BIOACTIVES FROM PROBIOTIC FOR DERMAL HEALTH UPON PHYSICAL TREATMENTS

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PRODUCTION OF DERMAL BIOACTIVES IN SELECTED LACTOBACILLI AND BIFIDOBACTERIA, WITH SPECIAL FOCUS ON SPHINGOMYELINASE ACTIVITY OPTIMISATION IN *LACTOBACILLUS RHAMNOSUS* FTDC 8313 IN THE PRESENCE OF MN^{2+} AND MG^{2+}

by

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LIST OF ABBREVIATIONS

Abbreviation	Caption
3D	Three dimensional
AHA	Alpha hydroxyl acid
Å	Armstrong
ANOVA	Analysis of variance
B.	*Bifidobacterium*
$CaSO_4$	Calcium sulphate
Ca^{2+}	Calcium ion
CCD	Central composite design
CFU	Colony forming unit
CTAB	Cetyltrimethylammonium bromide
Co^{2+}	Cobalt ion
E.	*Escherichia*
ELISA	Enzyme-linked immunosorbent assay
et al.	Latin *et* (‘and’) + *alii* (‘others’)
FEB	Free energy of binding
Glu	Glutamic acid
GRAS	Generally recognized as safe
hBD	Human beta-defensin
His	Histidine
HPLC	High performance liquid chromatography
HRP	Horse-radish peroxidase
H_2O_2	Hydrogen peroxide

H_2SO_4	Sulphuric acid
IgG3	Immunoglobulin G3
IL	Interleukin
kDa	Kilo-Dalton
KH_2PO_4	Potassium di-hydrogen phosphate
L.	*Lactobacillus*
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
Mg^{2+}	Magnesium ion
Mn^{2+}	Manganese ion
$MgSO_4$	Magnesium sulphate
$MnSO_4$	Manganese sulphate
$MgCl_2$	Magnesium chloride
MRS	de Mann Rogosa Sharpe
NaOH	Sodium hydroxide
NH	Nitrogen-hydrogen side chain
NMF	Natural moisturizing factor
OH	Hydroxyl group
P.	*Pseudomonas*
PBS	Phosphate buffered saline
PBST	Phosphate buffer saline with Tween 20
PDB	Protein Data Bank
pH	Potential of hydrogen
R^2	Coefficient of determination
RNase	Ribonuclease

RSM	Reconstituted skimmed milk
S.	*Staphylococcus*
SC	*Stratum corneum*
SMase	Sphingomyelinase
TLR	Toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNF	Tumor-necrosis factor
UV	Ultraviolet

PENGHASILAN BIOAKTIF DERMIS DARI LACTOBACILUS DAN BIFIDOBAKTERIUM TERPILIH, DENGAN TUMPUAN KHAS KE ATAS PENGOPTIMUMAN SPHINGOMYELINASE AKTIVITI *LACTOBACILLUS RHAMNOSUS* FTDC 8313 DENGAN KEHADIRAN MN^{2+} AND MG^{2+}

ABSTRAK

Laktobasilus dan bifidobakterium adalah antara genus probiotik yang paling lazim memiliki potensi yang terdokumentasi keatas kesihatan usus. Kajian terbaru mencadangkan bahawa potensi tersebut boleh menjangkaui kesejahteraan usus, contohnya untuk kesihatan kulit. Penyelidikan ini bertujuan untuk menilai penghasilan bahan bioaktif yang penting untuk kesihatan kulit dari laktobasilus dan bifidobakterium yang ditumbuhkan dalam susu. Kandungan asid lipoteikoik di dalam pecahan dinding sel adalah lebih tinggi ($P < 0.05$) dalam *Lactobacillus rhamnosus* FTDC 8313 dan *Bifidobacterium longum* BL 8643 dibandingkan dengan strain lain yang dikaji. Sementara itu, semua strain yang dikaji menunjukkan kepekatan peptidoglikan dinding sel yang sama. Keputusan kami menunjukkan bahawa laktobasilus mampu menghasilkan kepekatan asid hyaluronik dan diasetil yang lebih tinggi daripada bifidobakterium. Laktobasilus dan bifidobakterium yang dikaji juga menghasilkan sphingomyelinase (SMase) asid dan neutral, suatu enzim yang menjana seramida dan seterusnya mamainkan peranan penting dalam pembangunan sekatan fizikal pada stratum korneum (SC).

Aktiviti SMase yang dihasilkan oleh *L. rhamnosus* FTDC 8313 juga telah dioptimumkan dengan menggunakan ion logam dwivalen melalui metodologi gerak balas permukaan. Dengan menggunakan reka bentuk faktorial penuh untuk penyaringan faktor, hanya magnesium sulfat ($MgSO_4$) dan mangan sulfat ($MnSO_4$)

mempengaruhi aktiviti SMase secara signifikan ($P < 0.001$), manakala kesan kalsium sulfat ($CaSO_4$) adalah tidak ketara ($P > 0.05$). Satu matriks reka bentuk komposit pusat (nilai alfa ± 1.414) telah dijana dengan dua faktor bebas iaitu, $MnSO_4$ dan $MgSO_4$. Gerak balas permukaan tiga dimensi meramalkan aktiviti SMase mencapai nilai optimum 6.52 mU ml^{-1}, melalui kombinasi 3.85 mmol L^{-1} $MnSO_4$ 3.33 mmol L^{-1} $MgSO_4$. Data yang diperolehi daripada eksperimen pengesahan menunjukkan sisihan 1.07% daripada nilai yang diramalkan, mengesahkan ramalan dan mempamirkan kebolehpercayaan model regresi yang digunakan. Kesan ion logam dwivalen keatas ciri-ciri pertumbuhan dan penghasilan bahan bioaktif lain, iaitu asid hyaluronik, diasetil, peptidoglikan, asid lipoteikoik, asid laktik, dan asid asetik dalam rantau aktiviti SMase dioptimumkan juga dinilai menggunakan gerak balas permukaan tiga dimensi.

