamnosus* GG in pregnant mothers with a strong history of AD significantly reduced the frequency of developing atomic dermatitis in the offspring during the first 7 years of life (Kalliomaki *et al*., 2007). Although viable cells appear to have more beneficial effects than non-viable ones, a significant number of clinical studies have revealed that treatment and prevention of skin disorders with viable *Lactobacillus* and *Bifidobacterium* strains as either a single strain or in combination with other probiotic strains has been less impressive. This may be due in part to the decrement of viability and/or functionality of *Lactobacillus* and *Bifidobacterium* strains during storage. Meanwhile, the latter mounting studies have also suggested the potential use of non-viable cells, and bioactive compounds derived from these strains for dermal applications, via both oral and topical approaches (Cinque *et al*., 2011; Oh *et al*., 2006). Therefore, in contrast to viable cells, non-viable cells and/or bioactive compounds derived from *Lactobacillus* and *Bifidobacterium* strains may be a better option due to their stability at room temperature, and in cases in which the application of viable cells can lead to the risk of bacteremia.

Indeed, non-viable cells and/or bioactive compounds derived from *Lactobacillus* and *Bifidobacterium* strains have been demonstrated to exert dermal health-promoting effects. Daily oral administration of *L. rhamnosus* cell lysate for a month has been shown to aid children with resistant atopic eczema by decreasing irritation scores; while

daily topical application of cream containing *B. longum* sp lysate for 2 months has been reported to improve reactive skin by decreasing skin sensitivity and increasing skin barrier function (Hoang *et al*., 2010; Guéniche *et al*., 2010). Meanwhile, it is important to note that, lactobacilli and bifidobacteria produce bioactive compounds that inhibit the adhesion of Gram-positive and Gram-negative pathogenic bacteria to the intestinal epithelial cells, and thus protect intestinal epithelium against the development of infectious disease. Considering such beneficial effects, bioactive compounds produced from *Lactobacillus* and *Bifidobacterium* strains could also be applied in the field of dermatology to enhance skin health and treat skin infections.

Therefore, bioactive compounds produced by lactobacilli and bifidobacteria could exhibit antimicrobial activity against skin pathogens and/or improve dermal health. Moreover, production and potential mechanism of action by which bioactive compounds from lactobacilli and bifidobacteria can promote dermal health are not well understood with the studies currently available. Although bioactive compounds which are produced from natural resources as in this study generally have less toxicity, evaluation on the side effects and elimination of the toxicity (if present) via an *in vivo* model is deemed necessary to verify their safety. In addition, the efficacy of bioactive compounds produced from lactobacilli and bifidobacteria on dermal health via *in vivo* model is scarcely reported. Thus, more *in vitro* studies are needed to better understand the production and potential mechanisms of action of these bioactive compounds produced from lactobacilli and bifidobacteria on dermal health, as well as their efficacy and safety via *in vivo* studies.

1.2 Aim and Objectives for Research

The main aim of this study was to evaluate the effects of dermal bioactives from lactobacilli and bifidobacteria on skin pathogens and dermal health.

Specific and measureable objectives were:

1. To screen and select *Lactobacillus* and *Bifidobacterium* strains that are capable of excreting dermal bioactives.
2. To fractionate and characterize bioactives that are responsible for antimicrobial activity against skin pathogens for skin health.
3. To elucidate the mechanisms of action of selected bioactives against selected skin pathogen *in vitro*.
4. To evaluate the wound healing potential of selected bioactives on human keratinocytes *in vitro*.
5. To evaluate the safety and efficacy of topical cream containing selected bioactives via *in vivo* models.

CHAPTER 2

LITERATURE REVIEW

2.1 Probiotics

Probiotics are defined as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host" (Guarner *et al*., 2005). Although probiotics have been explored and consumed for centuries, they have only started to receive scientific popularity for the past two decades.

Several aspects which contribute a microorganism to be defined as a probiotic include i. must be alive when administered, ii. must deliver a measured physiological benefit that requires substantiation by studies performed in the target host, iii. not necessarily oral administered, but could encompass other applications, iv. restriction in term of mode of action are not defined, v. not excluded from pharmaceutical and therapeutic application, and vi. taxonomically defined strains (Sanders, 2003). A wide range of microorganisms has been identified to exhibit probiotic properties (Table 2.1). Lactic acid bacteria (LAB) and *Bifidobacterium* strains, in particular, have gained increasing attention as a major group of probiotic bacteria, mainly attributed to their proven potentials in the food industry, human nutrition and feed production. Probiotics are most often incorporated into food and beverage products as dietary adjuncts, aimed at promoting gastrointestinal health and modulating immune functions in the gut (Marini and Krutmann, 2012). Although major areas of concerns have been the potential for gut health, a growing number of studies have revealed suggestive evidences that probiotics may offer benefits beyond the gut.

Table 2.1: Major identified microorganisms associated with probiotic properties.

