EFFECTS OF PROTEIN-RICH FRACTION FROM *LACTOBACILLUS PLANTARUM* USM8613 AGAINST DERMAL *STAPHYLOCOCCUS AUREUS*

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by

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LIST OF ABBREVIATIONS

ACE	Angiotensin-I converting enzyme
AD	Atopic dermatitis
AHAs	A-hydroxy acids
AMPs	Antimicrobial peptides
AU	Arbitury unit
CFS	Cell free supernatant
CFU	Colony forming unit
CLSM	Confocal laser scanning microscope
CMA	Cow milk allergy
CME	Cystoids macular edema
C_T	Threshold cycle
DNA	Deoxyribonucleic acid
Eap	Extracellular adhesion protein
ELISA	Enzyme linked immunosorbent assay
EPS	Extracellular polymeric substances
FAME	Fatty acid methyl esterase
HA	Hyaluronic acid
hBD	Human beta-defensin
HPLC	High-performance liquid chromatography
IFN-γ	Interferon-gamma
Ig	Immunoglobulin

IL	Interleukin
LAB	Lactic acid bacteria
LD	Lethal dose
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
MMPs	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
MRS	De Man-Rogosa-Sharpe
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMMs	Microbial surface components recognising adhesive matrix molecules
NK	Natural killer cell
PAMPs	Pathogen-associated molecule patterns
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PIA	Polysaccharide intercellular adhesion
PLLA	poly-L-lactic acid
RT-PCR	Reverse-transcription polymerase chain reaction
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
SCORAD	Severity scoring of atopic dermatitis
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscope

sIgA	Secretory immunoglobulin A
SMase	shingomyelinase
TEM	Transmission electron microscope
Th	T-helper cell
TLRs	Toll like receptors
TNF-α	Tumor necrosis factor alpha
TSA/B	Trypticase soy agar/broth
VEGF	Vascular endothelial growth factor
VRE	Vancomycin-resistant *Enterococcus feacalis*
WT *S. aureus*	Wild type *Staphylococcus aureus*
Δ*atl S. aureus*	*atl*-null mutant strain of *Staphylococcus aureus*
EST	Expressed sequence tag
NGS	Next generation sequencing
dsDNA	Double stranded deoxyribonucleic acid
gDNA	Genomic deoxyribonucleic acid

KESAN FRAKSI YANG KAYA DENGAN PROTEIN DARI *L. PLANTARUM* USM8613 DALAM MELAWAN PATOGEN KULIT *STAPHYLOCOCCUS AUREUS*

ABSTRAK

Tiga puluh enam strain bakteria asid laktik telah diasingkan daripada tenusu, daging dan produk penapaian tempatan. Ekstrak extrasel (CFS) daripada *L. plantarum* USM8613, yang didapati daripada sosej yang ditapai, menunjukkan aktiviti perencatan dan penghasilan asid laktik yang lebih tinggi ($\rho < 0.05$) telah dipilih untuk analisis seterusnya. Kajian *ex-vivo* menunjukkan CFS daripada *L. plantarum* USM8613 berupaya merencatkan pembentukan biofilem dan pertumbuhan *S. aureus* pada kulit khinzir. Fraksi yang kaya dengan protein, lemak dan polisakarida yang diekstrak daripada CFS *L. plantarum* USM8613 telah diuji untuk aktiviti perencatan. Kajian menunjukkan fraksi yang kaya dengan protein memberi kesan yang lebih ketara berbanding dengan fraksi yang kaya lemak and polisakarida. Fraksi yang kaya dengan protein dari *L. plantarum* USM8613 juga berupaya merencatkan ($\rho <0.05$) pertumbuhan *S. aureus* klinikal dan pembentukan biofilem pada kulit khinzir. Rawatan topikal yang mengandungi 800 AU/mL fraksi yang kaya dengna protein daripada *L. plantarum* USM8613 didapati berupaya mengurangkan ($\rho < 0.05$) bilangan sel *S. aureus* ditapak luka tikus. Penghasilan IL-4, IL-6, IFN-Ɣ, TGF-β dan TNF-α, juga dipertingkatkan ($\rho < 0.05$) dengan rawatan fraksi yang kaya dengan protein tersebut. Fraksi yang kaya dengan protein juga berupaya meningkatkan ekspresi MMPs dan defensin-β. Keseluruhannya, kajian ini menunjukkan fraksi yang kaya deangan protein boleh menggalakkan penyembuhan luka dengan mengawal efektor sistem pertahanan badan yang terlibat

dalam penyembuhan luka. Kajian pengekspresan gen *S. aureus* menunjukkan gen *stress regulator* (*sigB*) dan autolysin utama (*atl*) telah meningkat ($\rho < 0.05$) sewaktu rawatan fraksi yang kaya dengan protein dan ini menyumbang kepada aktiviti perencatan fraksi yang kaya dengan protein daripada *L. plantarum* USM8613. Ekspresi gen faktor kevirulenan *S. aureus* (*hla, hlb, spaV*) telah disekat dengan rawatan fraksi yang kaya dengan protein daripada *L. plantarum* USM8613. Penggunaan strain mutan *atl*-null *S. aureus* mengesahkan lagi kesan perencatan fraksi yang kaya dengan protein daripada *L. plantarum* USM8613 dicapai dengan merangsangkan eskpresi gen autolysin utama, *atl* gen. Analisis genom keseluruhan menunjukkan *L. plantarum* USM8613 mempunyai genom bersaiz 3,258,106 bp yang mempamerkan adaptasi *L. plantarum* USM8613 untuk menggunakan pelbagai jenis sumber karbon dan asid amino daripada sekitar untuk kemandirian. Genom *L. plantarum* USM8613 mengandungi kesemua lima operon plantaricin dan kefungsian operon ini telah disahkan melalui analisis ekspresi gen. Secara keseluruhannya, hasil kajian ini menunjukkan keberkesanan fraksi yang kaya dengan protein daripada *L. plantarum* USM8613 dalam merencatkan pertumbuhan dan menyekat factor kevirulenan *S. aureus*, serta menggalakkan penyembuhan luka. Maka, fraksi yang kaya dengan protein dari *L. plantarum* USM8613 boleh digunakan sebagai bahan bioaktif dalam bidang dermatologi untuk merawat jangkitan *S. aureus* dan penjagaan luka.