Kesan ion logam dwivalen keatas aktiviti Smase juga dikaji selanjutnya dengan menggunakan simulasi molekular. Pengedokan molekul menunjukkan bahawa penambahan Mn^{2+} dan Mg^{2+} ke tapak aktif SMase meningkatkan afiniti penambatan antara SMase dan sphingomyelin berdasarkan tenaga bebas penambatan serta jarak interaksi antara residu mangkin, Glu53 dan His296. Sementara itu, penambahan Ca^{2+} mengakibatkan kehilangan interaksi antara residu Glu53 dengan sphingomyelin.

PRODUCTION OF DERMAL BIOACTIVES IN SELECTED LACTOBACILLI AND BIFIDOBACTERIA, WITH SPECIAL FOCUS ON SPHINGOMYELINASE ACTIVITY OPTIMISATION IN *LACTOBACILLUS RHAMNOSUS* FTDC 8313 IN THE PRESENCE OF MN^{2+} AND MG^{2+}

ABSTRACT

Lactobacilli and bifidobacteria are the most common genera of probiotics with documented potentials on gut health. Recent studies suggested that such potentials can be extended beyond gut well-being, such as that of dermal health. This study was aimed to evaluate the production of bioactives that are essential for skin health from either lactobacilli or bifidobacteria grown in milk. Lipoteichoic acid content in the cell wall fraction was higher ($P < 0.05$) in *Lactobacillus rhamnosus* FTDC 8313 and *Bifidobacterium longum* BL 8643 compared to the other strains studied. Meanwhile, all strains studied showed equal concentration of cell wall peptidoglycan. Our results showed that lactobacilli produced higher concentration of hyaluronic acid and diacetyl than bifidobacteria. Strains of lactobacilli and bifidobacteria studied also produced acid and neutral sphingomyelinase (SMase), enzymes that generate ceramides which subsequently play important roles in development of physical barriers in the *stratum corneum* (SC).

Sphingomyelinase activity in *L. rhamnosus* FTDC 8313 was also optimized using divalent metal ions via response surface methodology. Using a full-factorial design for factors screening, only magnesium sulphate ($MgSO_4$) and manganese sulphate ($MnSO_4$) significantly ($P < 0.001$) influenced SMase activity while the effects of calcium sulphate ($CaSO_4$) was insignificant ($P > 0.05$). A central composite design matrix (alpha value of ± 1.414) was generated with two

independent factors namely, $MnSO_4$ and $MgSO_4$. Three dimensional response surface predicted an optimum point with maximum SMase activity of 6.52 mU ml^{-1}, by a combination of 3.85 mmol L^{-1} of $MnSO_4$ and 3.33 mmol L^{-1} of $MgSO_4$. Validation data obtained showed a deviation of 1.07 % from the predicted value, ascertaining the predictions and the reliability of the regression model used. Effects of divalent metal ions on growth properties and production of hyaluronic acid, diacetyl, peptidoglycan, lipoteichoic acid, lactic and acetic acids in the region of optimized SMase activity were also evaluated using 3D response surfaces.

The effects of the divalent metal ions on sphingomyelinase activity were further evaluated via molecular simulations. Molecular docking demonstrated that the addition of Mn^{2+} and Mg^{2+} into the active site of SMase improved the binding affinity between the SMase and sphingomyelin based on it free energy of binding as well as the interaction distances between the important catalytic residues Glu53 and His296. Meanwhile, addition of Ca^{2+} appeared to result in a loss of interaction between the Glu53 residues with sphingomyelin.

CHAPTER 1- INTRODUCTION

1.1 Background

Lactobacilli and bifidobacteria are the most commonly documented genera of probiotics used for human consumption. Derived from the Greek word meaning “for life”, probiotics have been defined as “live microorganisms which, when administered in adequate amount confer a health benefit to the host” (FAO/WHO, 2001). Probiotics have been extensively reviewed for decades, emphasizing on improving general gut health such as alleviating intestinal disorders and maintaining a healthy gastrointestinal microflora.

Recently, more studies showed that probiotics may exert other health promoting effects beyond gut well-being, attributed to the rise of the gut-brain-axis correlations. The gut-brain-skin axis concept, as proposed by Arck *et al.* (2010) suggests that modulation of the microbiome by deployment of probiotics can also exert profound beneficial effects such as on skin inflammation and skin homeostasis. Some of these new benefits include skin health such as improving atopic eczema, atopic dermatitis, healing of burns and scars, skin rejuvenating properties, and improving skin innate immunity (Arck *et al*., 2010). Increasing evidences have also showed that bacterial compounds such as cell wall fragments, their metabolites and dead bacteria can elicit certain immune responses on the skin and improve skin barrier functions.

Increasing demand for natural formulations for skin care in the market indicate that there is an emerging new potential of probiotics in dermatology. It is estimated that the global probiotics market would grow at a compound annual growth rate of 13 % from 2009-2014 (Koncept Analytics, 2010). In general, non-intestinal applications of lactobacilli and bifidobacteria are few and there is little

information available on the use of generally recognized as safe (GRAS) microorganisms for production of bioactive metabolites for skin applications. Natural cell components and metabolites may be the preferred choice in cases where safety and side-effects are of concern. Moreover, cell components and metabolites are more stable than viable cells at room temperature, and are thus more suitable for various product developments (Ouwehand *et al*., 2010).

Medium optimization, *via* the addition of metal ions such as manganese (Mn^{2+}) and magnesium (Mg^{2+}) ion, has been documented to have an effect on increasing cellular growth and metabolite production (Fitzpatrick *et al*., 2001). These divalent metal ions are required as an essential cofactor for stimulating the activity of metalloenzymes such as aminopeptidases, dipeptidases, Mn-catalases, D-xylose isomerase, L-arabinose isomerase, ribozymes, etc. Sphingomyelinase (SMase) is one of the metalloenzymes responsible in generating a family of ceramides and phosphorylcholine from glucosylceramide and sphingomyelin precursors for the development of extracellular lipid bilayers in the SC (Jensen *et al*., 2005). Additions of the divalent metal ions into growth media have also been reported to result in increased growth of probiotics such as *Lactobacillus bifermantans* (Givry and Duchiron, 2008). For these reasons, by using response surface methodology, medium optimization *via* the addition of divalent metal ions can be carried out on the selected strains to observe its effect on production of dermal bioactives such as SMase. Briefly, response surface methodology can be outlined as a compendium of statistical methods to determine and solve multivariate equations by utilising quantitative data from experiments (Liong and Shah, 2005).

