Development of Probiotics Bio-actives Liposomes for Cosmetic Applications

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ABSTRACT

The *Lactobacillus paracasei* SD1 and *Lactobacilli rhamnosus* SD11 are found in the oral cavity of humans. They are used as a probiotic for oral health. In this study, it was intended to investigate the cosmetic potential of lyophilized cell free supernatant (LCFS) of *L. paracasei* SD1 and *L. rhamnosus* SD11 for dermal application. The cell free supernatant (CFS) of probiotic lactobacilli consisted of various bioactive compounds which confer valuable advantages to skin health. However, the LCFS of *Lactobacilli* have unpleasant colour and odour. These problems were solved by liposomal encapsulation technology. Therefore, the objectives of the present study were to investigate the antioxidant and antimicrobial activity of LCFS, to develop LCFS liposome, to formulate into a cosmetic product as well as to investigate their physicochemical and *in vitro* release properties.

The antioxidant activity was determined by DPPH radicle scavenging assay. The EC_{50} value for *L. paracasei* SD1 was 940 ± 0.1 µg/ml and for *L. rhamnosus* SD11 was 690 ± 0.02 µg/ml. Both strains exhibited antagonistic effects on *P. acne, S. aureus and S. epidermidis* when examined by agar well diffusion assay and broth microdilution assay. In combination, these two strains showed synergistic effects not only on antioxidant activity, but also on antimicrobial activity.

Liposomes were prepared with different compositions, ratios and total lipid contents. The optimal liposome formulation loading 5% w/v of actives which was composed of SPC:CHOL:TW 80 in 4:1:1 molar ratio with 80 µmol total lipid. It was

characterized by small particle size of 344 nm, low PDI of 0.19, high zeta potential value of -48.05 mV and satisfactory %EE of 69.45. The LCFS liposome showed stability in terms of physical appearance, vesicle sizes, zeta potential and %EE at different temperatures for 90 days. The result of *in vitro* release showed that the liposome could maintain the release of active compounds over 24 hours. Apart from that, it was observed that the liposome significantly reduced the cytotoxicity when tested with HaCaT cells.

For cosmetic formulation development, the cream containing 50% w/w liposome showed good stability. The *in vitro* release of liposome cream was higher than non-liposome cream at 24 hours with antioxidant activity of 58.87 ± 0.84% and 27.48 ± 0.80%, respectively ($P < 0.01$). Furthermore, the antimicrobial potency of the liposome cream was the same as the commercial products. Altogether, it can be concluded that the LCFS liposomes are worthy to develop as cosmetic or cosmeceutical products.

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LIST OF ABBREVIATIONS AND SYMBOLS

A	absorbance
CFS	cell free supernatant
CFU	colony forming unit (s)
°C	degree Celsius
CHOL	cholesterol from lanolin
cm^2	square centimeter (s)
Da	Dalton
DPPH	1,1-Diphenyl-2-picrylhydrazyl
EC_{50}	effective concentration of sample requires to scavenge free radical by 50%
EE	entrapment efficiency
et al	et alli, others
FT	freeze thaw
g	gram (s)
h	hour (s)
GI	gastro-intestine
GIT	gastro-intestinal tract
HPLC	high performance liquid chromatography
IL	interleukin

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

LAB	lactic acid bacteria
LCFS	lyophilized cell free supernatant
µg	microgram (s)
mg	milligram (s)
µl	micrometer (s)
µm	microliter (s)
ml	milliliter (s)
mm	millimeter (s)
µmol	micromole (s)
mV	milli volt (s)
M	molar
MW	molecular weight
MWCO	molecular weight cut-off
nm	nanometer (s)
%	percent
PBS	phosphate buffer solution, pH 7.4
PDI	polydispersity index
r^2	correlation coefficient
rpm	round (s) per minute

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

RT	room temperature
SPC	L-α-phosphatidylcholine from soybean
TW80	tween 80
UV	ultraviolet
w/v	weight by volume
w/w	weight by weight

CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

The skin covers the whole body and acts as a protective barrier between the body and the surrounding environment. The skin is inhabited by over 100 distinct species of microorganisms and most of them benefit the host. The skin microflora maintain the acidic pH of the skin and prevent the colonization of harmful pathogens to the skin (Anisari, 2014). Most skin disorders are the result of an imbalance of skin flora (Cinque et al., 2011).

Probiotics have been widely used as orally in gastro-intestinal (GI) disorders. Apart from that, clinical studies have already reported that probiotics provide profound advantages to the skin such as rejuvenating the skin, improving atopic dermatitis and healing burns and scars (Tavaria, 2017). Probiotics can be used in living form as well as in inactivated form in topical applications. Previous studies have reported that the cell free supernatant (CFS) of *Lactobacilli* contain various bio-active compounds which are beneficial for dermal applications (Lew et al., 2013; Lew and Liong, 2013).

Probiotics can be obtained from various sources such as fermented foods, GIT and the oral cavity of humans. In this study, the probiotics used were human oral derived probiotics, namely, *Lactobacillus paracasei* SD1 and *Lactobacillus rhamnosus* SD11. The probiotic potential and safe uses both *in vitro* and *in vivo* of *L. paracasei* SD1 and *L. rhamnosus* SD11 had already been investigated (Rungsri et al., 2017; Teanpaisan et al., 2015b, 2011; Wannun et al., 2016, 2014).

However, there are some factors that need to be considered when using CFS of *Lactobacilli* as a cosmetic ingredient such as its short shelf life, unpleasant

colour and odour. The CFS was therefore lyophilized to preserve the shelf life. The liposomal technology was applied to mask the unpleasant colour and odour of LCFS, as well as to improve the permeation of LCFS into the deep skin. Liposomes have been widely used as a delivery system in cosmetic, food and pharmaceutical products. Liposomes can entrap both hydrophilic and lipophilic substances and they are non-toxic, compatible with skin and enhance skin penetration.

Therefore, the objectives of this study are to:

1. Determine antioxidant activity and antimicrobial activity of LCFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 in alone or in combination
2. Develop probiotic bio-actives containing liposomes
   2.1 Evaluate their physicochemical properties
   2.2 Evaluate cytotoxicity of probiotic bio-actives containing liposomes
3. Develop probiotic bio-actives liposomes loaded cosmetic formulation and evaluate their physicochemical properties including antioxidant activity and antimicrobial activity
   3.1 Study *in vitro* release of both probiotic bio-actives liposomes and bio-actives liposomes loaded cosmetic formulations

CHAPTER 2

REVIEW OF LITERATURE

2.1 The skin microbiome

The skin is a dwelling place for different species of commensal microorganisms. The skin flora can be generally classified as resident microflora, transient microbes and temporary microorganisms (Huang and Tang, 2015). The composition of skin microflora is dynamic. It can be varied by host factors and environmental factors. The host factors are age, sex, anatomical site, gene, immune system, and pathobiology. For example, hormonal changes in puberty increased the production of lipids, and therefore lead to the proliferation of lipophilic microorganisms such as *Propionibacterium acne* (Grice and Segre, 2011).

The environmental factors are UV light, temperature, humidity, climate, hygiene, and the use of antibiotics. Interestingly, long term use of personal hygiene products, skin cares and cosmetics alter the conditions of skin such as pH and which in turn affect the homeostasis of the skin flora. The reason is that the acidic pH (5.4 to 5.9) of the skin favors the resident flora to attach to the skin (Cinque et al., 2011; Grice and Segre, 2011; Huang and Tang, 2015).

Common skin disorder such as acne and dermatitis are associated with an imbalance of skin microflora. Therefore, homeostasis of skin microbiome is essential for skin health (Grice and Segre, 2011; Huang and Tang, 2015).

2.2 Probiotic

FAO/WHO defined that “Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host”. Probiotics are widely used as orally in GI disorders such as lactose malabsorption, acute diarrhoeas, antibiotic associated diarrhoeas, Traveler’s diarrhoeas and inflammatory bowel disease. Moreover, it is also useful in prevention and treatment of: oral diseases such as dental caries; and allergic diseases such as atopic dermatitis, eczema; cancer prevention and diabetes. In addition, probiotics are involved in immunomodulation and reduced inflammation by promoting IL - 12 production (Chiba et al., 2010; Goldin and Gorbach, 2008).

Probiotics provide profound advantages to skin by balancing skin microflora, preventing skin aging and reducing skin inflammation. Topical probiotics are also able to prevent or reduce the altered microflora associated skin diseases such as acne, dermatitis, psoriasis (Cinque et al., 2011). Recently, the interest in topical applications of probiotics has tremendously increased. However, the development of dermal formulations of probiotics is on its way and still largely underdeveloped (Huang and Tang, 2015).

2.3 Beneficial effects of probiotics on skin

Lactic acid bacteria (LAB) could prevent UV radiation, repair skin damages, restore the skin health, improve the radiance of the skin’s complexion, and retard the skin aging (Gueniche and Castiel, 2011; Kludas and Heise, 1984). Different studies reported the antioxidant property of probiotics both *in vitro* and *in vivo* (Nyanzi et al., 2015; Xing et al., 2015). It has also been reported that LAB produced many bio-active compounds which are essential for the skin health such as lactic acid, acetic acid, hyaluronic acid, sphingomyelinase, diacetyl, lipoteichoic acid and peptidoglycan.