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ABSTRACT

Thirty-six strains of lactic acid bacteria were isolated from local dairy, meat and fermented products. Cell-free-supernatant (CFS) of *L. plantarum* USM8613, isolated from fermented sausage, was selected for subsequent analyses. The CFS exhibited a significantly stronger ($\rho < 0.05$) inhibitory activity against *S. aureus* and produced a higher amount of lactic acid as compared to all strains studied. *Ex-vivo* study demonstrated CFS from *L. plantarum* USM8613 inhibited the growth and biofilm formation of *S. aureus* on porcine skins. CFS of *L. plantarum* USM8613 was fractionated into protein-rich, lipid-rich and polysaccharide-rich fractions, and all fractions exhibited significant inhibitory activity, with a more prevalent effect from the protein-rich fraction. The antimicrobial and anti-biofilm effects of the protein-rich fraction were further confirmed with *S. aureus*-infected porcine skins. Topical application of ointment containing 800 AU/mL of the protein-rich fraction from *L. plantarum* USM8613 significantly reduced ($\rho < 0.05$) the cell counts of *S. aureus* in the wound site of *S. aureus* infected-rats. The production of IL-4, IL-6, IFN-Ɣ, TGF-β and TNF-α, and the expression of matrix metalloproteinases (MMPs) and β-defensin were also significantly elevated ($\rho < 0.05$) upon treatment with the protein-rich fraction. Altogether, it indicated that the protein-rich faction promoted wound healing by regulating the immune effectors involved in wound healing. Gene expression study of *S. aureus* showed the stress regulator gene (*sigB*) and the major autolysin gene (*atl*) were significantly up-regulated upon treatment with the protein-rich fraction and contributed

to the autolysis and cell death of *S. aureus* itself. Pathogenicity factors of *S. aureus* (*hla, hlb, spaV* genes) were also suppressed upon the protein-rich fraction treatment. The use of *atl* null mutant strain of *S. aureus*, which further justified the inhibitory effect of the protein-rich fraction from *L. plantarum* USM8613, was achieved via up-regulation of the major autolysin, *atl* gene. Genome-wide analysis revealed a genome size of 3,258,106 bp of *L. plantarum* USM8613, demonstrating the adaption of *L. plantarum* USM8613 to utilise a large variety of carbon and amino acid sources from the surroundings for survival. The genome of *L. plantarum* USM8613 contained all five plantaricin operons and the functionality of these operons was confirmed via gene expression analysis. Altogether, results in this research demonstrated the protein-rich fraction from *L. plantarum* USM8613 effectively inhibited the growth and suppressed the pathogenicity of *S. aureus*, and promoted wound healing. Therefore, the protein-rich fraction from *L. plantarum* USM8613 could be applied as a bioactive agent in the dermatological industry for the treatment of *S. aureus* infection and wound healing.

CHAPTER 1

INTRODUCTION

1.1 Background

Over the past 100 years, changes in society and technology have led to a change in lifestyle and resolved many basic life needs in many parts of the world. Meanwhile, these changes also brought about the renaissance of the old ones, creating new diseases and modification of existing dermatoses. Nowadays, the invention and extensive use of antibiotics have generated various antibiotics-resistant variants that gave rise to a new health risk (Padmanabhan & Fraser 2005; Amini *et al.* 2012, 2013). Lactic acid bacteria consist of Gram-positive, non-sporulating, microaerophilic bacteria that produce lactic acid as the main end product of carbohydrate fermentation. Lactic acid bacteria have a long history of use and play an important role in food industries due to their ability to exert various beneficial effects. For instance, the starter culture is used to improve the nutrient content, as well as preservatives to extend the shelf-life of food products (Caprice & Fitzgerald 1999; Jay 2000; Holzapfel *et al.* 2001). In addition to its additive effect to food content, the intake of lactic acid bacteria also confer health benefits to the host via improved gut ecosystem, reduced serum cholesterol and enhanced host immune system. Among them, members of the genera *Lactobacillus* are the most commonly and commercially used. Despite the long-term use of these beneficial lactobacilli in food industries, it was not until recently the use of lactobacilli had been extended to improve

dermal health. Several studies have suggested the use of these beneficial lactic acid bacteria to maintain cutaneous homeostasis and improve the regulation of the skin immune system (Kaliomake *et al*. 2001, 2003, 2007).

Various studies have reported that topical application where there is direct availability of the whole cells or metabolites from lactic acid bacteria to the skin could also improve dermal health (Krutmann 2009; Simmering & Breves 2009). Different approaches have been used by lactic acid bacteria to inhibit and out-compete the undesired species, for example competitive exclusion and production of various potent antimicrobial substances such as organic acids, bacteriocins, hydrogen peroxide and others (Oh *et al*. 2006; Gillor *et al*. 2008). Among the various potent antimicrobial substances produced by lactic acid bacteria, antimicrobial peptides have gained the most attention and are being extensively studied. Recent studies have revealed the ability of these proteinaceous compounds to exert wound healing properties in addition to its well-known antimicrobial effects, where nisin and plantaricin A were shown to exert significant antimicrobial property and immunomodulating effects in *S. aureus*-induced skin infections in mice (Marzani *et al*. 2012; Heunis *et al*. 2013).

Skin is the largest organ in the human body, providing a physical barrier that protects against dehydration and damage or insults from external aggression. The skin is continuously challenged by diverse environmental stresses such as changes in climate conditions, mechanical damages, and the exposure to chemical and physical factors such as ultraviolet radical, free radicals, toxins, allergens, and xenobiotics, which are the major factors that alter skin integrity, leading to immune system dysfunction, inflammation, photoaging, and a variety of hyperplasia (Krutmann *et al*. 1996;

Scharffeter-Kochanek *et al*. 2000). The skin is naturally populated by various microorganisms, dominated by health-promoting microorganisms known as commensal microorganisms, against harmful microorganisms. An alteration in the skin barrier functions increases the risks of infection by those harmful microorganisms. Among the various forms of skin infections such as impetigo, folliculitis, furunculosis, ecthyma, and cellulitis, *Staphylococcus aureus* is one of the most common causative agents.