The effects of the divalent metal ions on SMase activity were further investigated via molecular simulations. Molecular simulation can be described as the

process of docking a small molecule to a macromolecule with the objective of observing and studying interactions between these molecules. Molecular docking has developed by leaps and bounds in the last decades in terms of technology and computing power, and today, it is a common practice to utilize molecular simulation to complement conventional wet laboratory research. Emergence of molecular simulation has opened new possibilities and ways to learn molecular interaction between molecules.

1.2 Problem statement

While many studies and patents have been published on the use of probiotic extracts for topical application on the skin, the mechanisms or the exact compounds underlying the benefits of bacterial extract on the skin however, remain unclear. Increasing demand for probiotic dermal formulations further boost the urge to understand the exact mechanisms of action. The potential benefits of lactobacilli and bifidobacteria are, however, dependent on selection of strains. We hypothesized that certain strains of lactobacilli and bifidobacteria could exert dermal benefits *via* production of inhibitive and bioactive compounds. To my knowledge, little emphasis has been given on such properties of lactobacilli and bifidobacteria.

1.3 Aims and objectives of research

The main objective of this study was to evaluate the potential of lactobacilli and bifidobacteria in producing bioactives that are essential for skin defense and dermal health. This study was also aimed to optimize the production of the dermal bioactives and to further investigate the effects of the divalent metal ions on sphingomyelinase via molecular simulations. The specific objectives of this study were:

1) To screen and evaluate the production of dermal bioactives including lipoteichoic acid, peptidoglycan, hyaluronic acid, diacetyl, lactic acid, acetic acid and sphingomyelinase among 15 selected strains of lactobacilli and bifidobacteria.

2) To optimize the production of sphingomyelinase by *Lactobacillus rhamnosus* FTDC 8313 via response surface methodology upon addition of manganese ion, magnesium ion and calcium ion.

3) To evaluate the growth of *Lactobacillus rhamnosus* FTDC 8313 and production of lipoteichoic acid, peptidoglycan, hyaluronic acid, diacetyl, lactic acid, acetic acid upon sphingomyelinase optimization.

4) To evaluate the binding affinity and interaction between sphingomyelinase and sphingomyelin upon addition of manganese ion, magnesium ion and calcium ion via molecular simulations.

CHAPTER 2 – LITERATURE REVIEW

2.1 Definition and characteristics of probiotics

Derived from the Greek word “pro-bios” meaning “for life”, the word ‘probiotics’ was initially used as an antonym of the word ‘antibiotic’. Interest in probiotics was dated back to Metchnikoff in 1907, who suggested that several intestinal bacteria produce useful substances against premature aging. It was later in 1974 that probiotics were defined as “organisms or substances which contribute to intestinal microbial balance” (Parker, 1974). Since then, increasing interest on probiotics had mainly focused on improving general gut health. The definition of probiotics has been redefined throughout the years and it was defined as “live microorganisms that confer a health effect on the host when consumed in adequate amounts” (Guarner and Schaafsma, 1998). However, recent studies suggested that probiotics have great potentials beyond gut well-being and not only through oral consumption. Thus, one of the most accepted definition of probiotics is “live microorganisms which when administered in adequate amount confer a health benefit to the host” (FAO/WHO, 2001).

A notable number of microbial species and genera have been known to exhibit functional characteristics typically associated with probiotic properties (Table 2.1). For a microorganism to be defined as probiotic, it must possess the following requirements: 1) alive when administered, 2) have undergone controlled evaluation to document health benefits in the target host, 3) taxonomically defined microbe or combination of microbes (genus, species and strain level), and 4) safe for its intended use (Sanders, 2000).

Table 2.1 Microorganisms associated with probiotic properties

Lactobacillus	***Bifidobacterium***	***Enterococcus***	***Streptococcus***	***Lactococcus***
L. acidophilus	*B. adolescentis*	*E. faecalis*	*S. thermophilus*	*L. lactis subsp. cremoris*
L. brevis	*B. animalis*	*E. faecium*		*L. lactis subsp. lactis*
L. casei	*B. breve*			
L. curvatus	*B. infantis*			
L. fermentum	*B. longum*			
L. gasseri	*B. thermophilum*			
L. johnsonii				
L. reuteri				
L. rhamnosus				
L. salivarius				

Propionibacterium	**Yeast**	**Others**
P. freudenreichii	*Kluyveromyces lactis*	*Leuconostoc mesenteroides*
P. freudenreichii subsp. shermanii	*Saccharomyces boulardii*	*Pediococcus acidilactici*
P. jensenii	*Saccharomyces cerevisiae*	

(adapted from Caramia and Silvi, 2011)

2.1.1 *Lactobacillus*

Lactobacilli are among the most commonly studied genera of probiotics. The genus *Lactobacillus* represents the largest group within the family Lactobacillaceae, with 185 species and 28 subspecies listed to date (Euzéby, 2013). Originally isolated from the faeces of breast-fed infants in 1990, lactobacilli are characterized as gram-positive, facultative anaerobes, non-sporulating and non-flagelated rods or coccobacilli (Gomes and Malcata, 1999). They have been identified as part of the normal oral, intestinal and vaginal microflora (Dal Bello *et al*., 2006; Mijač *et al*., 2006). The most common *Lactobacillus* species isolated from human intestine includes *L. acidophilus, L. salivarius, L. casei, L. plantarum, L. reuteri* and *L. brevis* (Saito, 2004). Some species of lactobacilli has also been shown to be normal inhabitants of plant and vegetable material. Successful incorporation of lactobacilli into food system such as dairy, meat product, juice and fermented beverages, and in

grains and cereal products has proved the ability of lactobacilli to grow and persist in many diverse environments and conditions (Giraffa *et al*., 2010).