Lactobacillus	***Bifidobacterium***	***Lactococcus***	***Bacillus***	***Kluyveromyces***
L. acidophilus	*B. adolescentis*	*L. lactis* subsp. *cremoris*	*B. cereus*	*K. lactis*
L. brevis	*B. animalis* subsp. *lactis*	*L. lactis* subsp. *lactis*	*B. coaugulans*	
L. casei	*B. bifidum*	*L. raffinolactis*	*B. subtilis*	***Saccharomyces***
L. crispatus	*B. breve*			*S. boulardii*
L. curvatus	*B. infantis*	***Leuconostoc***	***Clostridium***	*S. cerevisiae*
L. delbrueckii subsp.*bulgaricus*	*B. lactis*	*L. mesenteroides*	*C. butyricum*	
L. fermentum	*B. longum*			
L. gasseri	*B. thermophilum*	***Streptococcus***	***Escherichia***	
L. johnsonii		*S. thermophilus*	*E. coli*	
L. paracasei	***Enterococcus***			
L. plantarum	*E. faecalis*	***Pediococcus***	***Propionibacterium***	
L. reuteri	*E. faecium*	*P. acidilactici*	*P. freudenreichii*	
L. rhamnosus			*P.jensenii*	
L. salivarius				

2.1.1 *Lactobacillus*

The genus *Lactobacillus* is the largest group in the family of Lactobacteriaceae, comprising 185 recognized species and 28 subspecies identified to date (Euzeby, 2013). Lactobacilli are characterized as Gram-positive, non-spore forming, non-flagelated rod or coccobacilli in shape bacteria that usually contain genomic guanine-cytosine (GC) varies from 32 to 51 mol % (Otieno, 2011). All *Lactobacillus* species are members of LAB, which able to produce lactic acid as a major end product of the fermentation of carbohydrates. Lactobacilli have been found in different location of the gastrointestinal tract, oral cavity, and vagina of healthy women microbiota (Reuter, 2001, Munson *et al*., 2004; Martin *et al*., 2007). Several studies have also reported that lactobacilli are among the most dominant bacteria distributed in the small intestine (Saito, 2004; Reuter, 2001; Molin *et al*., 1993). *Lactobacillus gasseri*, *L. reuteri* and *L. rhamnosus* have been identified as most commonly isolated *Lactobacillus* species from human small intestine (Reuter, 2001; Molin *et al*., 1993). Lactobacilli are not only found in the human body, but are also ubiquitous in environments where carbohydrate are available, such as fruits, vegetables, beverages, plant, plant materials, dairy products, fermented or spoiled food, sewage, manure, respiratory, gastrointestinal, and genital tracts of animals (Giraffa *et al*., 2010). The members of *Lactobacillus* are able to grow optimally at the temperature and pH varies widely from 30 ˚C to 45 ˚C and pH in the range of 5.5 to 7.0 (Hutkins, 2006). Clinical evidences have revealed potential uses of *Lactobacillus*-containing food and beverages on human health promoting effects (Reid *et al*., 2003). Thus, lactobacilli have generally considered as beneficial microorganisms, and often incorporated into daily diet.

2.1.2 *Bifidobacterium*

The genus *Bifidobacterium* was historically classified as a member of LAB, and in the genus *Lactobacillus* based on their correlation of the peptidoglycan (PGN) structure (Kandler and Lauer, 1974). However, further studies have discovered that bifidobacteria degrade hexoses using a peculiar metabolic pathway, *bifid shunt*, which is also known as fructose-6-phosphate pathway (Wolin *et al*., 1998). Fructose-6-phosphate, the key enzyme in this pathway, has also listed as one of the main character for the taxonomically classification at genus level (Biavati and Mattarelli, 2001). Therefore, in the 1970s, this group of bacteria has been reclassified into *Bifidobacterium*, which belongs to the family of Bifidobacteriaceae. Euzeby (2013) has reported that 47 species and 9 subspecies of the genus *Bifidobacterium* have been listed to date.

Bifidobacteria are Gram positive, non-motile, anaerobic and chemoorganotrophs bacteria, with genomic GC content between 42 and 67 mol % (Biavati and Mattarelli, 2001; Delcenserie *et al*., 2007; Otieno, 2011). They commonly occur as singly, chains, or clumps. They could also occur in various shapes, including short, curved rods, club-shaped rods, or bifurcated Y-shape rods (Gomes and Malcata, 1999). Bifidobacteria are able to grow optimally at temperatures ranging from 37 ˚C to 41 ˚C, and pH between pH 6.5 and 7.0 (Hutkins, 2006). Bifidobacteria have been successfully isolated from varies habitats in animals and human, such as feaces, honey bee intestine, rumen of cattle, and human vagina (Otieno, 2011). Bifidobacteria have been documented as the most dominant bacteria that colonize the gastrointestinal tracts of human and animals, especially in breastfed infants. *Bifidobacterium longum* biovar *infantis*, *B. breve* and *B. bifidum* are represent up to 91 % of intestinal microbiota in breastfed infant and 3 - 7 % in adults (Biavati *et al*., 2000). Therefore, *Bifidobacterium* have been focused, and considered as one of the most common genera used for human consumption.