S. aureus is a transient opportunistic skin pathogen of human and various animals. The ability of *S. aureus* to survive in various adverse conditions enables it to inhabit various niches and is easily transmitted via skin-formit contact (Amini *et al*. 2012, 2013; Tang *et al*. 2015). *S. aureus* is well-equipped with various virulence factors that causes mild to severe infections, ranging from cutaneous to systemic infections. In addition to virulence factors, *S. aureus* also contains several surface components such as Protein A and extracellular adhesion protein (Eap), which facilitate *S. aureus* to evade recognisation and phagocytosis and subsequently survive against the host immune system (Foster & McDevitt 1994; Chavakis *et al*. 2002; Lee *et al*. 2002). The invention and use of antibiotics to treat *S. aureus* have successfully controlled the threat. Recently, *S. aureus* was able to survive and be immune to the use of β-lactam antibiotics via the acquisition of the penicillin binding protein. The emergence of the antibiotics-resistant strains has further reduced the treatment and therapeutic options (Diekema *et al.* 2001; Foster 2005). Hence, there is a need for natural alternative compounds to treat *S. aureus* without causing the resistant issues.

To date, only limited studies have been conducted in direct topical application of extracts from lactic acid bacteria to exert antimicrobial activity against *S. aureus* and

improve dermal health. To further elucidate this assumption, more information regarding the production of the potential bioactive metabolites from lactic acid bacteria shall be gathered to fully understand the mechanism behind it. Moreover, the safety and efficacy of these bioactive metabolites need to be verified to provide a better understanding and compensate the scarce reports regarding the safety and efficacy issues.

1.2 Aim and Objectives for Research

The main aim of this study was to evaluate the potential use of bioactive metabolites from locally isolated lactic acid bacteria on improving dermal health and fight against *S. aureus*. The regulation of the target pathogen upon treatment was also examined. Hence, the specific objectives of this study were:

1. To isolate and identify potential lactic acid bacteria from local dairy, meat and fermented products.

2. To evaluate and characterise the antimicrobial and anti-virulence activities of the fractionated extracts from the selected lactic acid bacteria.

3. To evaluate the safety and efficacy of the fractionated extracts from the selected lactic acid bacteria via *in-vivo* models

4. To investigate the expression of regulatory pathways of *S. aureus* upon treatment with the fractionated extracts from the selected lactic acid bacteria

5. To determine the potential bioactive metabolites encoding genes in the selected lactic acid bacteria responsible for the inhibitory activity via whole genome study.

CHAPTER 2

LITERATURE REVIEW

2.1 Lactic Acid Bacteria

Over the past century, lactic acid bacteria (LAB) have gained much attention from various communities due to their ability to exert various beneficial factors. LAB consist of a group Gram-positive, non-sporulating bacteria that produce lactic acid as the major end product of carbohydrate fermentation. LAB utilise carbohydrates as the major carbon and energy source either through homofermentative or heterofermentative pathway. Homofermenters utilise carbohydrate via the Embden-Meyerhof-Parnas pathway to produce lactic acid as the major product of fermentation, while heterofermenters use the 6-P-gluconate or phosphoketolase pathway for carbohydrates fermentation resulting in lactic acid, acetic acid or ethanol, and carbon dioxide as end products (König & Fröhlich 2009). The most commonly recognised lactic acid producing bacteria are from the genera *Lactobacillus, Lactococcus, Bifidobacterium, Enterococcus, Leuconostoc, Pediococcus* and *Streptococcus* (Jay 2000; Holzapfel *et al.* 2001). Generally, LAB prefer to inhabit an area rich in nutrients, hence, they are widely distributed in dairy products, meats, plants, vegetables, fruits, fermented foods, beverages, decomposing materials, sewage, and also cavities of humans and animals such as mouth, genital, intestinal and respiratory tract as part of healthy microbiota (König & Fröhlich 2009).

LAB are commonly isolated from dairy products (Rodriguez *et al.* 2000; Martin *et al.* 2003). Raw milk is regarded as a source for isolation of new strains of LAB due to their potential to inhibit undesired microorganisms. For instance, LAB isolated from human breast milk can be potentially used as human probiotics due to their origin, history of safety, prolonged intake by infants, and adaptation to dairy substrates (Martin *et al.* 2003). Human gut and faecal samples are also regarded as a common source for isolation of LAB conferring health benefits (Pereira & Gibson 2002; Duncan *et al.* 2004). *L. plantarum* KC5b isolated from faecal sample of healthy human volunteers is regarded as a candidate probiotic due to its ability to remove a maximum of 14.8 mg of cholesterol per gram of cells from the culture medium (Pereira & Gibson 2002). Various LAB with probiotics characteristic also have been isolated from fermented meat products. Their presence in meat fermentations may improve the safety and stability of the product, and also enhance the sensory properties of the fermented meats (Lucke 2000; Papamanoli *et al.* 2003). Papamanoli *et al.* (2003) reported *L. sakei, L. curvatus* and *L. plantarum* strains isolated from naturally fermented dry sausages are able to grow in environments that mimic human gut and inhibit the growth of two common food spoilage bacteria, *Listeria monocytogenes* and *Staphylococcus aureus*.

Numerous studies and reviews about LAB have been extensively reported. The use of LAB in food industries had begun after the in depth study by L. Pasteur in lactic acid fermentation and the isolation of the first pure culture by J. Lister. The use of LAB as starter culture or preservative in food fermentation began in 1890 (König & Fröhlich 2009). The preservative effect of LAB is mainly due to the production of organic acids, especially lactic acid, which subsequently lowers the surrounding pH. The antimicrobial

effect of LAB is further enhanced by other antimicrobial compounds such as hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde, and bacteriocins (Klaenhammer 1988; Stiles & Hastings 1991; Klaenhammer 1993). In addition to their preservative action, the use of LAB in food industries is also due to their ability to enhance the texture, flavour, or nutrition of the foods. Among various lactic acid bacteria, member of the genera *Lactobacillus* was the most commonly used and studied.