Generally, there are two groups of species depending on the ability to ferment sugars; the homofermentative species which converts sugars mostly into lactic acid, and heterofermentative species which converts sugars into lactic acid, acetic acid, ethanol and carbon dioxide. However, some species of lactobacilli have the genetic and physiological wherewithal to ferment sugars by either pathway, and they are therefore referred to as facultative heterofermentative or obligate heterofermentative (Hutkins, 2006). The optimum temperature for growth of lactobacilli varies widely from 30˚C to 45˚C and pH 5.5 to 7.0 (Hutkins, 2006).

2.1.2 *Bifidobacterium*

Bifidobacterium strains, first isolated from faeces of breast-fed infant by Tissier in 1899, are major constituents of the microbiota that colonize the intestinal tract of animals and humans (Turroni *et al*., 2011). Due to the increasing evidence on their role in maintaining the gut health and well-being, bifidobacteria have become one of the most important probiotics used for human consumption. The genus *Bifidobacterium* is a member of the Bifidobacteriaceae family with 47 species and 9 subspecies listed so far (Euzéby, 2013). They are gram-positive, non-sporeforming, non-filamentous and non-motile (Delcenserie *et al*., 2007). Bifidobacteria were classified in the genus *Lactobacillus* until the 1970s, when they were discovered to be phylogenitically distinct from the lactic acid bacteria, and are in an entirely different phylum i.e. Actinobacteria. The main species present in human are *Bifidobacterium adolescentis, B. bifidum, B. infantis, B. longum* and *B. breve* (Roy, 2001). They are strictly anaerobic and catalase negative, with a temperature optima

between 37˚C and 41˚C and a pH optima for growth initiation between 6.5 and 7.0 (Hutkins, 2006). They may differ in shapes and occur as short, curved rods, club-shaped rods, or bifurcated Y-shaped rods (Gomes and Malcata, 1999). Bifidobacteria have also been reported to be isolated from a number of other environments such as anaerobic digester, sewage, honey bee intestine and fermented milk (Felis and Dellaglio, 2007). Their ability to utilize a wide array of carbohydrates, including non-digestible oligosaccharides that reach the colon, provides selective advantages in the colonic environment (Gibson *et al*., 1996). Bifidobacteria degrade hexoses through a peculiar metabolic pathway, the *bifid shunt*, or also known as fructose-6-phosphate pathway (Wolin et al, 1998).

2.2 Conventional health benefits of probiotics

The benefits of probiotics in regulating gut health have been explored and recognized for over a century. During the last decades, more evidence on new roles of probiotics for gut health has been proposed (Table 2.2). There are various evidence to support the verification of such effects, from *in vitro* animal and human studies. One of the established and potential health benefits of probiotic organisms regarding gut health includes alleviation of lactose intolerance. Another main application of probiotic organisms has been the treatment and prevention of diarrhea. Evidence on the efficacy of probiotics in preventing diarrhea of various causes such as acute rotavirus diarrhea (Teran *et al*., 2008), antibiotic-associated diarrhea (Szajewska *et al*., 2006), *Clostridium difficile* associated diarrhea (McFarland, 2006), radiation-induced diarrhea (Giralt *et al*., 2008) and traveler's diarrhea (McFarland, 2007) have been well explored.

By improving the intestinal microbial balance, probiotics have been reported to exert beneficial effects such as inhibiting growth of pathogenic microorganisms and preventing gut inflammation. The antimicrobial mechanisms of probiotics include competition for nutritional sources and adhesion sites in the intestines, secretion of antimicrobial substances and toxin inactivation (Collado *et al*., 2009). Probiotics have also been reported to exert important gut health promoting activities by increasing metabolic activity of the intestinal cells, stimulate immune response without harmful effects and enhancing intestinal integrity (Nissen *et al*., 2009).

Table 2.2 Role and benefits of probiotic bacteria in the gastrointestinal tract

Benefits of probiotic bacteria	**Reference**
Improved lactose tolerance	(Vesa *et al*., 1996)
Control inflammatory bowel disease	(Campieri and Gionchetti, 1999)
Improved mucosal immune function, mucin secretion and prevention of disease	(MacFarlane and Cummings, 2002)
Competitive exclusion of pathogens	(Lee *et al*., 2003)
Protection against infection	(Corr *et al*., 2007)
Preventing injury of the epithelial cell barrier	(Johnson-Henry *et al*., 2008)
Return to pre-antibiotic baseline flora	(Engelbrektson *et al*., 2009)
Reduction in irritable bowel disease symptoms	(MacFarlane *et al*., 2009)
Lowered incidence of diarrhea	(Collado *et al*., 2009)
Alleviate food allergy symptoms	(del Giudice *et al*., 2010)
Reduction in risk factors for colon cancer	(Liong *et al*., 2011)

2.3 New roles of probiotics

Recently, clinical studies have reported that probiotics may exert other health promoting effects beyond gut well-being. Probiotics work through several interrelated mechanisms to promote health at the molecular level. They favourably alter the composition and activities of the intestinal microbial community, thus potentially reversing major contributors to chronic disease.

Probiotics have been documented to lower blood cholesterol levels (Ooi and Liong, 2010). Past *in vivo* study showed that the administrations of probiotics successfully reduced serum/plasma total cholesterol levels in mildly to moderately hypercholesterolaemic subjects after 12 weeks (Ataie-Jafari *et al*., 2009). Also, Park *et al*. (2007) reported that hypercholesterolaemic rats showed a reduction of very low density lipoprotein, intermediate density lipoprotein and low density lipoprotein cholesterol after being fed with *L. acidophilus* ATCC 43121 for 21 days. It has been proposed that there were five possible mechanisms for the removal of cholesterol, namely assimilation of cholesterol during growth, binding of cholesterol to cellular surface, deconjugation of bile salt, disruption of cholesterol micelle and bile salt hydrolase activity (Lye *et al*., 2010).

Probiotics have also been documented to exert antihypertensive effects via the modulation of lipid profiles, insulin, rennin and sexual hormones (Yeo and Liong, 2010). Experimental evidence involving *in vivo* trials has exhibited positive results. In a double-blind, placebo-controlled, randomized study involving 94 hypertensive subjects, Jauhiainen *et al*. (2005) found that the consumption of 150 mL of *L. helveticus* fermented milk twice a day for 10 weeks successfully decreased systolic blood pressure and diastolic blood pressure by 4.1 mm Hg and 1.8 mm Hg, respectively. One of the proposed mechanisms leading to the antihepertensive effects

is that probiotics could convert milk protein into bioactive peptides such as angiotensin I-converting enzyme that plays an important physiological role in regulating blood pressure and fluid and salt balance in mammals (Yamamoto and Takano, 1999).