2.1.3 Conventional Health Benefits of Probiotics

Probiotics have a long history of safe use with fermented dairy products, since their beneficial effects on gastrointestinal health have been discovered. Indeed, maintenance of gastrointestinal health is crucial as approximately 70 % of all immune cells of the entire immune system are located in the gastrointestinal tract (Vighi *et al*., 2008). Accumulating evidences also indicate that intestinal microbiota interacts with both innate and adaptive immune system, affecting different aspects of gastrointestinal physiology and function (Purchiaroni *et al*., 2013). Lactobacilli and bifidobacteria are among the Gram-positive bacterial populations that commonly inhabit in healthy intestinal microflora, and thus they have been the focus that are used in most of the studies for exploring and evaluating the roles of probiotics in the maintenance of gastrointestinal health.

Probiotics have been found to alleviate lactose intolerance symptoms by increasing the digestibility of lactose that is present in human intestine. *In vitro* studies have demonstrated that *Lactobacillus* strains are capable of exhibiting β-galactosidase, phospho-β-galactosidase and phospho-β-glucosidase activities, which hydrolyze lactose by activating two lactose transportation systems, namely lactose-permease transportation and lactose-specific phosphoenolpyruvate-dependent phosphotransferase system (Honda *et al*., 2007). It has been described that if lactose maldigesters ingested sufficient amount of lactose, gastrointestinal symptoms may result, including abdominal discomfort, bloating, diarrhea, and flatulence (Vesa *et al*., 2000). Additionally, Gaón *et al.* (1995) have performed a clinical study to evaluate the efficacy of milk fermented with *L. acidophilus* and *L. casei* on alleviating lactose intolerance symptoms and lactose digestion with 18 lactase deficiency subjects. The oral administration of milk fermented with *Lactobacillus* reduced the development of symptoms, suppressed intestinal motility,

and decreased hydrogen production intake, thereby leading to an improvement in lactose digestion. He *et al*., (2008) have also conducted a human trial with 11 Chinese lactose maldigesters to evaluate the effects of yogurt supplemented with *B. animalis* and capsule encapsulated with *B. longum* on the colonic microbiota. The authors found that ingestion of yogurt and capsule containing *Bifidobacterium* increased the numbers of *Bifidobacterium* in the colonic microbiota and reduced symptoms in lactose maldigesters.

Probiotics have also been investigated for their roles in treating irritable bowel syndrome (IBS). IBS is a functional bowel disorder that has been associated with complex pathophysiology; include microscopic inflammation, alterations in gut motility, and visceral hypersensitivity (Aragon *et al*., 2010). The common features associated with IBS include discomfort of defecation, abdominal pain, bloating, and abnormal bowel habit. Probiotics are seen as a promising therapy to alleviate IBS symptom due to their ability to reduce gut and fluid motility. Probiotics have been reported to deconjugate and absorb bile acid, which would subsequently reduce the colonic mucosal secretion of mucin and fluids that lead to functional diarrhea (Camilleri, 2006). Additionally, lactobacilli and bifidobacteria could reduce inflammation by exhibiting antimicrobial activities. Probiotics inhibited the growth and colonization of pathogenic bacteria via three possible mechanisms, including ability of adherence, production of inhibitory substances and iron-siderophore (Fung *et al*., 2011). Administration of VSL #3, a mixture of 8 probiotic strains in male IBS rats, have revealed that probiotics significantly reduced visceral pain perception via resetting colonic expression of subsets of genes mediating pain and inflammation (Distrutti *et al*., 2013). The administration of *B. infantis* 35624- fermented malted milk drink (1×10^{10} live bifidobacteria per day) is also capable of normalizing the abnormal ratio of an anti-inflammatory to pro-

inflammatory cytokine (IL-10/IL-12), and resulting in significant reduction in IBS symptom scores (O'Mahony *et al*., 2005). Recent pilot study and meta-analysis also updated the significant reduction of common IBS symptoms, modulation of mucosa microbiota composition and immune functions in IBS patients treated with probiotics as compared to the placebo group (Ng *et al*., 2013; Ortiz-Lucas *et al*., 2013).

In addition to reduction of IBS, the potential protective roles of probiotics against tumor development in the colon have also been established. Previous studies reported that probiotics could modulate toxifying and detoxifying enzymes associated with carcinogenesis by producing short chain fatty acids that decrease the pH of the colon (Lankaputhra and Shah, 1998). Another possible mechanism to reduce the risk of colon cancer could be attributed to the cell wall skeleton of the LAB that can bind with mutagens (Zhang and Ohta, 1991). Administration of probiotics has also found to suppress nitroreductase and β-glucoronidase activities, thus reduced aberrant crypt foci counts in carcinogen-induced rats (Verma and Shukla, 2013). Probiotics also modulate immune response by decreasing the gene expression of programmed cell death in colorectal tissues of carcinogen-induced rats (Mohania *et al*., 2013). Clinical studies have shown promising results in colon cancer therapy, particularly on polypectomized (removal of a polyp) patients and patients undergoing elective colorectal surgery. Rafter *et al*. (2007) have evaluated the effects of symbiotic food containing *L. rhamnosus* LGG and *B. lactis* BB 12 in 12 weeks randomized, double-blind study involving 43 polypectomized patients. Symbiotic intervention significantly increased secretion of IL-2, changed feacal flora, decreased genotoxins, colorectal proliferation, and the capacity of fecal water to induce necrosis in colonic cells. Furthermore, Liu *et al*. (2011) conducted a double-blind study to determine the effects of perioperative administration of probiotics in 100 patients undergoing elective colorectal surgery. The authors found

that patients administered with probiotics significantly enhanced mucosal tight junction protein expression, increased transepithelial resistance, decreased transmucosal transmission of horseradish peroxidase, ileal-bile acid binding proteins and positive rate of blood bacterial DNA (risk of bacteremia).