2.1.1 *Lactobacillus*

The genus *Lactobacillus* was described as a heterogeneous group of "regular non-sporing Gram-positive rods" according to Bergey's Manual of Systematic Bacteriology (Sneath *et al.* 1986). Lactobacilli can be divided into three classes based on their fermentation characteristic: (1) obligate homofermentative; (2) facultative heterofermentative; and (3) obligate heterofermentative, as shown in Table 2.1.

The homofermenter gains energy via Embden-Meyerhof-Panas pathway while heterofermenter gains energy via 6-P-gluconate or phosphoketolase pathway. They live widespread in various fermentable materials (Pot *et al*. 1994; Hammes & Vogel 1995; Vandamme *et al*. 1996). Among the members of lactobacilli, the most commonly recognised are *L. delbrueckii, L. acidophilus, L. gasseri, L. casei, L. johnsonii, L. plantarum, L. reuteri, L. fermentum* and *L. brevis* that are used in various food processing industries such as meat fermentation, dairy products, bakery, and beverages fermentation (Pot *et al*. 1993; Stâhl & Molin 1994; Holzapfel *et al*. 1996). Lactobacilli have been extensively used due to their ability to exert various health promoting effects and improve food quality.

Table 2.1 Major division within the genus *Lactobacillus* based on fermentation characteristic (Collin *et al*. 1991; Schleifer & Ludwig 1995).

Group 1	Group 2	Group 3
Obligate homofermenters	Facultative heterofermenters	Obligate heterofermenters
L. acidophilus	*L. acetotolerans*	*L. brevis*
L. amylophilus	*L. agilis*	*L. buchneri*
L. amylovorus	*L. alimentarius*	*L. collinoides*
L. aviarius subsp. *araffinosus* subsp. *aviarius*	*L. bifermentans*	*L. fermentum*
L. crispatus	*L. casei*	*L. fructivorans*
L. delbrueckii subsp. *bulgaricus* subsp. *delbruekii* subsp. *lactis*	*L. coryniformis* subsp. *coryniformis* subsp. *torquens*	*L. fructosus*
L. farciminis	*L. curvatus*	*L. hilgardii*
L. gallinarum	*L. graminis*	*L. kefir*
L. gasseri	*L. hamsteri*	*L. malefermentans*
L. helveticus	*L. homohiochii*	*L. oris*
L. jensenii	*L. intestinalis*	*L. panis*
L. johnsonii	*L. murinus*	*L. parabuchneri*
L. kefiranofaciens	*L. paracasei* subsp *paracasei* subsp. *tolerans*	*L. parakefir*
L. kefirgranum	*L. paraplantarum*	*L. pontis*

Table 2.1 Continued

Group 1	Group 2	Group 3
Obligate homofermenters	Facultative heterofermenters	Obligate heterofermenters
L. mali	*L. pentosus*	*L. reuteri*
L. ruminis	*L. plantarum*	*L. sanfrancisco*
L. salivarius subsp. *salicinus* subsp. *salivarius*	*L. rhamnosus*	*L. suebicus*
L. sharpeae	*L. sake*	*L. vaccinostercus*
		L. vaginalis

2.1.2 Conventional Health Benefits from Lactic Acid Bacteria

Lactic acid bacteria (LAB) have a long history of use in food fermentation and consumption to improve gut health. They are also the predominant members that are usually associated as probiotics and constitute approximately one-third of the bacterial population in the intestinal tract. Hence, LAB have been used as a guideline for the stability of healthy intestinal microbiota and for the prevention and treatment of various diseases (Kruis *et al.* 2004; Sazawal *et al.* 2006; Gawronska *et al.* 2007; Reyed 2007).

The production of antimicrobial substances such as bacteriocins and hydrogen peroxide by LAB contribute to the antagonist activity against various antibiotic-resistant strains. The use of antimicrobial substances is preferred against antibiotics due to their

long history of safe use in foods. For example, the production of plantaricin ZJ008 by *L. plantarum* ZJ008 was reported to be effective against various *Staphylococcus* spp., including the methicillin-resistant strains. The possible mode of action of plantaricin ZJ008 is via pore formation, subsequently causing leakage of K^+ out of cells, thus contributing to bactericidal effect (Zhu *et al.* 2014). Meanwhile, hydrogen peroxide is a strong oxidiser produced by lactobacilli. For instance, hydrogen peroxide generated by *L. gasseri*, which is isolated from vaginal tract of cattle was reported to inhibit the growth of *S. aureus* (Otero & Nader-Macias 2006). Similar finding was reported by Pridmore *et al.* (2006), where the growth of *Salmonella* sp. was inhibited by the hydrogen peroxide produced by *L. johnsonii* NCC33. In addition, the production of these antimicrobial substances also confers a competitive advantage to the bacteriocin-producing bacteria, which further reduced the colonisation by antibiotic-resistant strains.

Despite direct action against the pathogenic strains, LAB also exert an indirect protective effects via stimulation of host immune system. The lipotechoic acid and peptidoglycan of LAB are detected by toll-like receptor 2 (TLR2) and peptidoglycan recognition proteins of the host immune system, leading to enhanced innate immunity and stimulation of immune response, such as initiating pro-inflammatory activities and enhancing the production of both cytokines and secretory immunoglobulin A (sIgA) (McDonald *et al.* 2005; Warchakoon *et al.* 2009; Brandt *et al.* 2013). The major role of cytokines is to activate the immune cells upon encountering pathogens and subsequently stimulate the immune response. Meanwhile, the main function of sIgA is in preventing the binding of foreign bacteria to the epithelial cells and penetration of harmful microorganisms (Erickson & Hubbard 2000). Upon encountering peptidoglycan from

LAB, the peptidoglycan recognition proteins will subsequently act as antibacterial molecules and activate the two-component systems, CssR-CssS or CpzA-CpxR. The activation of these systems will result in bacterial cell death via membrane depolarisation, increase the production of hydroxyl radical and cessation of DNA, RNA, and intracellular peptidoglycan synthesis (McDonald *et al.* 2005; Park *et al.* 2011). Furthermore, lipoteichoic acid isolated from *L. rhamnosus* GG was reported to enhance the pro-inflammatory activities in HEK293T cells by inducing IL-8 in intestinal cells and NF-$_K$B activation via TLR2/6 interaction (Claes *et al.* 2012).