Another potential role of probiotics includes treatment of urogenital infections (Abad and Safdar, 2009). Several mechanisms of action have been proposed for the potential beneficial effect. Urogenital infections in women are often caused by an alteration in the local flora with a transformation from predominance of lactobacilli to coliform pathogens (Sweet, 2000). Thus, the ability of probiotics to exhibit antimicrobial activity remains as one of the promising alternatives for the prevention or treatment of urogenital infections. Also, probiotics offer protection by producing biosurfactants, which interfere with growth and adhesion of pathogenic microorganisms such as *Escherichia coli* and *Enterococcus faecalis* to uro-epithelial cells (Velraeds *et al*., 1996).

Potential of probiotics in the treatment and reduction of allergic reactions have also been proposed (Michail, 2009). Allergic disorders are often associated with a shift of Th1/Th2 cytokine balance towards a Th2 response. Probiotics have been reported to potentially modulate the toll-like receptors and the proteoglycan recognition proteins of enterocytes, leading to activation of dendritic cells and suppression of Th2 responses (Winkler *et al*., 2007). Several *in vivo* as well as *in vitro* and animal studies have suggested the potential use of probiotics in allergic diseases such as atopic dermatitis (Kalliomaki *et al*., 2007) and allergic rhinitis (Giovannini *et al*., 2007).

Probiotics have also been suggested as alternatives for prevention of dental caries (Soderling, 2012). It has been reported that short-term consumption of *L.*

rhamnosus GG, *L. reuteri* and *B. lactis* BB-12 resulted in a reduction of *Streptococcus mutans*, a caries-promoting bacteria (Nase *et al*., 2001, Stamatova and Meurman, 2009). Also, the amount of dental plaque was reduced. In addition, results from human studies conducted by Nase *et al*. (2001) showed that *L. rhamnosus* GG fermented milk reduced caries occurrence in 3-4 years old children.

Studies on the effects of probiotic consumption in reducing the risks of cancers appear promising as many *in vitro* and *in vivo* studies have reported that probiotics reduce the risk, incidence and number of tumors in the colon, liver and bladder (Kumar *et al*., 2010). According to Saikali *et al*. (2004), probiotics may beneficially modulate several major intestinal functions: detoxification, colonic fermentation, transit, and immune status, which may accompany the development of colon cancer. Meanwhile, animal studies consistently show a reduction in chemically induced colorectal tumor incidence and aberrant crypt formation upon probiotic administration (Saikali *et al*., 2004).

Other documented potentials of probiotics include alleviation of postmenopausal symptoms (de Vrese, 2009), facilitation of mineral absorption (Scholz-Ahrens *et al*., 2007), amelioration of arthritis (Baharav *et al*., 2004) and exhibition of antioxidative effects (Songisepp *et al*., 2004).

2.4 Human Skin

2.4.1 Structure and function

The natural function of the skin is to protect the body by forming an effective barrier between the organism and the environment; preventing invasion of pathogens and fending off chemical and physical assaults, as well as the unregulated loss of water and solutes (Proksch *et al*., 2008). The skin is composed of two main layers; the epidermis and the dermis. The subcutaneous layer, located beneath the dermis, comprises mainly of loose areolar and fatty connective tissue (Figure 2.1). The epidermis is a thin protective outer layer and can be further divided into several stages of differentiation of cells. The dermis provides mechanical strength and is made up of dense fibroelastic connective tissue (collagen and elastic fibers) within a hydrated glycoprotein matrix (Sanders *et al*., 1999).

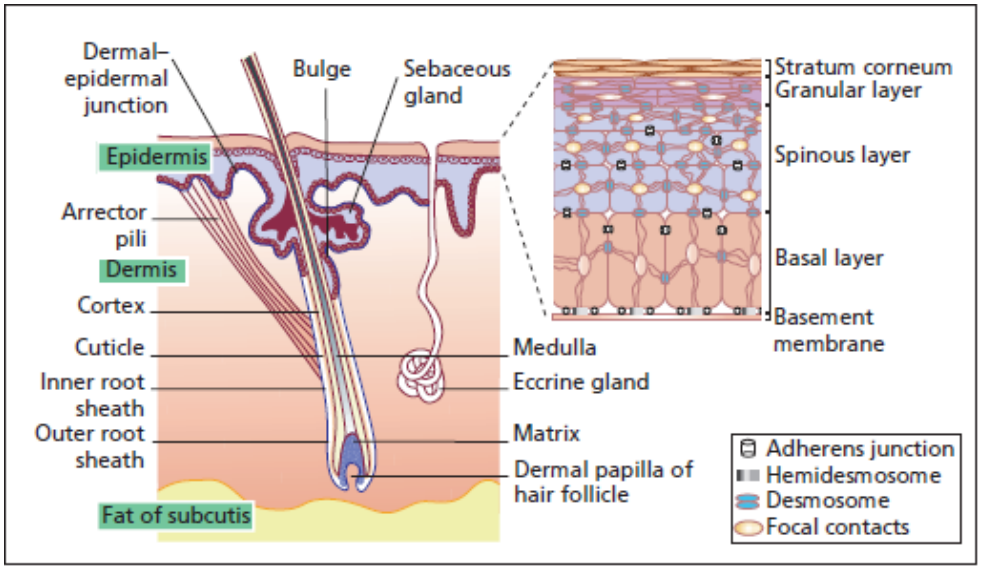

Figure 2.1: The skin and its appendages. (adapted from: McGrath *et al*., 2008)

The main barrier of the skin is located at the superficial layer of the epidermis, the SC. It can be depicted as flat, protein-rich hexagonal corneocytes embedded in a lipid-rich intercellular space. In the normal human skin, the corneocytes of SC are

extremely flat structures with widths of about 30μm and thickness of 0.3μm (Forslind *et al*., 2003). The major lipid classes in the SC are ceramides, free fatty acids and cholesterol and changes in the lipid composition will lead to a disturbed skin barrier (Proksch *et al*., 2008). It has been reported that the SC is not a dead tissue, but possesses multiple types of catalytic activities in both the cytosolic and membrane/extracellular compartments (Elias, 2007).