Other beneficial roles of probiotics in gastrointestinal health including the alleviation of inflammatory bowel disease, antibiotic-associated diarrhea, acute infection diarrhea, and postoperative complications have also been well documented (Fung *et al*., 2011, Sanders *et al*., 2013).

2.1.4 New Roles of Probiotics

There is an increasing evidence to indicate that contemporary studies have focused more on the possible deployment of probiotics for treating extra-intestinal disorders due to their ability to balance intestinal microbiota, which ameliorated the immune systems at local and systemic levels. Indeed, several promising new roles of probiotics have been proposed in the past 5 years (Table 2.2). Thus far, emerging evidences have outlined more promising and significant impact of probiotics on gut-brain-skin axis.

Dinan *et al*. (2013) have defined psychobiotics as living organisms, when ingested in adequate amounts, exerts beneficial effects in patients suffering from psychiatric illness. The potential novel use of probiotics as psychobiotics has recently been proposed due to their ability to manage stress-related psychiatric disorders. Preliminary studies have revealed that certain probiotic strains are capable of producing and delivering neuroactive substances, such as gamma-aminobutryic acid, at a concentration level which may alleviate symptoms of depression and anxiety (Barrett *et al*., 2012). Additionally, Messaoudi *et al*. (2011) have conducted both pre-clinical and

clinical studies to investigate the anxiolytic-like activity of a probiotic formulation containing *L. belveticus* R0052 and *B. longum* R0175 in rats, and its possible psychotropic-like effects via a double-blind, placebo-controlled, randomized parallel study that involving 66 healthy volunteers. The authors found that administration of probiotic formulation for 2 weeks significantly reduced anxiety-like activity in rats, while administration of probiotic formulaiton for 30 days mitigated psychological distress (somatization, depression, anger-hostility, anxiety) in volunteers. Tillisch *et al*. (2013) also reported that consumption of fermented milk with probiotics for 4 weeks could change midbrain connectivity by reducing intrinsic activity of resting brain (affective, viscerosensory, and somatosensory cortices) in healthy women. Regarding the potential mechanistic pathway, it has been reported that consumption of probiotics may influence systemic cytokines and thus improved mood disturbance and fatigue which were induced by systemic administration of lipopolysaccharide endoxtoxin (Lakhan and Kirchgessner, 2010). Another possible mechanism by probiotics involved the production of antimicrobial compounds such as short chain fatty acids, which prevented the stress-induced alteration to overall intestinal microbiota (Logan *et al*., 2003). The beneficial effects of probiotics on mental health may also be due to their ability to modulate neurotrophic chemicals including brain-derived neurotrophic factor (Logan and Katzman, 2005). All biochemical and behaviour evidences have led to the suggestion that they could be used as a psychotropic agent.

In addition to the psychobiotic properties, potential roles of probiotics in the maintenance of skin health have also been highlighted. Preliminary studies have suggested that probiotics could produce dermal bioactives such as bacteriocins and lipoteichoic acid (LTA), and thereby inhibiting the growth of skin pathogens and/or enhancing skin defense system (Tan *et al*., 2014). *In vitro* studies have further

demonstrated that keratinocytes treated with lysates from *Lactobacillus* and *Bifidobacterium* strains could increase tight-junction barrier function via modulation of protein components such as claudin 3, while *L. helveticus*- fermented milk enhanced keratin-10 mRNA expression subsequently promoted cell differentiation (Baba *et al*., 2006; Sultana *et al*., 2013). Feeding of *B. breve* strain Yakult to ultraviolet-induced hairless mice also decreased transepidermal water loss, suppressed oxidation levels of proteins and lipids by preventing the generation of reactive oxygen species (Ishii *et al*., 2014). On the other hand, Jones *et al*., (2012) also found that topical application of an adhesive gas permeable patch containing nitric oxide gas-producing probiotic increased wound closure and accelerated wound healing in New Zealand white rabbit model of ischaemic and infected wounds. Clinical studies have reported on the promising effects of probiotics on dermal health. Guéniche *et al*., (2009) conducted a randomized, double blind placebo-controlled trial to determine the immunomodulatory effects of probiotics in 57 volunteers upon exposure to ultraviolet (2 x 1.5 minimal erythema dose). The authors reported that volunteer ingested *L. johnsonii* NCC 533 daily for 8 weeks significantly increased the production of regulating cytokines and growth factor such as TGF-β, which lead to the preservation of cutaneous immune homeostasis. Recently, K *et al*., (2014) also found that consumption of probiotics for 6 months could interact with neuropeptide S receptor 1 gene SNP hopo546333, and thus reduced the risk of IgE-associated atopic eczema in early childhood. Altogether, current available evidences have illustrated the dermal potential of formulations containing living probiotics and/or probiotic-derived bioactives for skin maintenance.