In addition, the administration of certain LAB could ease antibiotic-associated diarrhoea and inflammatory bowel diseases such as ulcerative colitis and Crohn's disease via regulating the intestinal microbiota and stabilise antibiotic induced dysbiosis as demonstrated by *Lactobacillus* GG (Zhang *et al.* 2005). Fung *et al.* (2011) suggested the possible approaches of LAB to inhibit the growth of intestinal pathogen that leads to inflammation via three possible mechanisms: the production of inhibitory substances, adherence to mucosal layer, and iron-siderophore. This indicates the ability of some LAB to protect gastrointestinal tract against the invasion of pathogens and subsequently lower the risk of infections, suggesting the potential use of lactobacilli as an alternative for antibiotic treatment, thus reducing the occurrence of antibiotic resistant.

LAB also have been found to alleviate lactose intolerance symptoms. Lactose maldigester may experience abdominal discomfort, bloating, diarrhea, and flatulence upon ingestion of sufficient amount of lactose (Vesa *et al.* 2000). Honda *et al.* (2007) demonstrated the ability of lactobacilli to exhibit β-galactosidase, phosphor-β-galactosidase and phosphor-β-glucosidase activities that hydrolyse lactose via activating

two lactose transportation systems, namely lactose-permease transportation and lactose-specific phosphoenolpyruvate-dependent phosphotransferase system. Another study also demonstrated oral administration of *L. acidophilus* and *L. casei*-fermented milk by 18 lactase deficiency subjects alleviated the lactose intolerance symptoms, leading to an improvement in lactose digestion (Gaón *et al.* 1995).

Another health benefit from the consumption of LAB is the reduction of serum cholesterol level. Several possible mechanisms were used to exert hypocholesterolemic effect such as assimilation by growing cells or through binding to the cell surface or incorporation into the cell membrane (Liong & Shah 2005a, 2005b). Serum cholesterol could also be reduced via bile salt hydrolase (BSH) to deconjugate bile salt and the resulting free bile salts have limited re-absorption in the gut and more easily to be excreted in the faeces due to poor solubilisation in the gastrointestinal tract. As a result, the demand for synthesise of new bile salt increases to replace those lost in faeces, resulting in the serum cholesterol lowering effect where cholesterol is the precursor for bile acids. Various *in-vivo* studies have been conducted, indicating the serum cholesterol lowering property of LAB, as shown by Shah (2007) where the administration of probiotic fermented milk (10^9 bacteria per mL) to hypercholesteromic human subject was capable of reducing 50% of serum cholesterol level.

Apart from serum cholesterol lowering property, LAB also contain blood pressure lowering ability. This ability is achieved through the production of release bioactive peptides, the angiotensin-I converting enzyme (ACE) inhibitory peptides that play a crucial role in the rennin-angiotensin system. Several *in-vitro* and *in-vivo* studies have been conducted to illustrate the blood pressure lowering on hypertension patients.

For example, Ong and Shah (2008) reported that the addition of *L. casei* and *L. acidophilus* in cheese production had a significantly higher production of ACE inhibitory peptides compared with those without the addition of probiotics. Similar findings were also observed in the studies by Donkor *et al.* (2007) and Rhyänen *et al.* (2001). In addition, an *in vivo* study by Jauhiainen *et al.* (2005) illustrated the consumption of *L. helveticus*-fermented milk twice a day for 10 weeks could decrease systolic blood pressure and diastolic blood pressure by 4.1 mm Hg and 1.8 mm Hg, respectively.

LAB are also postulated to possess anti-carcinogenic effect via various approaches. Gomes and Malcata (1999) postulated that lactobacilli decreased the risk of tumour development by reducing the production of bacterial pro-carcinogenic enzymes such as β-glucuronidase, nitroreductase and urease. The anti-carcinogenic effects also attributed to the production of short-chain fatty acids that lower the colonic pH, and subsequently suppressing the growth of pathogenic microorganisms that are involved in the production of tumour promoters and pro-carcinogenic (Liong 2008). Another studies suggested that tumour suppressing ability is attributed to the binding of mutagens to the cell wall skeleton of LAB and the binding of heterocyclic amines by intestinal probiotics (Zhang & Ohta 1991; Orrhage *et al.* 1994). Cabana *et al.* (2007) reported that the anti-carcinogenic effect from LAB could be accredited to their ability to enhance the intestinal detoxification, transit and immune status, while Singh *et al.* (1997) indicated the anti-carcinogenic effect could attribute to suppression of *as-p21* oncoprotein expression. In addition, LAB, which possessed anti-neoplastic activity were also shown to play an important role in the prevention of colorectal cancer (Boyle *et al.* 2006).

Regardless of various approaches, several studies have been conducted indicating the capability of LAB to exert various degrees of anti-mutagenic activity in the *Salmonella typhimurium* mutagenic assay (Renner & Münzner 1991; Hosoda *et al.* 1992; Abdelali *et al.* 1995).

LAB that colonise the gastrointestinal tract are also responsible in producing various nutrients to the host such as vitamins, which are essential for the microorganisms' growth and metabolism (Hooper *et al.* 2002). Various vitamins such as folic acid, niacin, thiamine, riboflavin, pyridoxine, cyanocobalamin, and vitamin K have been reported to be synthesised by certain lactobacilli, which are slowly absorbed by the host body (Gomes & Malcata 1999). Several studies have reported the ability to synthesise B-vitamins via *L. lactis* and *L. bulgaricus* fermentation and higher production of folic acid, niacin, biotin, pantothenic acid, vitamin B_6 and vitamin B_{12} compared with unfermented counterpart (Hugenholtz & Kleerebezem 1999; Kleerebezem & Hugenholtz 2003). However, the synthesis ability and concentration of vitamins produced are strain dependent. For example, some strains are only capable of synthesising biotin but not riboflavin (Biavati & Mattarelli 2006).