Although the physical barrier consists mainly of SC, the nucleated epidermal layers also contributes to the barrier through tight, gap and adherens junctions, as well as through desmosomes and cytoskeletal elements (Proksch *et al*., 2008). While removal of SC by tape-stripping resulted in a low to moderate increase of transepidermal water loss, the loss of entire epidermis leads to a severe disturbance in barrier function. Besides preventing excessive water loss, the nucleated epidermal layers also prevents the entry of harmful substances into the skin (Honari, 2004).

2.4.2 Skin microbiota

Skin, as the largest human organ, is constantly exposed to microorganisms in the environment. As a result, it harbors a plethora of different groups of microorganisms that make up the human skin microbiota. For the past decades, many studies have focused in defining the microbial inhabitants of human skin, especially on descriptive features such as their association with infection, their interactions with other microbes and their stability over time (Rosenthal *et al*., 2011). To date, the number of bacteria identified from human skin has expanded significantly, and will probably continue to increase as genotyping techniques advance.

Bacterial skin populations can be categorized as transient (contaminant, non-reproducing), temporary resident (not typically resident, yet can colonize) and

resident (growing, reproducing). According to a recent review by Kong and Segre (2012), the 'normal' resident skin flora includes *Staphylococcus, Corynebacterium, Propionibacterium* and *Pseudomonas* as determined by traditional cultivation methods. Using the 16S rRNA gene-based methods, the presence of *Corynebacterium* spp., *Propionibacterium* spp. and *Staphylococcus* spp. was confirmed to exist on human skin. However, it has to be kept in mind that whether the microorganism is pathogenic or not depends on the profile of the human immune system rather than ''the inherent properties of the microbe'' itself (Cogen *et al*., 2008).

Staphylococcus epidermidis, the most common clinical isolate of the cutaneous microbiota, is a Gram-positive coccus found in clusters. As a major inhabitant of the skin and mucosa it is thought that *S. epidermidis* comprises greater than 90% of the aerobic resident flora (Cogen *et al*., 2008). *S.epidermidis* is harmless under normal condition, if not as a mutual on the skin's surface. But for immune-compromised host, it is recognized as an important pathogen involved in nosocomial bloodstream infections, cardiovascular infections, and infections of the eye, ear, nose and throat (Vuong and Otto, 2002).

Staphylococcus aureus is known widely as one of the leading human pathogen, particularly those strains that produce superantigenic toxins. *S. aureus* causes clinical disease ranges from minor and self-limited skin infections to invasive and life-threatening diseases. Examples of *S. aureus* skin infections include folliculitis, furuncles, impetigo and subcutaneous abscesses (Iwatsuki *et al*., 2006). However, studies have suggested that colonization by *S. aureus* is certainly not synonymous with infection. In fact, *S. aureus* found on healthy human skin and in nasal passages are acting as a commensal rather than a pathogen (Cogen *et al*., 2008).

Coryneforms are gram-positive pleomorphic bacilli included as skin commensals and are classified as either lipophilic or nonlipophilic. This group includes *Corynebacterium sp., Propionibacterium sp., Dermabacter sp.,* and *Brevibacterium sp. Propionibacterium acnes* is by far the most dominant anaerobic coryneform on the skin and is abundant on the skin of the scalp, forehead and back. *Dermabacter* and *Brevibacterium sp*. prefer glabrous, human skin such as the toe webs and may cause 'foot odor' (Chiller *et al*., 2001).

Pseudomonas aeruginosa is another common bacterial species which is considered as normal constituent of a human's natural microflora and lives innocuously on human skin and in the mouth. However, they are able to infect practically any tissue with which they come into contact. *P.aeruginosa* occasionally causes dermatitis or deeper soft-tissue infections. Dermatitis occurs when skin comes into contact with infected water such as in hot tubs (Cogen *et al*., 2008).

There are many elements that potentially contribute to the composition of microbial communities in skin. External factors include ambient humidity, seasonal weather conditions, previous antibiotic treatment, clothing type, use of lotions, creams, cleansers, deodorants or anti-perspirants, hygiene frequency and other environmental surfaces interaction. Intrinsic factors such as age, genetic makeup and host immune system also influence the composition of skin microbiota.

2.4.3 Skin defense mechanisms

Although our skin is permanently threatened by various potential pathogenic microorganisms, it is surprisingly highly resistant against infections. The skin's innate immunity, often defined as a rapid, first line defense system is responsible in providing protection against infection and degradation. The defense system

comprises both physical and chemical constitutive protection and the response process is elicited once the basic barrier is breached (Gallo and Nizet, 2008).

Skin as a protective organ covers the body and accomplishes multiple defensive functions. The structural integrity of the body surfaces, i.e., the skin, forms an effective barrier to initial lodgment or penetration by microorganisms. The intact skin also represents a barrier to the uncontrolled loss of water, proteins, and plasma components (Darlenski *et al*., 2008). Most of the defensive (barrier) functions of the epidermis localize to the SC, which limits pathogen colonization through its low water content, acidic pH, resident (normal) microflora, and surface-deposited antimicrobial lipids (Elias, 2007). The SC is the outermost layer of the epidermis that results from the terminal differentiation of the keratinocytes. It forms the primary layer of protection from the external environment and is composed of highly cross-linked proteinaceous cellular envelopes with extracellular lipid lamellae consisting of ceramides, free fatty acids, and cholesterol (Proksch *et al*., 2008).