Those bio-active compounds are found in CFS, lysate and cell wall or cell membrane of lactobacilli or bifidobacteria (Lew et al., 2013; Lew and Liong, 2013).

Lactic acid, alpha-hydroxy acid, is also known as natural moisturizing factor. Lactic acid derived from micro-organisms is purer compared to the chemically synthesized one. Lactic acid is widely used in cosmetic and dermal formulations as emollient, moisturizer, exfoliator, peeling agent, whitening agent as well as to reduce wrinkles, to treat and prevent photoaging. Moreover, it also has antimicrobial activity that protects against especially *Staphylococcus aureus* (Lew et al., 2013; Lew and Liong, 2013).

Acetic acid prevents invasion of several pathogenic microorganisms including *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Many studies reported that acetic acid is used in superficial infections and burns. Diacetyl produced from *Lactobacilli* also exert antimicrobial activity on dermal pathogens which are gram negative bacteria and fungus (Lew et al., 2013; Lew and Liong, 2013).

Hyaluronic acid obtained from a bacteria source contains lower contamination than from an animal source. The hyaluronic acid acts as a natural moisturizing factor which possesses the ability to bind water 1,000 times of its molecular weight. Hyaluronic acid has been widely used in dermatology because of its beneficial effects such as controlling epidermal water loss, preventing aging, improving skin hydration and elasticity, and healing wounds (Lew et al., 2013; Lew and Liong, 2013).

Sphingomyelinase is an enzyme which is required to promote ceramide production in skin cells. Dermatological disorders such as decreasing skin barrier functions, atopic dermatitis, and irritant dermatitis are related to a decrease of ceramide level in stratum corneum (Lew et al., 2013; Lew and Liong, 2013).

Diacetyl produced from *Lactobacilli* also exert antimicrobial activity on dermal pathogen which are gram negative bacteria and fungus (Lew et al., 2013; Lew and Liong, 2013). Lipoteichoic acid and peptidoglycan are the important components

of cell walls of gram-positive bacteria. They stimulate the innate immune system of the host by not only initiating production of antimicrobial peptides such as human beta-defensins and cathelicidins, but also secreting a variety of cytokines and chemokines. Therefore, LAB could improve barrier functions of skin and increase dermal cellular defence against bacterial infections (Lew et al., 2013; Lew and Liong, 2013).

2.4 *Lactobacillus paracasei* SD1

L. paracasei SD1 is obtained from the oral of caries-free humans. It is facultative anaerobic, gram positive, rod shape bacteria, which is included in *Lactobacillus* genus. The previous study reported that CFS of *L. paracasei* SD1 consisted of the hydrophilic bacteriocin, namely, paracasin SD1 (MW_24,028.2 Da). It possesses broad spectrum antimicrobial effects, inhibits against gram positive bacteria, gram negative bacteria and yeast. It is relatively heat stable; however, the activity is completely lost at 120°C. The optimal pH for paracasin SD1 for antimicrobial activity is the acidic pH ranges from 5.0 to 6.0. The safe uses of *L. paracasei* SD1 for both short term and long term as well as both *in vitro* and *in vivo* have already been investigated (Teanpaisan et al., 2015b; Teanpaisan and Piwat, 2014; Wannun et al., 2014).

2.5 *Lactobacillus rhamnosus* SD11

L. rhamnosus SD11 is included in *Lactobacillus* genus which is gram positive, rod shape bacteria, and facultative anaerobe. It is one of the commensal microbes in human oral cavities which is capable to protect against various oral pathogens. The CFS of *L. rhamnosus* SD11 contained an antimicrobial substance called fermencin SD11 (MW_ 33,593.4 Da). It is a broad spectrum antimicrobial bacteriocin, active against gram positive and negative bacteria as well as yeast. The antimicrobial activity of fermencin SD11 is stable within pH 3.0 to 7.0, and in temperature between

60 to 80 °C. The activity is completely lost after heating at 100°C for 10 mins and pH above 7.0 (Wannun et al., 2016). The probiotic potential and safe use of *L. rhamnosus* SD11 for both short term and long term as well as both *in vitro* and *in vivo* was already investigated (Rungsri et al., 2017; Wannun et al., 2016).

2.6 Liposomes

In 1960, Bangham first introduced liposome. After that, the uses of liposome have increased and expanded in various fields such as cosmetics, food and agricultural industry and in pharmaceutical applications.

Liposomes formed spontaneously when certain lipids are hydrated in aqueous media (Wagner and Vorauer-Uhl, 2011). Liposomes are synthetic vesicles in which an inner aqueous core is entirely enclosed by lipid bilayer membranes. The liposome bilayers may be composed of phospholipids, cholesterols and or surfactants/additives. The lipid bilayer membranes of liposomes are similar to the biological membranes since both of them are made with phospholipids. The phospholipids may be neutral, negatively charged or positively charged. In liposome preparation, the most commonly used phospholipids are phosphatidycholine (PC). It is amphipathic molecules which have hydrophilic polar head group, phosphocholine, and hydrophobic tail group, fatty acid chains. Phosphatidycholine, also called lecithin, can be obtained from both natural and synthetic (New, 1990).

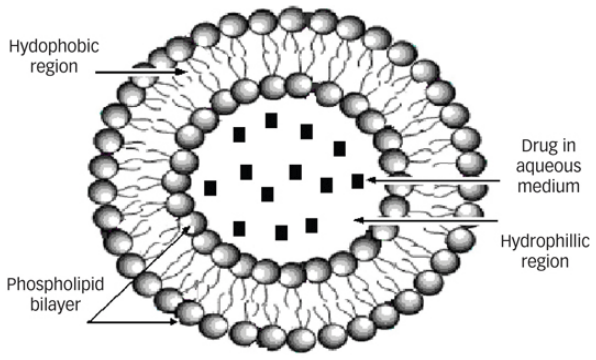

Figure 2.1. Basic liposome structure (Aparajita and Ravikumar, 2014)

Liposomes are classified according to their structural properties and or their preparation methods.

(i) Based on structure (Dua et al., 2012)

- Multilamellar vesicles (MLV) - > 0.5 µm
- Oligolamellar vesicles (OLV) - 0.1 to 1.0 µm
- Unilamellar vesicles (ULV) - all size ranges
- Multivesicular vesicles - > 1.0 µm
- Small unilamellar vesicle - 20 to100 nm
- Giant unilamellar vesicle - > 1.0 µm
- Large unilamellar vesicle - > 100 nm

(ii) Based on method of preparation (Dua et al., 2012)

- Dehydration rehydration method
- Small unilamellar vesicle/ Oligolamellar vesicles by reverse phase evaporation method
- Multivesicular vesicles by reverse phase evaporation method
- Vesicles prepared by Extrusion Technique
- Frozen and Thawed Multivesicular vesicles
- Stable plurilamellar vesicles

Liposomal delivery system provides various advantages as follow (Akbarzadeh et al. 2013).

1. Liposomes can deliver hydrophilic, hydrophobic and amphiphatic compounds.
2. Liposomes can mask unpleasant colour and odour of compounds.
3. Liposomes can improve the stability of the encapsulated compounds from the undesirable external environment.
4. Liposomes are non-toxic, biocompatible and biodegradable.
5. Liposomes can deliver the active compounds to the targeted site.
6. Liposomes reduce toxicity and or irritation of the active compounds.

Apart from above all advantages, liposomes are quite unstable. It may due to phospholipids which can cause oxidation or hydrolysis, and sometimes due to leakage of encapsulated drugs or fusion of liposome molecules (Akbarzadeh et al., 2013). Therefore, the applications of liposome as a delivery system are depending on various factors such as colloidal stability, chemical composition and surface properties.

2.7 Encapsulation of probiotic in liposome

Presently, many cosmetic companies are interested developing probiotics containing products, because LAB are capable to produce beneficial effects on endogenous microbiota of the skin. However, the survival of probiotics in cosmetic formulations are influenced by various factors such as temperature, pH, water content, oxygen content, preservative and the packaging condition (Huang and Tang, 2015; Teanpaisan et al., 2015a; Yuan Kun Lee, 2009).

The patent publications have reported that the probiotics are used in living form, lyophilized form or dead form in the topical applications (Gueniche and

Castiel, 2011; Kludas and Heise, 1984). It also had been reported that the CFS of LAB consisted of various bio-active compounds which are beneficial to skin health (Lew and Liong, 2013). The LAB is found in different parts of human body such as skin, oral, nares, GIT and vagina. Among them, there were limited studies of human oral origin LAB compared to the other sources. Hence, it is worthwhile to investigate the potential use of CFS of human oral derived *Lactobacilli*, namely, *L. paracasei* SD1 and *L. rhamnosus* SD11, for cosmetic applications.

However, the drawbacks of CFS such as short shelf life, unpleasant colour and odour cause limitations when using it for cosmetic formulation. Therefore, the CFS was lyophilized to maintain long shelf life, and encapsulated to mask its colour and odour. There are several techniques, methods and materials for encapsulation such as liposomes, freeze-dried liposomes, spray drying, spray cooling, spray coating, emulsification, coacervation and extrusion. Each technique has its own advantages and disadvantages (B Haffner et al., 2016). In this study, liposomal technology was used to encapsulate the LCFS of oral *Lactobacilli* for the aesthetic purpose.