Table 2.2 New roles and benefits of probiotic bacteria beyond the gut.

	Roles and/or benefits	Condition/ Location	Reference
1	Suppress arthritic inflammation	osteoarthritis	So *et al*. (2011)
2	Reduce risk factors for cardiovascular diseases	cardiovascular diseases	Ebel *et al*. (2014)
3	Inhibit JUNV infection	viral infection	Martinez *et al*. (2012)
4	Decrease body and fat pad weights	obesity	Park *et al*. (2013)
5	Protect against asthma	respiratory system	Yu *et al*. (2010)
6	Reduce plasma, aortic, and hepatic lipid profile	hypercholesterolemia	Mohania *et al*. (2013)
7	Modulate lung immune functions	lung	Forsythe (2014)
8	Protect against free radicals-induced disorders	metabolic disorders	Ghoneim and Moselhy (2013)
9	Modulate bone health	bone	Rodrigues *et al*. (2012)
10	Protect urogenital tract	renal	Vujic *et al*. (2013)

2.2 Human Skin

2.2.1 Skin Structure and Function

Skin is the largest organ of the human body and functions as a primary physical barrier that protects the host's underlying tissues from external environmental influences such as bacterial infection, desiccation, ultraviolet irritation, physical as well as chemicals assaults, and excessive water loss. The human skin can be basically divided into two main layers, the dermis and the epidermis (Figure 2.1). The dermis is the thick inner layer and consists mostly of fibrous and amorphous connective tissues, such as elastic and collagen fibers that provide mechanical support, pliability, elastic

and tensile strength to the skin (Prost-Squarcioni *et al*., 2008). The epidermis is the outer layer of dense epithelial keratinocytes, which undergo keratinization to maintain the integrity of epithelial tissues and serve as an effective protective barrier (Presland and Dale, 2000).

The effective physical barrier is predominantly located in the outermost layer of epidermis, the stratum corneum (SC). The stratum corneum is 10 - 20 μm thick, and formed when keratinocytes from the stratum basale begin to differentiate, migrate towards the upper layers (stratum spinosum, and stratum granulosum), and transform into continuous sheets of flattened and anucleated cells (corneocytes), at the end of the keratinization process (WHO, 2009). Corneocytes are composed mainly of insoluble bundled keratin filaments that are surrounded by cornified envelope proteins filled with inoculcrin, loricrin, filaggrin and cornified lipid envelope, which are important for the mechanical stability and chemical resistance of the cells (Proksch and Jensen, 2012). Corneocytes are embedded in a hydrophobic lipid-rich intercellular space that is composed of ceramides, free saturated fatty acids, cholesterol, and organized as lamellar lipid layers, which increased the cohesion between the cells, and thereby contributes to making the epidermis a competent barrier (WHO, 2009). In the normal human epidermis, the balanced processes of cellular proliferation and desquamation facilitated the reduction of cohesion between corneocytes, and resulted in a complete renewal of stratum corneum (Proksch and Jensen, 2012).

In addition to stratum corneum, nucleated epidermis, in particular the desmosomes and tight junctions also contribute to the barrier function of skin. It has been demonstrated that desmosomal and adherence junction proteins such as E-cadherin is essential due to their ability to retain a functional epidermal water barrier by stabilizing the adhesion between the cells (Tunggal *et al*., 2005). Furthermore, the

presence of tight junction protein such asoccludins and claudins could lead to a proper separation between apical and basolateral part of a cell, which prevented the alteration of epidermal function (Furuse *et al*., 2002). The importance of nucleated epidermal layers in preventing the entry of harmful substances into the skin has also been reported (Baroni *et al*., 2012). Taken together, stratum corneum and nucleated epidermis plays an irreplaceable role in maintaining the skin barrier function.