Over the last decades the simple two-compartment model (‘‘brick-and-mortar”) of the SC structure evolved to a concept presenting SC as a system with a regulated metabolic activity and as a biosensor for external factors such as regulating proteolytic activity, DNA synthesis, and lipid synthesis (Darlenski *et al*., 2008). Besides its physical barrier against invading microorganisms, the skin has the ability to produce a number of antimicrobial peptides and proteins that participate in the innate host defense (Niyonsaba and Ogawa, 2005). These antimicrobial agents are strongly active against a wide spectrum of various pathogens such as bacteria, viruses and fungi. The resulting local accumulation of antimicrobial proteins offers a fast and very efficient way to prevent microbes from establishing an infection (Harder and Schroder, 2005). In addition to their antimicrobial properties, they also

contribute to cutaneous wound healing and perform a series of immunomodulatory functions, thus acting not only as proinflammatory agents but also as a key link between the innate and the adaptive immune system (Niyonsaba *et al*., 2007). Some skin antibacterial agents from the epithelium such as defensins and cathelicidins have the ability to chemoattract neutrophils or other inflammatory cells after their migration to sites of infection or injury (Niyonsaba and Ogawa, 2005).

Defensins are one of the most common types of antimicrobial peptides participating in the host response against bacterial infections and are expressed in multiple tissues in the body (Menendez and Finlay, 2007). They are small cationic peptides of 3—5 kDa (mature form peptides) and are characterized by six cysteine residues forming three intramolecular disulfide bridges. Defensins are divided into α- and β-defensins based on the distribution of the cysteines and linkages of the disulfide bonds (Niyonsaba and Ogawa, 2005). To date, six human α-defensins have been identified, mainly packaged in azurophil granules of neutrophils or secreted by intestinal Paneth cells (Oppenheim *et al*., 2003). Human β-defensins on the other hand, are constitutively expressed in various mucosa and epithelial cells where they can be up-regulated in response to infectious and inflammatory stimuli. So far, six human β-defensins (hBDs), hBD-1 through hBD-6, have been identified but only hBD-1, hBD-2 and hBD-3 are expressed in the skin (Guaní -Guerra *et al*., 2010; Sørensen *et al*., 2005).

hBD-1 is a 3.9 kDa peptide of 36 amino acid residues and is the first isolated human β-defensin. It is expressed constitutively in epidermal keratinocytes and shows antimicrobial activity against predominantly Gram-negative bacteria like *E. coli* and *P. aeruginosa* (Pivarcsi *et al*., 2005). The expression of hBD-1 is also

inducible in keratinocytes of human skin by microbe-derived molecules such as lipopolysaccharide (LPS) and peptidoglycan (Sørensen *et al*., 2005).

The second human β-defensin, hBD-2, consists of 41 amino acids. It is a 4.3-kDa peptide, primarily isolated from extracts of lesional scales of psoriatic skin and is expressed in the skin as well as in urinary, gastrointestinal, and respiratory epithelia (Guaní -Guerra *et al*., 2010). hBD-2 is inducible upon stimulation of epithelial cells by contact with microorganisms such as *P. aeruginosa* or cytokines such as TNF-alpha and IL-1 beta (Schröder and Harder, 1999). Purified staphylococcal cell-wall components, LTA and peptidoglycan has been suggested to stimulate the production of hBD-2 via Toll-like receptor 2 (TLR2) (Dziarski and Gupta, 2005). It has also been suggested that a diminished expression of antimicrobial peptides such as hBD-2 might be one reason to explain why patients with atopic dermatitis often suffer from recurrent skin infections caused particularly by *S. aureus* (Niyonsaba and Ogawa, 2005).

hBD-3 is a 5.1-kDa antimicrobial peptide with 45 amino acid residues (Harder and Schroder, 2005). The same authors have also mentioned that hBD-3 demonstrated a salt-insensitive broad spectrum of potent antimicrobial activities against many potentially pathogenic microbes including the multiresistant *S. aureus*. This molecule is expressed either constitutively or induced upon a challenge, and a growing evidence indicates the involvement of such molecules in adaptive immunity as well (Dhople *et al*., 2006). Also, hBD-3 induces cytokine production from human keratinocytes and stimulates monocyte migration (Niyonsaba and Ogawa, 2005).

Another major family of antimicrobial peptides with potential importance in human skin defense belongs to cathelicidins. Cathelicidins share a conserved cathelin pro-domain and a variable antimicrobial peptide domain that is released from the

parent molecule by proteolytic processing (Zanetti *et al*., 2000). However, only cathelicidin hCAP18 or its mature antimicrobial peptide LL-37 is typically upregulated in human keratinocytes. LL-37 exhibited a broad-spectrum antimicrobial activity against a variety of Gram-positive and Gram-negative bacterial, fungal and viral pathogens (Niyonsaba and Ogawa, 2005). It has been suggested that keratinocyte synthesis of cathelicidin play an important role in skin immunity. It forms an active part of the antimicrobial defense barrier that is independent of circulating immune cells (Braff *et al*., 2005). Using a mouse model, it has also been suggested that cathelicidin LL-37 not only contributes to clearance of bacteria, but also offers systemic protection against the detrimental effects of massive microbial invasion (Nizet *et al*., 2001). In addition, LL-37 also plays a part in epithelial cell proliferation and re-epithelialization of human skin wounds (Heilborn *et al*., 2003).

Lysozyme is a peptidoglycan N-acetyl-muramoylhydrolase, also known by the trivial name muramidase. Lysozyme is expressed in human skin and the level was found three-fold higher in the epidermal portion than the dermal one (Niyonsaba and Ogawa, 2005). The substrate of lysozyme is peptidoglycan, which is a major component of bacterial cell wall. Hydrolysis of peptidoglycan results in cell lysis (Niyonsaba and Ogawa, 2005). Although lysozyme is mainly a Gram-positive bacteria-killing antimicrobial peptide, it is also active against Gram-negative bacteria such as *E. coli* and *P. aeruginosa (*Schroder and Harder, 2006). Apart from its antimicrobial properties, lysozyme has been demonstrated to play a role in the control of inflammation when lysozyme M-deficient mice developed much more severe lesions than wild-type mice (Ganz *et al*., 2003).

Keratinocytes also produce RNase 7, a 14.5-kDa antimicrobial ribonuclease that exhibits antimicrobial activity against various pathogenic microorganisms,

including *S. aureus*, *P. aeruginosa*, *Propionibacterium acnes*, *Candida albicans* and *E. coli* (Harder and Schroder, 2005). RNase 7 is constitutively expressed in normal healthy skin and is further inducible upon microbial stimulation, suggesting that it may play an important role in the innate immune defense system of human epithelia (Harder and Schroder, 2002).