Until present, the topical formulation of probiotic is still yet to be fully developed. Therefore, this study aims to develop probiotic bio-actives liposomes for cosmetic formulations by using LCFS of *L. paracasei* SD1 and *L. rhamnosus* SD11.

CHAPTER 3

Materials and Methods

3.1 Materials

3.1.1 Probiotic strains

Human oral probiotics, *L. paracasei* SD1 and *L. rhamnosus* SD11, were obtained from the previous study (Piwat et al., 2010). The strains were stored at -80 °C at the Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Thailand.

3.1.2 Chemicals and reagents

1. Absolute ethanol (Labscan Asia Co., Ltd, Bangkok, Thailand)
2. Ascorbic acid (Sigma-Aldrich, China)
3. Cellulose acetate membranes (Spectra/Pro ®3 Dialysis membrane, MWCO 3500 Dalton, Spectro Laboratories, Inc., CA, USA)
4. Cetearyl Ethylhexanoate (Namsiang International Co., Ltd, Bangkok, Thailand)
5. Ceterayl octanoate Lanol 1688 (ADINOP Co., Ltd., Bangkok, Thailand)
6. Cholesterol from lanolin (Sigma-Aldrich, Singapore)
7. Disodium hydrogen orthophosphate anhydrous (Univar®, New South Wales, Australia)
8. Glycerine (P.C. Drug Center Co., Ltd, Bangkok, Thailand)
9. Isopropyl palmitate (P.C. Drug Center Co., Ltd, Bangkok, Thailand)
10. L-α-phosphatidylcholine from soybean (Sigma-Aldrich, USA)

11. Lactic acid (L6661) (Sigma-Aldrich, USA)
12. Mineral oil light (Namsiang International Co., Ltd, Bangkok, Thailand)
13. 1,1-Diphenyl-2-picrylhydrazyl (Sigma-Aldrich, Germany)
14. Phenoxyethanol (P.C. Drug Center Co., Ltd, Bangkok, Thailand)
15. Phosphoric acid (85%) (Labscan Asia Co., Ltd, Bangkok, Thailand)
16. Polyoxyethylene (80) sorbitan monooleate, Tween 80 (Sigma-Aldrich, Switzerland)
17. Propylene glycol (P.C. Drug Center Co., Ltd, Bangkok, Thailand)
18. Polyacrylamide/ c 13,14 Isoparaffin/ Laureth-7-Seppic, Sepigel 305 (ADINOP Co., Ltd., Bangkok, Thailand)
19. Sodium chloride (Carlo Erba, Milan, Italy)
20. Sodium dihydrogen orthophosphate (Univar®, New South Wales, Australia)
21. Stearic acid (P.C. Drug Center Co., Ltd, Bangkok, Thailand)
22. Triethanolamine (P.C. Drug Center Co., Ltd, Bangkok, Thailand)
23. Tocopherol acetate (Namsiang International Co., Ltd, Bangkok, Thailand)
24. Triton X 100 (Loba chemie, India)

3.2 Instruments

1. Electrical balance, AB 135-S (Mettler Toledo, Switzerland)
2. Constant climate chamber, HPP260 (Memmert Gmbh, Germany)
3. Hot air oven, DIN 12880-KI (Memmert Gmbh, Germany)
4. Magnetic stirrer, MR 3000D (Heidolph, Germany)
5. Microplate reader (SPECTROstar^{Nano}, BMG LABTECH Gmbh, Germany)
6. Modified Franz diffusion apparatus, 57-6 M (Hanson, USA)
7. pH meter (Mettler Toledo, Switzerland)
8. Refrigerated centrifuge, 5922 (Kubota, Japan)
9. Rotary evaporator, N-1000 series (Eyela, Japan)
10. Sonicator (HT Crest, S.V. Medico Co., Ltd, USA)
11. Ultracentrifuge, Optima^{TM} L-100XP (Beckman, USA)
12. Viscometer, LVT (Brookfield dial reading, USA)

3.3 Methods

3.3.1 Preparation and lyophilization of CFS

L. paracasei SD1 and *L. rhamnosus* SD11 were cultured on MRS (Difco™, USA) at 37°C for 24 hours under anaerobic condition (80% N_2, 10% H_2, and 10% CO_2). A single colony of each strain was cultured in MRS broth and incubated anaerobically for 24 hours. The cultures were centrifuged at 8,000 rpm for 10 minutes to remove bacterial cells. The obtained CFS was frozen overnight at -80°C prior to freeze-drying by using vacuum freeze dryer (Scanvac CoolSafe™, Denmark) for 48 hours at -110°C. The lyophilized CFS was kept at -20°C until further use.

3.3.2 DPPH radical scavenging activity assay

3.3.2.1 Determination of antioxidant activity of LCFS of each strain

The LCFS of each strain was dissolved in deionized water and carried out two-fold serial dilution to obtain the final concentrations ranging from of 0.08 – 1.25 mg/ml. Sample solutions (100 µl of each strains) were mixed separately with freshly prepared DPPH solution (6×10^{-5} M in absolute ethanol) in 96 wells-plate. The mixtures were shaken and incubated for 30 minutes at room temperature with light protection and the absorbance was measured at 517 nm. Ascorbic acid was used as standard. The percentage of DPPH radical scavenging activity was calculated as followed:

$$\text{Scavenging activity (\%)} = [1-(A_{sample}/A_{control})] \times 100 \quad (1)$$

where A_{sample} is the absorbance of the sample solution and DPPH solution and $A_{control}$ is the absorbance of deionized water and DPPH solution.

The calibration curves between % scavenging activity and the concentration of each strain were plotted. The regression analysis was carried out and the antioxidant activity of both strains was reported in terms of EC_{50} which means the concentration of antioxidants required to scavenge 50% of DPPH radical.

3.3.2.2 Determination of antioxidant activity of LCFS in combination

The antioxidant activity in combination of LCFS of two strains was determined by slight modification of the previous method (Liu et al., 2008). As showing in Table 4.2, the LCFS of two lactobacillus strains were mixed in various concentrations. Each combination (50 µl of *L. paracasei* SD1 and 50 µl *L. rhamnosus* SD11) was mixed with an equal volume of freshly prepared DPPH solution (6×10^{-5} M in absolute ethanol). The experimental procedures were the same as 3.3.2.1. The percentage of DPPH radical scavenging activity was calculated with the equation (1) in 3.3.2.1.

Synergistic effect (SE) of antioxidant activity of the combined strains was calculated using the following equation:

$$SE = ESC/TSC \quad (2)$$

where ESC is the experimental scavenging capacity and TSC is the theoretical scavenging capacity. Synergistic effect was only shown when the result of SE is greater than 1.

The experimental scavenging capacity of combined strains is calculated by:

$$\%ESC = 100 - \{[Abs_{sample} - Abs_{blank}] \times 100]/Abs_{control}\} \quad (3)$$

where Abs_{sample} is the absorbance of the sample solution and DPPH solution, Abs_{blank} is the absorbance of the sample solution and ethanol, and $Abs_{control}$ is the absorbance DPPH solution and deionized water.

The theoretical scavenging capacity (TSC) is calculated by:

$$\%TSC = 100-[(100-ESC_1) \times (100-ESC_2)/100] \quad (4)$$

where ESC_1 and ESC_2 are experimental scavenging capacity of the individual strain.

3.3.3 Antibacterial assay

3.3.3.1 Pathogens and growth conditions

Propionibacterium acne ATCC 6691, *Staphylococcus aureus* ATCC 29213 and *Staphylococcus epidermidis* ATCC 12228 were cultured on blood agar (Difco™, USA) which was supplemented with 5% v/v blood. The strains were incubated anaerobically (80% N_2, 10% H_2, and 10% CO_2) at 37 °C for 24 hours.

3.3.3.2 Agar well diffusion assay

One millilitre of each tested pathogen (10^8 CFU/ml) was mixed with 20 ml of melted BHI (Difco™, USA). The mixture was poured into a plate containing metal cups (6 mm diameter). The metals cups were removed as soon as the agar was solidified. Eighty microliters of LCFS was added into each well. Distilled water was used as negative control. The plate was then incubated anaerobically (80% N_2, 10% H_2, and 10% CO_2) at 37°C for 24 hours. The antibacterial activity was evaluated by measuring the inhibition zone in millimetres. Each experiment was duplicated.

3.3.3.3 Broth microdilution assay

The MIC and MBC were investigated by broth microdilution assay according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). First, two-fold serial dilutions of LCFS with BHI broth (Difco™, USA) were carried

out in 96 wells plate to obtain the final concentrations ranging from 0.04 – 10 mg/ml. Next, 100 µl of each pathogen (10^8 CFU/ml) was added into each well and incubated at 37°C in appropriate conditions. The final volume in each well was 200 µl. The pathogens suspensions were used as positive control and BHI broth was used as negative control.

The MIC was recorded at the lowest concentration of the agent that completely inhibited the growth of pathogens. The MBC was evaluated by using the lowest concentration of the wells that did not show visible growth by sub-culturing the wells contents (10 µl) on blood agar and incubating overnight at 37°C in appropriate conditions.