2.1.3 The Use of Lactic Acid Bacteria Beyond Gut Health

Beyond altering and improving the intestinal health, recent emerging studies have shown that LAB could exert health effects beyond gut health, such as dermal health aspects (Table 2.2), as supported by the gut-brain-skin axis hypothesis of Arck *et al.* (2010).

LAB have been reported to act as an immunomodulator that regulate the production of cytokines and growth factors such as tumour necrosis factor-alpha, interferon-gamma, transforming growth factors, and antibodies (IgA and IgE) for improving skin health. For instance, administration of *L. rhamnosus* GG increased the secretion of cytokines such as IL-10 and interferon-gamma in cow milk allergy and atopic dermatitis lesions (Pessi *et al.* 2000; Pohjavuori *et al.* 2004). Recently, the use of LAB has been extended as topical application that directly acts on the skin. Clinical studies have reported the promising effects of topical application of whole cell or bioactive metabolites from LAB by resuming the host skin homeostasis. *In vitro* studies have demonstrated lysate treatment from lactobacilli and bifidobacterium have increased the tight-junction barrier function of keratinocytes via modulating the protein components such as claudin 3. Furthermore, *L. helveticus*-fermented milk was shown to promote cell differentiation by enhanced the keratin-10 mRNA expression (Baba *et al.* 2006; Sultana *et al.* 2013). Animal study by Jones *et al.* (2012) reported that topical application of an adhesive gas permeable patch containing nitric oxide gas-producing LAB promoted wound closure and subsequently accelerated wound healing in New Zealand white rabbit model of ischaemic and infected wounds. Altogether, the current available evidences illustrated the potential use of either whole cell or bioactive metabolites derived from LAB for improving dermal health.

Table 2.2 Clinical evidences of topical applications of whole cell and/or bioactive metabolites from LAB to improve dermal health.

Methods	Remarks	Authors
Adult male BALB/c mice were infected with *S. aureus* (10^8 CFU/mL) and treated with nisin-containing nanofibre dressing for seven days	Viable cell number of *S. aureus* in nisin group was significantly decreased (10^2 CFU/wound) and accelerated excisional wound closure without observable adverse effects	Heunis *et al.* (2013)
29 healthy females aged 25-55 years old with mild acne lesions were treated with oil in water formulation containing 5 % *L. plantarum* extract twice per day for two months	All participants receiving *L. plantarum* extract treatment significantly reduced skin erythema by 57 % and skin redness by 7.5 %	Muizzuddin *et al.* (2012)
20 healthy females aged 18-50 years old were treated with milk lotion containing 3 % *L. plantarum* fermented rice powder twice per day for one month	No erythema was observed and nine females showed skin brightening effect with improve pigmentary deposit.	Sawaki *et al.* (2010)
29 healthy females aged 25-55 years old with sensitive skin were treated with cream containing 1 % *Lactobacillus* extract twice per day for two months	All participants treated with cream containing 1 % *Lactobacillus* extract treatment significantly reduced lactic acid sting by 27 % in the first month, and 39 % in the second month	Sullivan *et al.* (2005)
17 healthy Caucasian volunteers aged 24-47 years old were treated with cream containing 0.5 g sonicated *Strep. thermophilus* cells twice per day for seven days	The lipid barrier of all volunteers was improved, with the stratum corneum ceramide levels significantly increased from 0.25 – 36 pmol total ceramide/µg protein to 639 pmol total ceramide/µg protein	Di Marzio *et al.* (1999)

2.2 Human Skin

The skin is the largest organ of the body, consisting of roughly 15 % of the total body weight and covering an area of 1.7 m^2. The skin serves as the primary physical barrier that protects the body (underlying tissues) against external environment, as "*It keeps the outside out and inside in*", as mentioned by Zaidi and Lanigan (2010). The skin is constantly exposed to external stresses such as physical, chemical, immune pathogen, ultraviolet radiation and free radicals, which damages the skin. Furthermore, internal influences such as hormonal changes, immunological status, food intake, and physiological stresses could disturb the gastrointestinal homeostasis, which later reflects on the skin (Guéniche *et al.* 2009). The skin is also a major participant in thermoregulation and functions as a sensory organ and performs endocrine functions such as vitamin D synthesis and peripheral conversion of prohormones (Menon 2002).

2.2.1 Skin Structure and Function

The skin consists of two distinct layers; the outermost epidermis layer and the inner dermis layer. Beneath both epidermis and dermis layers is the subcutaneous fat layer (Fig 2.1). The subcutaneous fat layer consists of mainly lobules of fat cells and connective tissue septa, which are traversed by nerves and blood vessels and, continuous with the collagen of the dermis. This subcutaneous fat layer serves as a heat insulator, storage for nutritional energy and cushion that protects the body against trauma.