In addition to the antimicrobial peptides mentioned above, human skin has also been shown to produce other antimicrobial peptides such as elafin, psoriasin, dermicidin, antileukoprotease, adrenomedullin, and nuetrophil gelatinase-associated lipocalin for protection of the skin against microbial infection (Niyonsaba and Ogawa, 2005).

2.5 Probiotic applications for dermal health

Probiotics have also been documented to exert dermal potentials such as improving atopic eczema, atopic dermatitis, healing of burns and scars, having skin rejuvenating properties, and also improving the skin's innate immunity. The gut-brain-skin axis concept, as proposed by Arck *et al*. (2010) suggested that modulation of the microbiome by deployment of probiotics can exert profound beneficial effects such as on skin inflammation and skin homeostasis. Accordingly, a significant improvement on the course of atopic dermatitis has been reported in 230 infants subjected to probiotic-supplemented elimination diets in a randomized double-blind manner for four weeks (Viljanen *et al*., 2005). Meanwhile, a study involving 56 patients with acne showed that the consumption of a *Lactobacillus*-fermented dairy beverage for 12 weeks improved clinical aspects of acne (Kim *et al*., 2010). It has been suggested that healthy skin may also benefit from the oral ingestion of probiotic bacteria (Krutmann, 2009). In a double-blind, randomized clinical study involving 72

healthy women with dry and sensitive skin, it was reported that the SC barrier function significantly improved upon consumption of fermented dairy product as compared to placebo product (Krutmann, 2009). Also, supplementation of hairless mice with *L. johnsonii* has been reported to provide protection of the skin immune system against ultraviolet B radiation-induced immunosuppressive effects. Similarly, using a human study, it has been suggested that oral consumption of probiotics may represent a novel approach to protect the skin immune system against ultraviolet radiation.

While approaches based on the oral application of probiotic strains appear promising, human clinical studies suggested that probiotic exert dermal benefits not only through the gastrointestinal route but also upon topical applications. Using an *in vitro* study, Iordache *et al*. (2008) has demonstrated that cell free supernatant of lactic acid bacteria with probiotic potentials including *L. plantarum, L. casei and Enterococcus faecium* inhibited the expression of soluble virulence factors by opportunistic dermal pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* and decreased their adherence capacity to the cellular substrate represented by HeLa cells. Using an *ex vivo* human skin explants model, (Gueniche *et al*., 2010) found a statistically significant improvement ($P < 0.05$) following application of cell lysate from *B. longum sp.* versus placebo in various parameters associated with inflammation including decrease in vasodilation, oedema, mast cell degranulation and tumor necrosis factor-alpha (TNF-α) release. The potentials of probiotics in dermal application have also been evaluated in several in-vivo studies (Table 2.3).

Table 2.3. In-vivo potential of probiotics in dermal applications

Treatment	Strains involved	Experimental design	Dose and duration	Effects	ref
Atopic eczema/ dermatitis	*L. rhamnosus* GG ATCC53103	Randomized, double-blind, placebo-controlled clinical trial to 230 infants with atopic eczema/dermatitis syndrome	Administration of 5 x 10^9 CFU; twice daily for 4 weeks	· Greater reduction in Severity Scoring of Atopic Dermatitis (SCORAD) of IgE-sensitized infants as compared to the placebo group	Viljanen *et al*. (2005)
Resistant childhood atopic eczema	*L. rhamnosus* TB	Open label non-randomized clinical observation of 14 cases of pediatric patients (ages of 8 months to 64 months)	Administration of cell lysate (300 mg to 500 mg daily) for 1 month	· Marked improvements in eczema symptoms scores, daytime irritation and nighttime disturbances scores	Hoang *et al*. (2010)
Sensitive skin	*B. longum sp*	Randomized, double-blind, placebo-controlled clinical trial to 66 female volunteers with reactive skin	Topical application of cream containing 10% Cell lysate; twice a day for 2 months	· Decrease skin sensitivity score · Strengthen skin's natural barrier	Guéniche *et al*. (2010)
Wound healing	Probiotic mixture in kefir	Randomized, double-blind, placebo-controlled clinical trial to 56 male Wistar rats with burn wounds infected with *Pseudomonas aeruginosa* (ATCC 27853)	Topical application of gel containing 50% kefir extract; twice a day for 1 week	· No microbial contaminations were observed in the burn wounds; · Wound size significantly lower ($P < 0.01$) as compared to base gel and untreated groups as well as silver sulfadiazine treated group.	Huseini *et al*. (2012)

2.6 Dermal bioactives from probiotics

There is increasing evidence that bacterial compounds such as cell wall fragments, their metabolites and dead bacteria can elicit certain immune responses on the skin and improve skin's barrier function. Cell free supernatant of lactic acid bacteria with probiotic potentials have been demonstrated to exert antimicrobial and immunomodulatory activity, suggesting the use of probiotic in non-viable forms (Iordache *et al*., 2008). Natural cell components and metabolites may be the preferred choice in cases where the delivery of live cells is not possible. Moreover, cell components and metabolites are more stable than viable cells at room temperature, and are thus more suitable for topical applications.

2.6.1 Lipoteichoic acid

Lipoteichoic acid (LTA) is a group of membrane-anchored molecules formed by linking a hydrophilic polyphosphate polymer to a glycolipid (Fischer, 1988). It is a structural component of the cell walls of Gram-positive bacteria and plays a vital role in the growth and physiology of the bacteria. Lipoteichoic acid has been reported as one of the immune-stimulating component not only to pathogenic but also non-pathogenic Gram-positive bacteria. Previous studies showed that LTA can function as an important pathogen-associated molecular pattern, leading to production of proinflammatory cytokines, nitric oxide, activation of nuclear transcription factor (NF-κB) and other proinflammatory mediators (Lebeer *et al*., 2012). Inflammatory response is an attempt by the host's body to restore and maintain homeostasis following an infection or injury. LTA from pathogenic Gram positive bacteria such as *S. aureus* can however cause excessive inflammation, leading to the development of systemic inflammatory response syndrome such as