3.3.3.4 Antibacterial activity of combination

The antibacterial activity after the interactions between LCFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 were determined by the checkerboard method. Briefly, 50 µl of LCFS of *L. paracasei* SD1 was mixed with an equal volume of LCFS of *L. rhamnosus* SD11 in 96 wells plate. Two-fold serial dilutions were carried out with BHI broth, the final concentrations ranged from 2 MIC to 1/16 MIC. Then, 100 µl of each pathogen was added into each well. The final volume in each well was 200 µl. The plate was incubated in the same conditions as in the previous determination of individual MIC.

The fraction inhibitory concentration (FIC) index was calculated as followed:

FIC index = (MIC of supernatant A in combination/MIC of supernatant A alone) + (MIC of supernatant B in combination/MIC of supernatant B alone) (5)

where supernatant A is LCFS of *L. paracasei* SD1 and supernatant B is LCFS of *L. rhamnosus* SD11. The synergistic effect occurred when FIC index was ≤ 0.05, no difference when FIC index was > 0.5 to 4, and antagonistic when FIC index > 4 (Stefanovic et al., 2011).

3.3.4 Analysis of lactic acid

Lactic acid was analysed using HPLC Agilent 1100 series which is equipped with a quaternary pump, various wavelength detector (VWD), auto sampler. Agilent Chemstation. Hypersil ODS C_{18} was used as a column (4.0 x 250 mm, 5 µm) and degassed H_3PO_4 (0.1%) was used as the mobile phase with a flow rate of 1.0 ml/min (25°C). Injection volume was 20 µl and the absorbance was detected at UV 210 nm. All samples were filtered with 0.22 µm syringe filter membrane before they were injected.

3.3.5 Preparation of liposomes

The LCFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 were mixed at a ratio of 1:1 for liposome preparation. To cover all bioactivities and dilution process, 2% or 5% w/v of the LCFS mixture were incorporated into liposome according to EC_{50} value and MIC values (at least 100 x EC_{50} value and 10 MIC value) (Taofiq et al., 2016). The LCFS loaded liposomes were prepared by the modified ethanol injection method (Maitani et al., 2001). Briefly, the oil phase which contained lipid and cholesterol were dissolved in absolute ethanol and sonicated at 50°C for 30 minutes until homogeneous. The water phase consisted of 2% or 5% w/v of LCFS mixture, tween 80 and water. The temperature of both phases was maintained at 50°C before mixing. Then, two phases were mixed in a round bottom flask bottle and the absolute ethanol was removed by rotary evaporator under reduced pressure at 50 ± 5 °C.

Table 3.1 Ratios and compositions of LCFS liposomes

Code	Compositions	Ratio	Total lipid (μmol/ml)	LCFS mixture
L-1	SPC:CHOL	4:1[a]	20	2% w/v
L-2			40	
L-3			60	
L-4			80	
L-5	SPC:TW80	84:16[b]	20	
L-6			40	
L-7			60	
L-8			80	
L-9	SPC:CHOL:TW80	4:1:1[a]	20	
L-10			40	
L-11			60	
L-12			80	
L-13	SPC:CHOL:TW80	4:1:1[a]	40	5% w/v
L-14			60	
L-15			80	

[a] molar ratio, [b] weigh ratio

3.3.6 Characterization of liposomes

3.3.6.1 Physical appearance

Physical appearances of liposome formulations were visually observed including colloidal appearance, colour, phase separation and precipitation.

3.3.6.2 Size, polydispersity index and zeta potential determination

Physicochemical properties of liposomes such as size, polydispersity index (PDI) and zeta potential were determined by using Zeta Potential Analyzer (ZetaPLAS, Brokheaven, USA). Vesicle size and PDI were investigated by dynamic light scattering at 25°C with scattering angle of 90 degrees. Zeta potential value was obtained from measurement of electrophoretic mobility of liposomes at 25°C. Before measurement, all liposome samples were diluted with milli-Q water.

3.3.6.3 Entrapment efficiency

Entrapment efficiency of liposomes was investigated by using ultracentrifugation method. The free drug from liposome formulation was separated by ultracentrifugation at 40,000 rpm for 2 hours at 4°C using Ultracentrifuge (Optima^{TM} L-100XP, Beckman, USA). The supernatant containing free active was collected and diluted with distilled water. The total amount of active was determined by disrupting liposomes with Triton X 100 (10%) at ratio of 1:9 of sample and Triton X 100 (10%). All samples were examined by DPPH assay as described in 3.3.2.1. The entrapment efficacy was calculated by the following equation:

$$\text{Entrapment efficiency (\%)} = (T-F)/T \times 100 \qquad (6)$$

where T is antioxidant activity of total active, and F is antioxidant activity of free active. Ascorbic acid was used as standard. Since the CFS of probiotic lactobacillus contained

various bio-active compounds, the DPPH assay is an indirect method to measure the entrapment efficacy of LCFS loaded liposome.

3.3.7 Cytotoxicity test on human keratinocytes

Human keratinocytes (HaCaT) were grown in high glucose Dulbecco's modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine, 10% Fetal Bovine Serum (Gibo, USA), 1% Penicillin and 1% Streptomycin at 37 °C, 5% CO_2 in a humidified atmosphere.

The cytotoxicity test was examined with sulforhodamine B (SRB) assay which is depending on the ability of bright pink aminoxanthene dye (SRB) to bind with basic amino acid residues in protein components of cells under acidic conditions. The amount of dye extracted from stained cells was directly proportional to the cell mass. Briefly, the cells ($1x10^5$ cells/well) were seeded in a 96 wells plate and incubated at 37°C, 5% CO_2 for 24 hours. The cells were then treated with or without tested liposome formulations for 48 hours. The cells were fixed with 40% w/v Trichloroacetic acid and then incubated at 4°C for 1 hour. After removing the medium, the cells were rinsed with distilled water. The microplates were then dehydrated at room temperature, stained with 0.4% SRB solution, washed 4 times in 0.1% acetic acid and re-dehydrated at room temperature. The protein bound cells were lysed in 10mM Tris-buffer and the optical density was measured at 510 nm. Blank liposome was used as blank and 5% actives in distilled water was used as positive control. The percentage of cell viability was calculated by following equation:

$$\% \text{ Cell viability} = (A_{sample}/A_{control}) \times 100 \quad (7)$$

where A is the absorbance at 510 nm.

3.3.8 Stability of LCFS liposomes

The selected liposomes were evaluated for their stability at 4±1°C, 30±1°C and 45±1°C with 75% RH for 3 months in a constant climate chamber (Memmert Gmbh, Germany) (Amnuaikit et al., 2018; Limsuwan et al., 2016). The physicochemical chemical properties such as physical appearance, particle sizes, zeta potential and entrapment efficiency were evaluated at 0, 30, 60 and 90 days. All evaluations were done in triplicate.

3.3.9 Antioxidant activity of LCFS liposome after in vitro release test

3.3.9.1 *In vitro* release study

The *in vitro* release of LCFS was studied by comparing the antioxidant activities of LCFS liposomes and LCFS solutions. The cellulose acetate membranes (Spectra/Pro ®3 Dialysis membrane, MWCO 3500 Dalton, Spectro Laboratories, Inc., CA, USA) was boiled two times with distilled water to remove coating wax. After that, the membrane was soaked in distilled water, stored in a cool place (2 - 4°C) and used within 7 days. The diffusion area was 1.77 cm^2 and the receptor compartment was filled with 12 ml of receptor fluid which was composed of phosphate buffer solution (pH 7.4) and absolute ethanol at a ratio of 70:30. The modified Franz diffusion cell was set at 37 ± 1°C with a magnetic stirred at 300 rpm. The hydrated cellulose acetate membrane was placed between donor and receptor compartments. After equilibrium for 30 minutes, 1ml of each sample was put to the donor compartment. At 0.5, 1, 2, 4, 6, 8, 12 and 24 hours, the receptor fluid was withdrawn for analysis and immediately replaced with equal volume of pre-thermostated (37°C) fresh receptor fluid.

3.3.9.2 Antioxidant evaluation

Each sample (100 µl) was mixed with an equal volume of DPPH solution (6 x 10^{-5} M in ethanol) in 96 wells plate. After incubating for 30 minutes at room temperature, the mixture was measured by using microplate reader at the absorbance of 517 nm. The measurement was triplicated. The sample and control used in each experiment were as followed:

Control : 100µl of receptor fluid + 100µl of 6 x 10^{-5} M of DPPH in absolute ethanol

Control blank : 100µl of receptor fluid + 100µl of absolute ethanol

Sample : 100µl of sample + 100µl of 6 x 10^{-5} M of DPPH in absolute ethanol

Sample blank : 100µl of sample + 100µl of absolute ethanol

The percentage of DPPH radical scavenging activity was calculated with the equation (1) in 3.3.2.1.

3.3.10 Cosmetic formulation

3.3.10.1 Formulation of cream base

The cream base was prepared by beaker method. Each ingredient was accurately weight and added into two separate beakers, one for oil phase and another for water phase. Then, water phase was poured into oil phase slowly with constant stirring by hand until it congealed and was kept in a glass container at room temperature overnight before further study. The cream bases were evaluated for stability by freeze thaw cycle (each cycle composed of 4 ± 2°C for 24 hours followed with 45 ± 2°C for 24 hours) for 5 cycles and after storage at room temperature for 30 days. Their physical properties including colour, texture, phase separation, pH and viscosity were observed

before and after storage. The cream base with the best appearance and promising stability was selected to develop LCFS liposome cream.