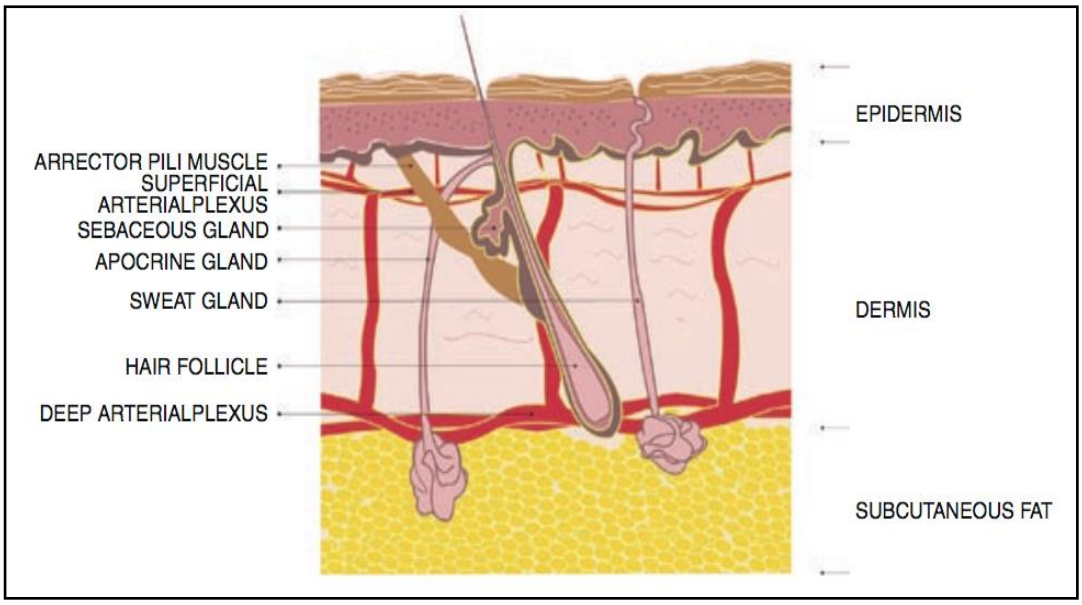

Fig 2.1 Structure of the skin: apocrine glands are found only in the axillae, periareolar region, periumbilical area, and anogenital region. Sebaceous glands and hair follicles are not found in the palms and soles. Arrector pili muscles are not found on the face. Reprinted from Zaidi and Lanigan (2010); with permission from Springer (Licence number: 3720130089766)

The middle dermis layer is the tough fibrous layer, consisting of collagen fibres, elastic fibres, fibroblasts, dermal dendrocytes, mast cells, histiocytes, blood vessels, nerves, lymphatics, and ground substances such as glucosaminoglycans (Prost-Squarioni *et al.* 2008; Zaidi & Lanigan 2010). The collagen fibres that span within the dermis provide tough mechanical support to the skin while the elastic fibres loosely arranged in all directions help in the elastic recoil of the skin. Meanwhile, the blood vessels serve two major purposes, to help maintain body temperature and to supply nutrients to the skin layers. The nerve fibres are responsible for cutaneous sensations such as heat, cold, pain, pressure with one end of the nerves extending to the epidermis layer, while the other end of the nerves end in specialised effectors in the dermis. The ground substances such as glucosaminoglycans has a remarkable important role by assisting the passage of

nutrients, hormones, and fluid molecules through the dermis. The glucosaminoglycans also support the collagen and elastic tissues, and water holding capacity to prevent desiccation (Zaidi & Lanigan 2010). Despite being highly vascular, the dermis layer also contains pilosebaceous unit, sweat glands, dermal adipose cells, mast cells, and infiltrationg leucocytes (Menon 2002).

Overlaying the dermis layer is the avascular outer epidermis layers which are composed primarily of keratinocytes that undergo keratinisation, which then turn into an effective protective barrier and maintain the integrity of the epithelial tissues (Presland & Dale 2000; Menon 2002; Zaidi & Lanigan 2010). Meanwhile, other prominent cells are melanocytes, Langerhans cells, and Merkels cells. The epidermis obtains its nutrients from the dermis blood vessels, as the epidermis does not have any blood vessels. The keratinocytes are arranged in different levels of epidermis. The stratified epidermis is approximately 100 to 150 μm thick with four distinct layers, namely the stratum germinatum (basal cell layer), stratum malpighian, stratum granulosum, and outermost stratum corneum layers (Fig 2.2). There is an additional epidermis layer that is only present in the palms and soles, namely stratum lucidim that spans between stratum corneum and stratum granulosum (Zaidi & Lanigan 2010).

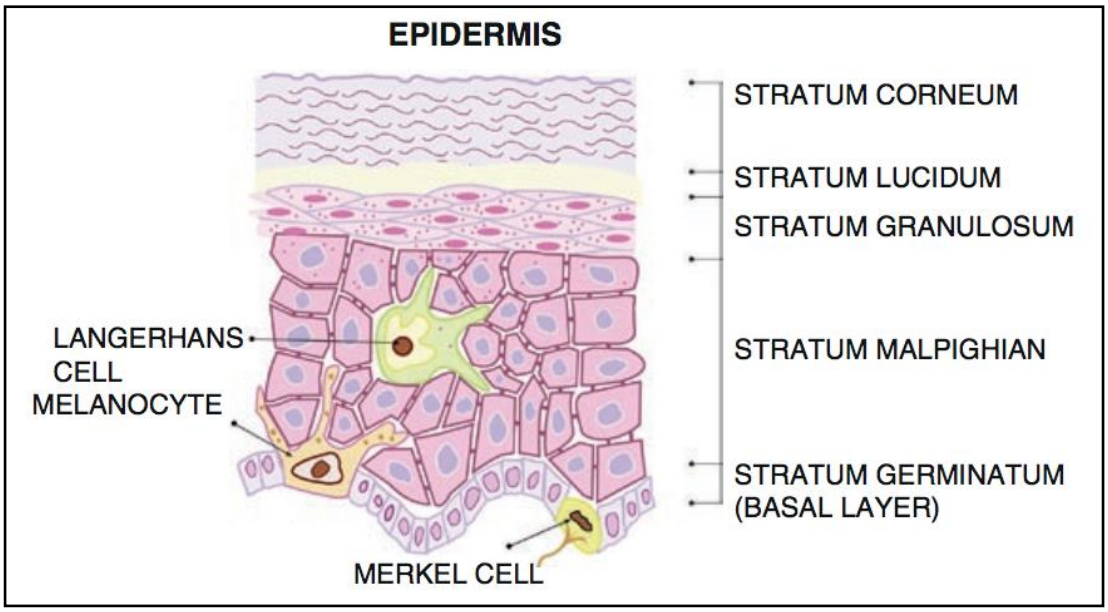

Fig 2.2 Epidermal layers: stratum corneum - anucleated cells; stratum lucidum - present only in palms and soles; stratum granulosum - epidermal nuclei start disintegrating; stratum malpighian - thickest and strongest layer; stratum germinatum - the only cells which undergo division. Epidermal cells: keratinocytes - the main cells of the epidermis, present in every layer of the epidermis; melanocytes - dendritic pigment producing cells, seen with a halo around them under ordinary staining, due to the lack of desmosomes. Present amongst the basal cells; langerhans cells - dendritic immunologically competent cells, also seen with a halo around them, due to the absence of desmosomes. Present in the stratum malpighian; merkel cells - present only in hairless skin; related to the sense of touch. These cells can only be seen under an electron microscope. Present amongst the basal cells. Reprinted from Zaidi and Lanigan (2010); with permission from Springer (Licence number: 3720130089766)