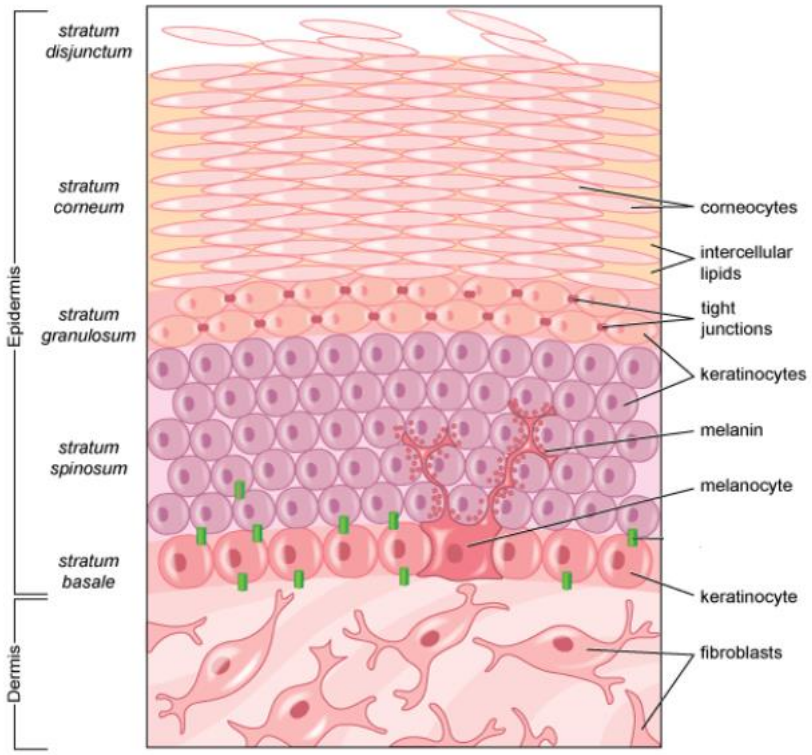

Figure 2.1 Skin layer and its regular resident cells. Reprinted from Beutler Lab (2011).

2.2.2 Skin Microbiota

In addition to physical barrier function, human skin also acts as an intricate habitat, harbouring a dynamic and diverse population of microorganisms, which is known as the skin microbiota (Hannigan and Grice, 2013). The advanced technology in DNA sequencing and metagenomics have provide new insights on the studies of human skin microbiota, by facilitating a greater identification method of the microorganisms;

and thorough investigation approach on the interaction between skin microbiota and skin diseases. Archaea, bacteria, fungi, and viruses constitute the skin microbiota. Grice *et al*. (2009) have characterized the topography diversity of healthy human skin microbiota via the use of 16S rRNA gene phylotyping. A total of 19 phyla were found from the samples of twenty diverse skin sites in 10 healthy humans, and the identified microorganisms were mostly classified into four bacterial phyla, Actinobacteria (51.8 %), Bacteroidetes (6.3 %), Firmicutes (24.4 %), and Proteobacteria (16.5 %).

Bacterial population on human skin can be categorized as resident (reproducing, growing), temporary resident (not typically resident, yet can colonize), and transient (contaminant, non-reproducing). Consistent with previous studies, 16S RNA gene phylotyping also listed *Staphylococcus epidermidis, S. aureus, Corynebacterium diphtheria, C. jeikeium,* and *Propionibacterium acnes* as normal resident of cutaneous bacteria (Cogen *et al*., 2007; Findley *et al*., 2013). Despite these bacteria are abundant populations of the normal skin microbiota, Grice *et al*. (2009) have reported that the bacterial communities are distributed in a range of physiologically and topographically distinct niches, with sebaceous sites being the most stable. Sebaceous sites were predominantly *Staphylococcus* spp and *Propionibacterium*, whereas moist sites were found to be predominantly resided by *Staphylococcus* and *Corynebacterium.*

Skin microbiota is not only limited to bacteria, fungi also represent as a major population in the normal human skin. Findley *et al*. (2013) have explored topographical map of the fungal diversity on 14 skin sites in 10 healthy adults, using intervening internal transcribed spacer 1 region and 18S rRNA sequencing methods. Authors found that eleven core-body and arm sites were dominated by 11 *Malassezia* species, and sites on the feet shown the richest fungal diversity among all the body sites. Recently, a whole metagenomic analysis also discovered the cutaneous viral population- human

polyomaviruses in the healthy individuals (Foulongne *et al*., 2012).

Advanced molecular analyses revealed that skin microbiota may intervene in the disruption of skin homeostasis, subsequently raise the risk for dermatological diseases. Although *S. aureus* is one of the normal residents on human skin, it is likely in part contributed to AD, which is a chronic inflammatory skin disease that frequently occurs in children. Park *et al*. (2013) have reported that AD patients were heavily colonized by *S. aureus*. The *S. aureus* colonization rates in acute and chronic skin lesions of 687 AD patients (188 infants, 267 children, and 232 adults) were 71 % and 35 % higher as compared to 247 control urticarial patients without any skin lesions. *Enterococcus* and *Corynebacterium* were also significantly higher in the lesions than non-lesional skin of AD in Saudi children (Bilal *et al*., 2013). On the other hand, evolving evidences have suggested the potential role of *P. acnes* in acne vulgaris, which is a common skin disorder associated with abnormal sebum production, bacterial proliferation and inflammation. A current study has identified 71 strains of *P. acnes* in different skin sites of acne patients, and acne-associated genes were also found to be located in different chromosomal loci of the bacterial genome, thereby highlighting that there may contribute to acne pathology (Fitz-Gibbon *et al*., 2013). Dysbiosis of the skin microbiota have been implicated in the pathogenesis of psoriasis which may cause excessive growth of skin cells and chronic inflammation. Fahlen *et al*. (2012) have compared bacterial microbiota in skin biopsies from normal and psoriasis patients with massive parallel pyrosequencing targeting the 16S rRNA gene and the variable regions V3-V4. Results shown that *Streptococcus* spp, including *S. pyogenes* was present at significantly higher level in psoriasis, whereas staphylococci and propionibacteria were significantly lower in psoriasis as compared to normal skin.