Table 3.2 Ingredients of cream base

Phase	**Ingredients**	**%w/w**		
		Rx1	**Rx2**	**Rx3**
A	Cetearyl Ethylhexanoate	-	12.0	-
	Isopropyl palmitate	-	-	2.0
	Lanol 1688	12.0	-	-
	Mineral oil	5.0	5.0	-
	Stearic acid	-	-	23.0
B	Glycerin	8.0	10.0	2.0
	Propylene glycol	2.0	-	-
	Sepigel 305	10.0	10.0	-
	Triethanolamine	-	-	1.4
	Vitamin E acetate	2.0	2.0	2.0
	Phenoxyethanol	0.5	0.5	0.5
	Water to	100.0	100.0	100.0

3.3.10.2 Formulation of cream containing LCFS liposomes

The LCFS liposome creams were prepared by using suitable cream base from Table 3.2. To cover all bioactivities and dilution process, 20% or 50% w/w of the LCFS liposome were incorporated into the selected cream base to obtain 1% and 2.5% w/w LCFS (at least 50 x EC_{50} value and 5 MIC value) (Taofiq et al., 2016). Cream bases containing LCFS liposome were prepared as the same procedures described in 3.3.10.1. The LCFS liposome was added into the cream base at 45°C with constant stirring. The mixture was stirred until homogenous and congealed at room temperature.

3.3.10.3 pH measurement

Cosmetic formulation (1 g) was diluted with distilled water (10 ml). The pH of the diluted formulation was measured with pH meter. The measurements were done in triplicate.

3.3.10.4 Viscosity measurement

The viscosity of each formulation was measured with Brookfield viscometer (Brookfield dial reading, Model LVT, USA). The measurement was performed by using the spindle number F at the rotation speed of either 3 or 12 rpm depending on the formulations. The measurements were done in triplicate.

3.3.10.6 Antioxidant evaluation

Cosmetic formulation (1 g) was mixed with absolute ethanol (10 ml). The mixture was sonicated for 45 minute and centrifuged again for 15 minutes. The supernatant was then evaluated for antioxidant activity using DPPH assay as described in 3.3.2.1.

The sample and control used in each experiment were as followed:

Control : 100µl of absolute ethanol + 100µl of 6 x 10^{-5} M of DPPH in absolute ethanol

Control blank : 100µl of absolute ethanol + 100µl of absolute ethanol

Sample : 100µl of sample + 100µl of 6 x 10^{-5} M of DPPH in absolute ethanol

Sample blank : 100µl of sample + 100µl of absolute ethanol

3.3.11 Stability study of cosmetic formulation

Each formulation was evaluated for its stability by freeze thaw cycle (each cycle composed of 4 ± 2°C for 24 hours followed with 45 ± 2°C for 24 hours) for 5 cycles and after storage at room temperature for 90 days. The physical properties including colour, texture, phase separation, pH and viscosity were observed before and after freeze thaw cycle as well as in each month. Moreover, the antioxidant activity of the formulation was evaluated. The experiments were done in triplicate.

3.3.12 Antibacterial evaluation of cosmetic formulation

Antibacterial activities of selected liposome creams were evaluated by agar well diffusion assay. The pathogens and the growth conditions were the same as in 3.3.3.1. The experimental procedures were the same as in 3.3.3.2 except the creams were loaded into the well at brimful volume capacity by using syringe. Blank cream and blank liposome were used as blank, distilled water was used as negative control and the three commercial products were used as positive control. The antibacterial

activity was evaluated by observing the presence of inhibition zone. Each experiment was done in triplicate.

3.3.13 In vitro release study

The *in vitro* release of LCFS from the formulation was studied by comparing the antioxidant activities of LCFS liposome cream and LCFS cream. The experimental procedures were the same as in 3.3.9. Each formulation (1g) was put on to the donor compartment. Each experiment was done in triplicate.

3.3.14 Statistical analysis

The data were expressed as mean ± standard deviation (S.D), and analysed either with pair t-test or one-way analysis of variance (ANOVA) and the level of significant difference was set at $P < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 DPPH radical scavenging activity assay

4.1.1 Antioxidant activity of each strain

The antioxidant activity of LCFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 were evaluated by DPPH radical scavenging assay. Ascorbic acid was used as standard. The linear corresponding curve of ascorbic acid, *L. paracasei* SD1 and *L. rhamnosus* SD11 were displayed in Figure 4.1 and 4.2.

The LCFS of *L. paracasei* SD1 scavenged DPPH radical 6.96 ± 0.67%, 10.06 ± 0.58%, 19.73 ± 0.00%, 35.20 ± 2.90% and 64.99 ± 7.81% while *L. rhamnosus* SD11 scavenged 8.27 ± 0.56%, 16.17 ± 1.48%, 27.14 ± 0.32%, 49.44 ± 0.32% and 83.84 ± 2.43% respectively at the concentration of 0.08, 0.16, 0.42, 0.63 and 1.25 mg/ml. As shows in Figure 4.2, the DPPH quenching capacity of both strains increased with increasing concentration. Previous studies also reported that the antioxidant activity of culture supernatant LAB was depending on concentration (Bharti et al., 2017; Tsai et al., 2013).

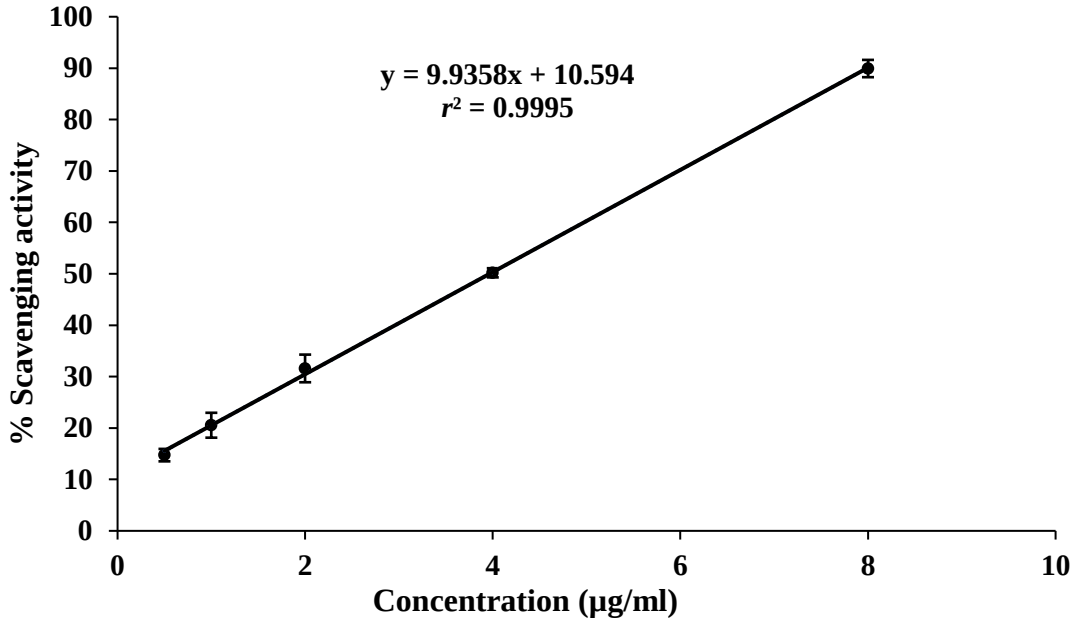

Figure 4.1 Linear regression analysis of concentrations against % scavenging activity of Ascorbic acid.

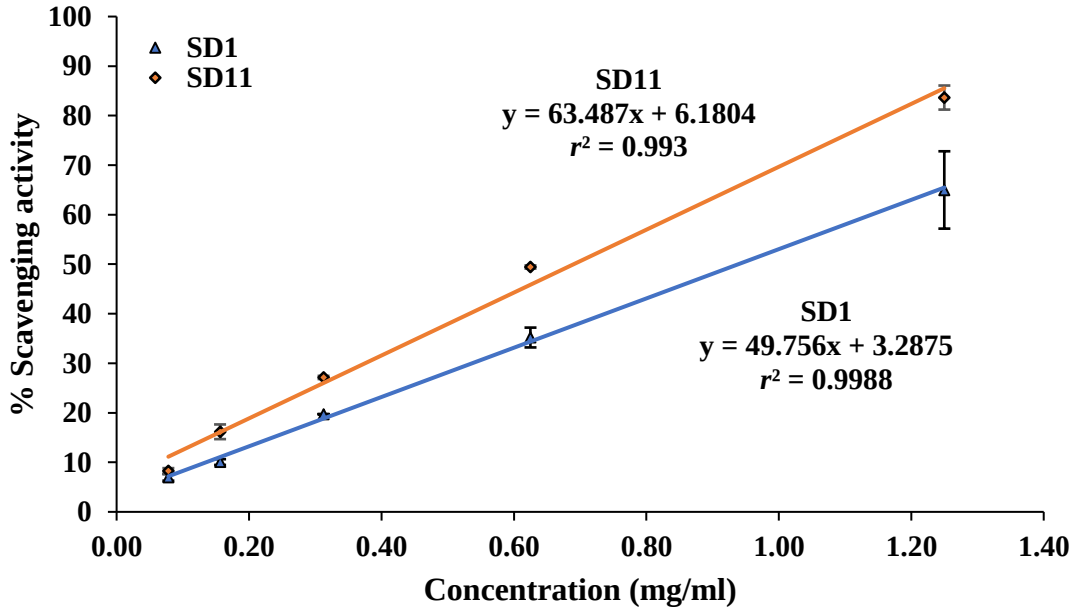

Figure 4.2 Linear regression analysis of concentrations against % scavenging activity of *L. paracasei* SD1 and *L. rhamnosus* SD11.