Stratum germinatum is located at the innermost layer in the epidermis and presents as a single layer overlay the basement membrane. They are the only cells of the epidermis that divides, subsequently migrating towards upper layers and transform into continuous sheets of flattened and anucleated corneocytes at the outermost stratum corneum layer (Menon 2002). The upper stratum corneum that shed from the skin surface in the form of microscopic scales is balanced by the cells of the basal layer.

Typical human stratum corneum has about 18 to 21 layers of corneocytes. The strong mechanical stability and chemical resistant in corneocytes were attributed to the insoluble bundled keratin filaments that are surrounded by cornified envelope proteins filled with inoculcrin, loricrin, filaggrin, and cornified lipid envelope (Proksch & Jensen, 2012). These cells overlap each other and are held together by firm lipid-rich cement composed of ceramides, free saturated fatty acids, and cholesterol that is organised as lamellar lipid layers, making stratum corneum prevent the loss of fluids from the body and entry of microorganisms and chemicals into the body (Menon 2002; WHO 2009; Zaidi & Lanigan 2010).

The loss of integrity of a portion of skin as a result of injury or illness need to be recovered as soon as possible to prevent bacterial infections and further fluid loss. Wound healing is a dynamic biological process that can be divided into three phases, namely inflammation, proliferation, and maturation phases that involve various soluble mediators, blood cells, extracellular matrix, and parenchymal cells (Singer & Clark 1999). Wound healing process begins with the blood clot formation that re-establishes hemostasis. This has provided an extracellular matrix for cell migration, such as neutrophils and macrophages to cleanse the wound area from foreign particles and bacteria. In addition, various cytokines and growth factors such as IL-1, TGF, TNF-α, and macrophage-derived growth factors were expressed to initiate the proliferation phase for the formation of new tissue in wounds (Clark 1996; Riches 1996). During proliferation phase, angiogenesis occurs and a provision extracellular matrix is formed by fibroblast through excreting collagen and fibronectin. Concurrently, epithelial cells continue to proliferate to form a new cover tissue on top. During the maturation phase,

the expression of growth factors and cytokines begin to cease and the cells that are no longer needed undergo the apoptosis process (Garg 2000; Chang *et al.* 2004; Midwood *et al.* 2004).

2.2.2 Skin Microflora

Despite acting as a physical barrier, the skin is also an intricate habitat for many bacteria. The use of DNA sequencing and metagenomics enable the identification of skin microorganisms and interaction between skin microflora and skin diseases. A total of 19 phyla were found from 20 diverse skin sites of 10 healthy humans and identified via 16S rRNA gene phylotyping. Most of the identified microorganisms were classified into Actinobacteria (51.8 %), Firmicutes (24.4 %), Proteobacteria (16.5 %), and Bacteriodetes (6.3 %) (Grice *et al.* 2009). The anatomic location, local humidity, amount of sebum and sweat production, and host's hormonal status and age greatly influence the type and density of bacteria (Aly *et al.* 1991).

Skin microflora can be grouped as commensal, symbiotic, or parasitic relative to the host. The use of 16S rRNA gene phylotyping demonstrated *Staphylococcus sp.*, *Micrococcus sp.*, *Corynebacterium sp.*, and *Propionibacterium sp.* are the common residents of the skin (Chiller *et al.* 2001; Findley *et al.* 2013). Gram-negative bacteria such as *Pseudomonas sp.*, *Klebsiella sp.*, and *Vibrio sp.* are not typical resident skin microflora and often associated with cutaneous infections. However, moist intertriginous areas allow the growth of *Acinetobacter sp*. The growth of commensal bacteria was supported by the skin by utilising the skin surface sebum as nutrients, which in turn maintain skin acescence and prevent the invasion of transient pathogenic bacteria both

directly and indirectly (Chiller *et al.* 2001). For instance, the binding of commensal *S. epidermidis* to keratinocytes prevent adherence of virulent *S. aureus*, while fatty acid released by *Propionibacterium acnes* from lipid breakdown acidify the environment and subsequently inhibit the growth of *Streptococcus pyrogenes* (Hentges 1993).

In addition to bacteria, fungi also represents a major population in normal human skin. Topographical mapping using intervening internal transcribed spacer 1 region and 18S rRNA sequencing revealed that 11 core-body and arm sites of 10 healthy adults were dominated by 11 *Malassezia* sp., with feet sites demonstrated richest fungal diversity as compared to other body sites (Findley *et al.* 2013). Furthermore, a whole metagenomic analysis by Foulougne *et al.* (2012) has discovered the cutaneous viral population, the human polyomaviruses in healthy individuals.

Alteration in the balance of microflora and skin homeostasis might subsequently lead to dermatological diseases. For instance, study by Fadeyibi *et al.* (2013) demonstrated that the Gram-negative bacteria, *Pseudomonas aeruginosa*, was dominating the infected burn wounds in burnt patients. The distribution of the bacteria in skin biopsies was different between normal and psoriasis patients. Via pyrosequencing targeting the 16S rRNA and variable regions V3-V4, the number of *Streptococcus pyrogene* was significantly higher while the number of staphylococci and propionibacteria was significantly lower in skin biopsies of psoriasis patients as compared to normal skin (Fahlen *et al.* 2012).

Despite being one of the common residents on the human skin, *Staphylococcus aureus* also take part in atopic dermatitis (AD), a chronic inflammatory skin disease.