Additionally, bacteria are also known as the most common microorganisms associated with wound infections. Previous studies have reported that *Escherichia coli*, *Klebsiella* spp, *S. aureus*, *Micrococcus luteus*, and *Enterococcus faecalis* are most frequently found in both post-operative wounds and minor wound infections (Ranjan *et al*., 2010; Malic *et al*., 2009; Giacometti *et al*., 2000). Fadeyibi *et al*. (2013) have also demonstrated that 53.6 % of the infected burn wounds in burns patients were infected with Gram-negative bacteria, *Pseudomonas aeruginosa*.

2.2.3 Skin Defense Mechanisms

Human skin is more than a mere physical protective barrier against environmental challenges; it also has a formidable function to protect the epidermal integrity via a panoply of defense mechanisms, aimed at controlling invading microbial pathogens. The skin defense mechanisms consist of innate immunity, which mediates the initial rapid elimination of pathogens; and adaptive immunity, which generates highly specific second line of defense as well as immunological memory (Kang *et al*., 2006). Although both of the innate and the adaptive immune systems have distinct function, there is coordinated effort between these systems, which defines the effective immune responses.

The innate immune system in skin consists of a range of pre-existing readily mobilized cells, and preformed nonspecific and broadly specific effector molecules (Oppenheim *et al*., 2003). When pathogenic bacteria succeed in breaching the skin barrier, toll like receptors (TLRs) and other pattern recognition receptors that are expressed by readily mobilized cells start to recognize pathogen-associated molecule patterns (PAMPs). Keratinocytes have been shown to express TLRs 1, 2, 4, 5 and 9, which can recognize exogenous PAMPs including lipopeptides (TLR 1,2), phenol-

soluble modulin (TLR 2), PGN (TLR 2), lipopolysaccharide (LPS) (TLR 2,4), flagellin (TLR 5), and hypomethylated CpG (TLR 9) from pathogenic bacteria, through myeloid differential factor 88 dependent pathway (Miller, 2008). These recognitions result in the activation of nuclear factor-κB (NF-κB), and subsequently release and/or stimulate the production of effector molecules, in particular, antimicrobial peptides (AMPs) and antimicrobial enzymes. AMPs are pivotal defense molecules of the cutaneous innate immune system, which act as endogenous antibiotics against a broad spectrum of pathogenic bacteria (Gallo and Huttner, 1998). Two major families of small cationic AMPs (< 100 amino acids, 3 - 5 kDa) that are synthesized and/or released from keratinocytes and neutrophils are the β-defensins and the cathelicidins. The cationic and amphiphilic characteristics of the β-defensins and cathelicidins have been suggested to contribute to the antimicrobial action, by disrupting the membrane integrity and altering the intracellular function of pathogenic Gram-negative and Gram-positive bacteria (Oppenheim *et al*., 2003). In human skin, β-defensin-1 (hBD-1, 36 amino acids, 3.9 kDa) is constitutively synthesized by keratinocytes, whereas hBD-2 (41 amino acids, 4.3 kDa), hBD-3 (45 amino acids, 5.1 kDa) and the cathelicidins are presented at lower level in keratinocytes, but can be upregulated during inflammation and accumulated at sites of infection through release by neutrophils (Braff *et al*., 2005).

Accumulative studies have demonstrated the roles of human β-defensins and cathelicidins as AMPs in skin defense. hBD-1 has been shown to exhibit antimicrobial activity against Gram-negative bacteria such as *P. aeruginosa* and *E. coli* (Pivarcsi *et al*., 2005). Although hBD-1 is constitutively expressed, Sorensen *et al*. (2005) have reported that expression of hBD-1 in epidermal keratinocyte cultures can be increased upon stimulation with LPS, PGN or SpeB, a cysteine proteinase from *S. pyogenes*. On the other hand, Dinulos *et al*. (2003) found that hBD-2 could have potent antimicrobial

activity against skin pathogenic bacteria, particularly high adherent strains of *S. pyogenes* and *S. aureus*, but not skin commensal bacteria such as *S. epidermidis*. The expression of hBD-2 was also induced consistently by tumor necrosis factor alpha (TNF-α), IL-1β, and both Gram- positive and negative bacteria including *S. aureus, E. coli,* and *P. aeruginosa* (Schroder and Harder, 1999; Dinulos *et al*., 2003). Furthermore, Lai *et al*. (2010) have reported that a sterile non-toxic small molecule (< 10 kDA) of *S. epidermidis* activated TLR 2 signaling, subsequently enhancing the hBD-2 mRNA expression, and increasing the capacity of cell lysates to inhibit the growth of *S. aureus* and group A *Streptococcus* (GAS). Meanwhile, hBD-3 demonstrated a broad spectrum of antimicrobial activity against potent pathogen bacteria, including methicillin-resistant *S. aureus* (MRSA) (Harder and Schroder, 2005). An *in vitro* study indicated that *S. aureus* and LTA-induced the expression of hBD-3 through TLR 2 signaling and activation of mitogen-activated protein kinase (Menzies and Kenoyer, 2006). hBD-3 has also shown the ability to bind to lipid II-rich sites of cell wall biosynthesis of MRSA, which may lead to perturbation of the biosynthesis machinery and result in localized lesions in the cell wall (Sass *et al*.. 2010). Another important AMPs on skin, human cathelicidin antimicrobial protein 18 (hCAP18) or its mature form, AMP LL-37 (37 amino acids, 4.5 kDa) has also exhibited rapid antimicrobial activity against *S. aureus, E. faecalis* and *P. aeruginosa* (Nizet and Gallo, 2003). Although the production of LL-37 is very low in normal keratinocytes, its production can be dramatically increased in response to wounding and upon challenge with *S. pyogenes* (Dorschner *et al*., 2001).