From the linear plots, the EC_{50} values were calculated and the results were summarized in Table 4.1. In DPPH scavenging assay, low EC_{50} value indicates potent antioxidant activity. Based on the EC_{50} value, both strains possessed more than 100 folds lower antioxidant capacity compared to that of ascorbic acid. When comparing the two strains, antioxidant activity of *L. rhamnosus* SD11 was higher than that of *L. paracasei* SD1. It was because the antioxidant activities of probiotic lactobacillus were strain specific (Chooruk et al., 2017).

Table 4.1 EC_{50} of LCFS of *Lactobacilli* and ascorbic acid

Sample	EC_{50} (µg/ml)
LCFS of *Lactobacillus paracasei* SD1	940 ± 0.1
LCFS of *Lactobacillus rhamnosus* SD11	690 ± 0.02
Ascorbic acid (standard)	3.97 ± 0.16

Data are means ± S.D. (n=3).

4.1.2 Antioxidant activity of combined strains

The antioxidant activities such as experimental scavenging capacity, theoretical scavenging capacity and synergistic effect of the various combinations are shown in Table 4.2. Basically, the radical scavenging activity is directly proportional to the concentration of antioxidants. However, that basic theory did not correlate to high synergistic response. For example, the sample with the highest combined concentration (sample 11) showed no synergistic response. It has been reported that not all combinations were capable of producing synergistic effect, and oppositely, it could result in antagonistic effect (Peyrat-Maillard et al., 2003).

A synergistic effect is that when two or more compounds are used together, it produces an enhanced effect rather than the cumulative effect of those compounds when used individually. In this study, sample 7, 8, 9 and 10 showed synergistic effects. Among them, sample 7 produced the highest synergistic activity ($P < 0.01$). It was suggested that the combined concentration plays an important role in obtaining synergistic effect (Liu et al., 2008).

Table 4.2 Various compositions and concentrations of LCFS of *Lactobacilli* and their experimental scavenging capacity, theoretical scavenging capacity and synergistic effects

Sample	**Final concentration(mg/ml)**		**%ESC**	**%TSC**	**SE[a]**
	SD1	**SD11**			
1	0.94	-	49.52 ± 3.17	-	-
2	1.88	-	72.88 ± 0.20	-	-
3	3.76	-	94.04 ± 0.35	-	-
4	-	0.69	50.00 ± 3.29	-	-
5	-	1.38	78.63 ± 0.37	-	-
6	-	2.76	93.86 ± 0.33	-	-
7	0.94	0.69	95.38 ± 0.28	74.78 ± 1.84	1.28 ± 0.03
8	0.94	1.38	97.50 ± 1.42	89.21 ± 0.85	1.09 ± 0.01
9	0.94	2.76	99.23 ± 0.88	96.91 ± 0.10	1.02 ± 0.01
10	1.88	0.69	92.12 ± 0.39	86.46 ± 0.43	1.07 ± 0.00
11	3.76	0.69	95.20 ± 0.66	97.03 ± 0.06	0.98 ± 0.01

[a] SE > 1: synergistic effect; SE < 1: no synergistic effect
Data are mean ± S.D. (n=3).

For liposome formulation, the two strains were combined in 1:1 ratio. The linear regression analysis was carried out and the graph is illustrated in Figure 4.3. The EC_{50} value was 190 ± 0.01 µg/ml. It was observed that the EC_{50} value of combination was significantly lower than that of individual strain ($P < 0.01$). It was because of the synergistic response of the two strains.

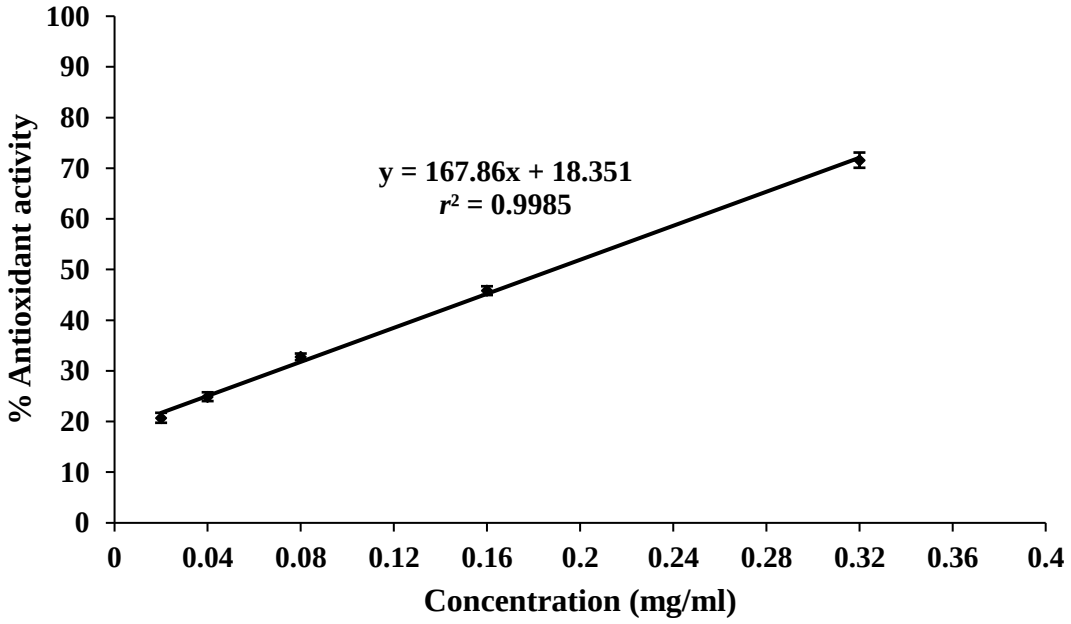

Figure 4.3 Linear regression analysis of concentrations against % antioxidant activity of combination of *L. paracasei* SD1 and *L. rhamnosus* SD11 in the ratio of 1:1

4.2 Antibacterial activity

4.2.1 Agar well diffusion assay

P. acne, *S. aureus* and *S. epidermidis* are common skin pathogens which cause acne, dermatitis, atopic dermatitis and other skin related problems. The antibacterial potential of LCFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 on these skin inflammatory bacteria were screened by agar well diffusion method. The resultant clear zones indicated that both strains have antagonistic activities against all tested bacteria (Table 4.3). It was in good agreement with the previous findings; *L. paracasei* SD1 and *L. rhamnosus* SD11 displayed antagonistic effect on gram positive bacteria (Wannun et al., 2016, 2014).

4.2.2 Broth microdilution assay

The MIC and MBC values are presented in Table 4.3. The results implied that *L. paracasei* SD1 and *L. rhamnosus* SD11 possess equal inhibitory efficacy against *P. acne*, *S. aureus* and *S.epidermidis*. It might be because the probiotics are of the same lactobacillus species or isolated from the same origin. It is commonly known that the antimicrobial activities of LAB are influenced by different parameters. One of the most dominant factors influencing the antimicrobial activity of probiotics is the production of bacteriocin. Previous studies have already reported in detail concerning purification and characterization of bacteriocins, which were found in supernatant of *L. paracasei* SD1 and *L. rhamnosus* SD11 (Wannun et al., 2016, 2014).

The other parameter is pH. The pH of both strains was pH 5 which is the optimal pH to achieve antimicrobial activity. The previous studies also revealed that the strongest antagonistic effects occur in acidic pH ranges from 5 to 6, while the activity is completely lost in alkaline pH for both strains (Wannun et al., 2016, 2014).

The production of short chain fatty acids such as lactic acid, acetic acid and butyric acid also influenced the antimicrobial activity. The concentration of lactic acid in LCFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 was analysed by HPLC. Lactic acid can diffuse easily in non-dissociated form through the bacterial cell membrane, lowering the pH and disturbing cellular enzymatic activities of bacteria. Although the *L. paracasei* SD1 produced lower concentration of lactic acid than *L. rhamnosus* SD11 (Figure 4.4), the antimicrobial efficacy of both strains was the same (Table 4.3). As described above, the antimicrobial activity of LAB is depending on different factors than lactic acid production.