Besides their microbicidal functions, hBDs and cathelicidins could extend their roles as “alarmins” to other aspects of immunity in inflamed skin and/or wound repair process. Niyonsaba *et al*. (2007) have performed a study to investigate whether hBDs participate in cutaneous inflammation and wound healing. Authors found that hBD-2

and hBD-3 stimulated the production of pro-inflammatory cytokines and chemokines (IL-6, IL-10, and monocyte chemoattractant protein-1). They also demonstrated that hBD-2 and hBD-3 elicited intracellular Ca^{2+} mobilization, induced phosphorylation of epidermal growth factor receptor, and signal transducer and activator of transcription, subsequently increasing keratinocyte migration and proliferation. Although hBD-1 is structurally closer to hBD-2 and hBD-3, it has no effect on the release of cytokines and cell proliferation. In contrast, LL-37 has shown strong chemotactic activity for cluster of differentiation 4 expressed on the surface of helper T lymphocytes (CD^{4+} T lymphocytes) (Agerberth *et al*., 2000). LL-37 bridges the innate and adaptive immune system, by influencing dendritic cells (DCs) differentiation, and enhancing secretion of T helper-1 (Th-1) inducing cytokines via the activation of CD^{4+} T lymphocytes (Davidson *et al*., 2004). *In vitro* studies suggest that LL-37 could also stimulate angiogenesis on endothelial cells via activation of formyl peptide receptor 1, increase proliferation and formation of vessel-like structure (Koczulla *et al*., 2003). Therefore, LL-37 plays several crucial roles in re-epithelialization and wound healing on human skin.

Another effector molecule with potential importance in human skin defense is lysozyme (14 - 15 kDa), an antimicrobial enzyme. Lysozyme is a PGN N-acetyl-muramoylhydrolase, also known as muramidase. The substrate of lysozyme is PGN, which is an abundant component responsible for the rigidity of the bacterial cell wall. Lysozyme has been reported as a lytic enzyme that cleaves the bond between N-acetylglucosamine and N-acetylmuramic acid of the PGN in the Gram-positive bacterial cell wall, and results in cell lysis (Niyonsaba and Ogawa, 2005). Previously, Ogawa *et al*. (1971) have found lysozyme in human skin, and the content was three-fold higher in the epidermal than the dermal layer. Gram-positive bacteria have a thick layer of PGN

whereas Gram-negative bacteria have a thin layer of PGN surrounded by outer membrane that acts as a protective barrier (Masschalck and Michiels, 2003). Therefore, lysozyme is more active against Gram-positive pathogenic bacteria. However, Masschalck and Michiels (2003) have also reported that the antimicrobial spectrum of lysozyme can be extended till Gram-negative bacteria via the use of outer membrane permeabilizing agent such as ethylenediaminetetraacetic acid (EDTA) or polycations. In addition to their role as antimicrobial, lysozyme has also been shown to enhance phagocytic activity of polymorphonuclear leukocytes, and control skin inflammation (Ibrahim *et al*., 2001; Ganz *et al*., 2003).

The activation of TLRs not only produces AMPs and lysozyme, but also bridge innate and adaptive immunity. PAMP recognition by TLRs on DCs, mediates DCs maturation and initiates adaptive T cell and B cell immunity (Lai and Gallo, 2009). In normal uninflamed skin, DCs are well positioned in both epidermis (Langerhan cells) and dermis (dermal DCs), and incapable of initiating T cell immunity. Using a mice model of skin infection, Igyarta *et al*. (2011) have shown that DCs become activated and migrated from the site of injury or infection to regional lymph nodes, resulting in the generation of antigen-specific $Th\text{-}_{17}$ and $Th\text{-}_{1}$ cells. These T helper cells responses are essential for the host to orchestrate sufficient defensive mechanism to control inflammation. $Th\text{-}_{17}$ cells produce IL-17 and enhance host defense against extracellular pathogenic bacteria at the epidermal surface, whereas $Th\text{-}_{1}$ cells produce interferon gamma and enhance cell-mediated immunity against intracellular pathogenic bacteria (Tesmer *et al*., 2008). The activation of DCs also stimulates B cells to proliferate, differentiate into plasma cells and secret immunoglobulins, which are used by the host's immune system to identify and neutralize pathogenic bacteria (Wykes and Macpherson, 2000).