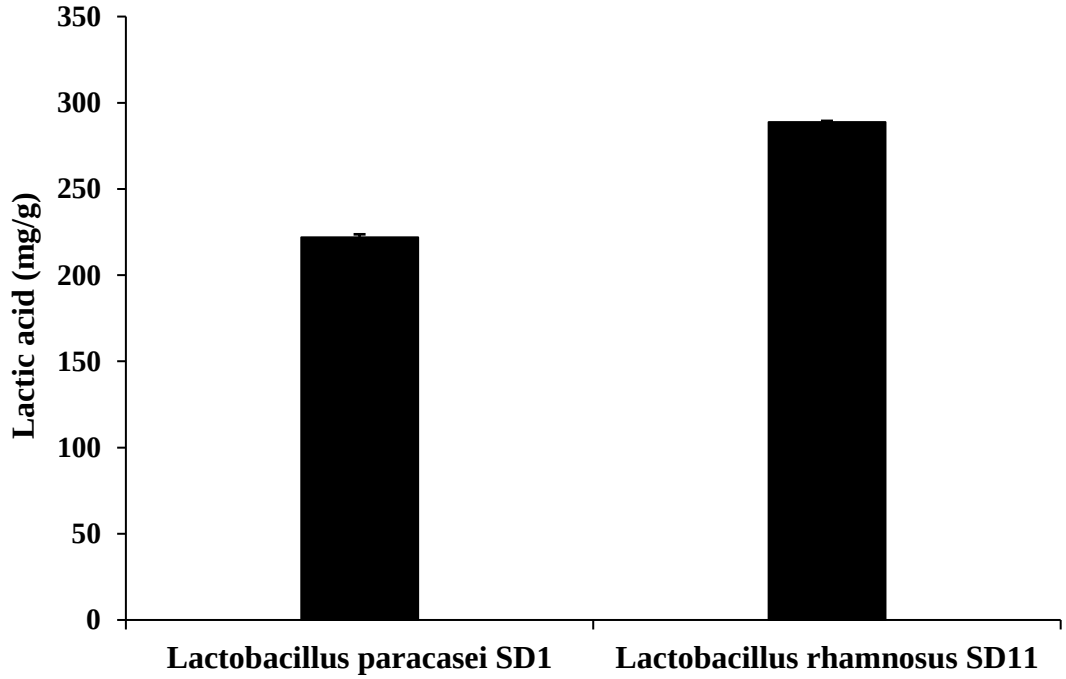

Figure 4.4 Lactic acid concentration of LCFS of *L. paracasei* SD1 and *L. rhamnosus* SD11

4.2.3 Antibacterial activity of combination

The combination mixtures of the two strains showed synergistic effects on *P. acne*, *S. aureus* and *S. epidermidis* (Table 4.3). The CFS of lactobacillus consisted of various bio-active compounds which contributed to antimicrobial activity such as bacteriocin, proteins, short chain fatty acids, hydrogen peroxides and the other cell wall fragments. Therefore, the synergistic effect seems to be the outcome of summation of the activities of bacteriocins, organic acids and hydrogen peroxide.

Table 4.3 Antimicrobial activity of concentrated supernatant of *L. paracasei* SD1 and *L. rhamnosus* SD11 on gram-positive bacteria

Pathogens	Inhibition zone (mm)		MIC (mg/ml)		MBC (mg/ml)		MIC in combination (mg/ml)		FIC index[a]
	SD1	SD11	SD1	SD11	SD1	SD11	SD1	SD11	
P. acne ATCC 6691	16.0 ± 0.0	16.0 ± 0.1	12.5 ± 0.0	12.5 ± 0.0	12.5 ± 0.0	12.5 ± 0.0	1.6 ± 0.0	1.6 ± 0.0	0.3 ± 0.0
S. aureus ATCC 29213	15.0 ± 0.0	15.0 ± 0.0	12.5 ± 0.0	12.5 ± 0.0	25.0 ± 0.0	25.0 ± 0.0	3.2 ± 0.0	1.6 ± 0.0	0.4 ± 0.0
S. epidermidis ATCC 12228	15.0 ± 0.0	15.0 ± 0.0	12.5 ± 0.0	12.5 ± 0.0	25.0 ± 0.0	25.0 ± 0.0	3.2 ± 0.0	1.6 ± 0.0	0.4 ± 0.1

[a] FIC index ≤ 0.5 synergy; FIC index > 0.5 to 4 indifference; FIC index > 4 antagonism
Data are mean ± S.D. (n=2).

4.3 Physicochemical characterization of LCFS liposomes

4.3.1 Physical appearance

The physical appearances of the formulations are presented in Figure 4.5. The brown colour of agar media had faded and the unpleasant odour was masked after encapsulating the LCFS into liposomes.

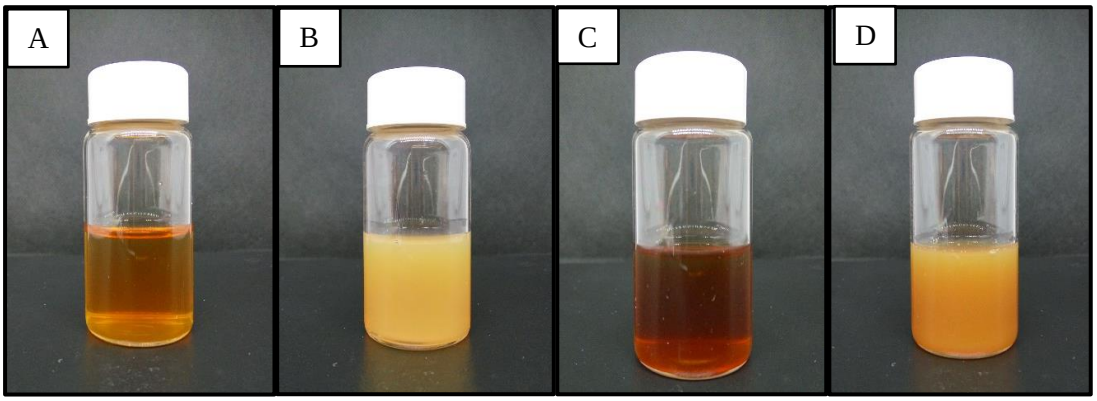

Figure 4.5 Physical appearance of solutions and liposomes containing LCFS mixture of *L. paracasei* SD1 and *L. rhamnosus* SD11 (A) 2% w/v solution (B) 2% w/v liposome (C) 5% w/v solution (D) 5% w/v liposome.

4.3.2 Size, PDI and zeta potential

The results of size, PDI and zeta potential were summarized in Table 4.4. The particle sizes of all formulations were found in the range of 334 to 630 nm in diameters. It was clearly observed that the particle sizes were decreased when the total lipid contents were increased from 20 µmol to 80 µmol. Moreover, smaller particle sizes were observed in the formulations containing surfactant although they contained the same total lipid contents. It was noticed that the concentrations of surfactant also dictated the vesicle sizes of liposomes. The surfactant concentration was inversely proportional to the particle sizes. In addition, higher surfactant concentration lead to consistency of size distribution, reduction of interfacial tensions and therefore improved the stability of liposomes (Bnyan et al., 2018).

Zeta potential of the formulations were negatively charged and generally between -30mV to -50 mV. The net negative charge of liposome was due to the orientation of phospholipid carboxyl head group of negatively charged SPC. High values of negative charges on the surface of the vesicles inhibit the agglomeration of liposomes by creating repulsive forces between vesicles (Bnyan et al., 2018).

4.3.3 Entrapment efficiency of LCFS loaded liposomes

The entrapment efficiency was influenced by the compositions of liposome, total lipid contents and the percentage of active incorporated into liposome. It was commonly reported that inclusion of cholesterol in liposome system increased the rigidity of membrane, prevented leakage of drugs, stabilized the liposome and increased %EE. Conversely, L-3 and L-4 which were composed of SPC and CHOL showed low %EE and precipitated after one week. The reason was that %EE of hydrophilic drugs depend on the internal aqueous volume and the concentration of drugs inside the aqueous core. It was suggested that cholesterol reduced the internal

aqueous volume of liposome thereby resulting in low entrapment for hydrophilic drugs (Eloy et al., 2014).

It was observed that the surfactant concentration was directly proportional to %EE. Although liposome with high surfactant concentration which were composed of SPC and TW 80 (L-8) showed relatively high %EE, it was not able to load high concentrations of active (5% w/v) (data not shown). This might be due to the unstable vesicular system since unsaturated double bond interaction of surfactant on the lipid bilayer caused pores within the membranes. It can be overcome by the addition of cholesterol into vesicle system. The cholesterol can fill the pores formed by the interaction of surfactant and vice versa surfactant reducing the rigidity of cholesterol and providing flexibility to the membrane (Bnyan et al., 2018; Glavas-Dodov et al., 2005). Therefore, the liposomal formulation composed of SPC, CHOL and TW80 was the optimal system to incorporate lyophilized CFS mixture.

Regardless of the compositions, %EE was increased as the total lipid contents was increased. However, it was noted that the successful encapsulation of 5% w/v actives was possible only when the total lipid content was high. This was because the high lipid content caused large internal volume for encapsulation of drug. The %EE was increased when the percentage of active loaded into liposome was increased. Although L-12 and L-15 consisted of the same compositions and total lipid content, the incorporation of different percentage of active into liposome produced different %EE.

The aim of encapsulation of probiotic CFS was for aesthetic purposes, to mask the unpleasant colour and odour of CFS. The optimal formulations were selected based on the criteria of particle sizes $\leq$ 500 nm, PDI $\leq$ 0.3, high zeta potential values and relatively high %EE (Amnuaikit et al., 2018; Limsuwan et al., 2017). Therefore, L-12 and L-15 were selected as optimal formulations for further studies.

Table 4.4 Physicochemical properties and entrapment efficiency LCFS liposomes

Code	Particle size (nm)	PDI	Zeta potential (mV)	%EE	Appearance
L-1	ND	ND	ND	ND	ppt
L-2	ND	ND	ND	ND	ppt
L-3	629.7 ± 9.4	0.35 ± 0.01	-38.73 ± 0.12	24.90 ± 2.26	ppt after one week
L-4	442.6 ± 16.4	0.21 ± 0.01	-45.81 ± 3.30	26.52 ± 1.94	ppt after one week
L-5	ND	ND	ND	ND	ppt
L-6	475.9 ± 4.2	0.31 ± 0.01	-37.95 ±3.12	14.32 ± 1.71	ppt after one week
L-7	367.1 ± 22.2	0.12 ± 0.08	-44.83 ± 0.99	35.61 ± 1.46	Pale yellow
L-8	344.8 ± 3.4	0.19 ± 0.06	-45.26 ± 2.38	43.37 ± 2.19	Pale yellow
L-9	ND	ND	ND	ND	ppt
L-10	405.9 ± 13.3	0.17 ± 0.08	-34.46 ± 2.99	33.40 ± 1.20	ppt after one week
L-11	362.1 ± 6.7	0.18 ± 0.01	-47.80 ± 2.33	36.14 ± 0.33	Pale straw colour
L-12	349.3 ± 9.2	0.03 ± 0.02	-47.60 ± 1.89	46.71 ± 7.02	Pale straw colour
L-13	ND	ND	ND	ND	ppt
L-14	333.6 ± 2.5	0.12 ± 0.02	-46.94 ± 3.58	46.28 ± 0.96	Straw colour
L-15	344.1 ± 1.1	0.19 ± 0.03	-48.05 ± 1.53	69.45 ± 2.34	Straw colour

Data are mean ± S.D. (n=3).
ND means not determined.
ppt means precipitate.

4.4 Cytotoxicity of LCFS liposomes on HaCaT

Cytotoxicity test was carried out to prove the safe use of LCFS liposomes for cosmetic applications. The results are illustrated in Figure 4.6. The percentage of cell viability of 5% w/v active solution was 47% which means it contributes to moderate cytotoxicity potential according to ISO 10993-5:2009(E). It might be due to acidic pH since *Lactobacillus* consists of short chain fatty acids which were produced during fermentation process. After incorporating the LCFS into liposome, the cytotoxicity was significantly decreased. Furthermore, the blank liposome itself did not show significant cytotoxicity. It was noted that the % cell viability was not significantly different between blank liposome and active loaded liposomes. Therefore, it was clearly observed that the liposomes remarkably reduced cytotoxicity of LCFS of *L. paracasei* SD1 and *L. rhamnosus* SD11.

Table 4.5 Percentage of cell viability and level of cytotoxicity (López-García et al., 2014)

% Cell viability	**Cytotoxicity level**
more than 80%	non-cytotoxicity
80% to 60%	mild cytotoxicity
60% to 40%	moderate cytotoxicity
less than 40%	severe cytotoxicity

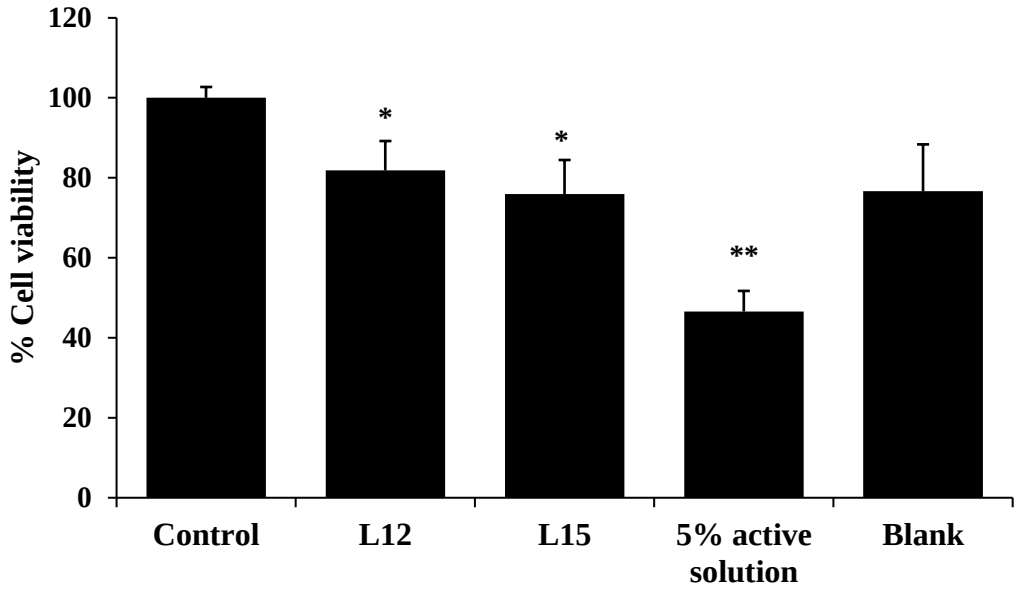

Figure 4.6 Cell viability of L-12 and L-15

Data are mean ± S.D. (n=3).

*$P < 0.05$ and ** $P < 0.01$ compared to the control.

4.5 Stability of LCFS liposomes

The selected liposomes (L-12 and L-15) were kept at 4±1°C, 30±1°C and 45±1°C with 75% RH for 3 months to study their stability in terms of physical appearances, sizes, zeta potential and %EE. The physical appearance of all formulations remained unchanged over 3 months except the L-15 where the colour became darker at 45±1°C as shown in Figure 4.7.

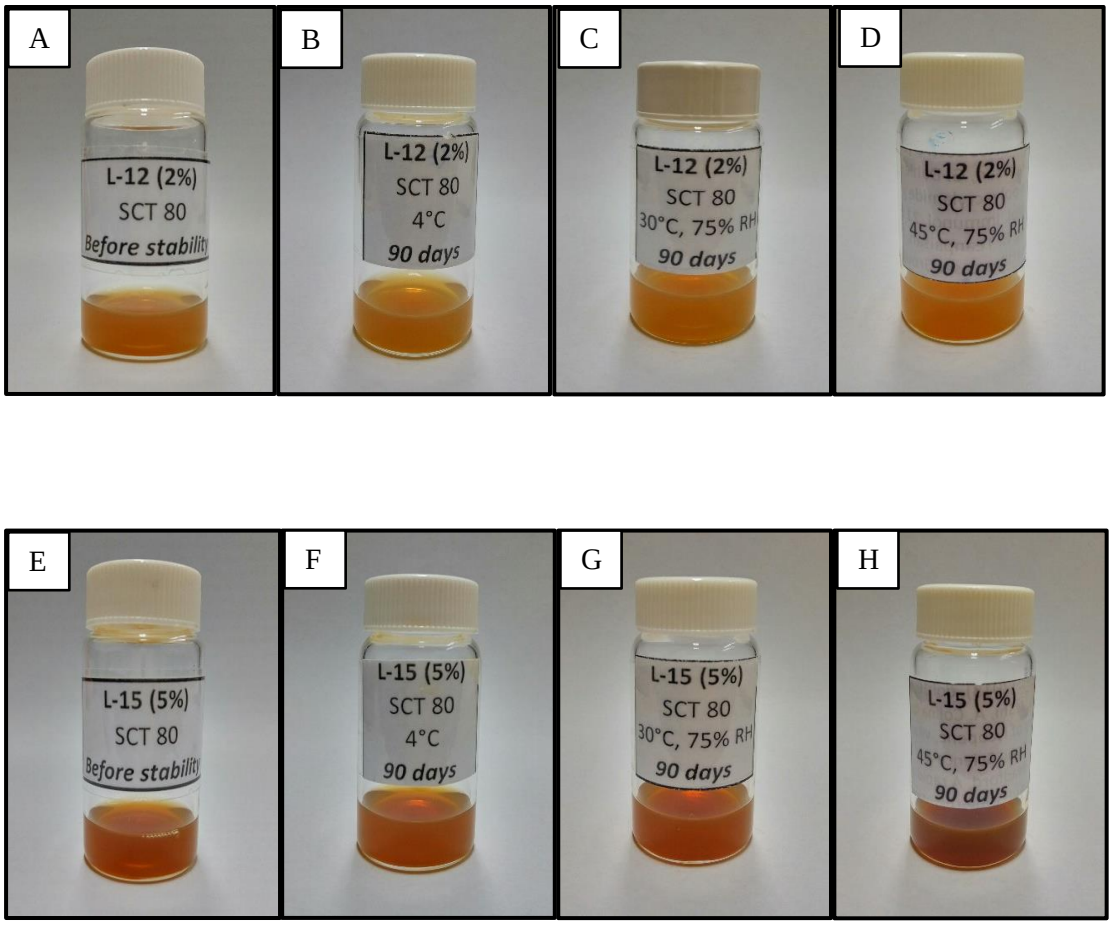

Figure 4.7 Physical appearance of LCFS liposomes containing *L. paracasei* SD1 and *L. rhamnosus* SD11 mixture before and after storage at different temperatures (A - D) 2% w/v CFS and (E - H) 5% w/v CFS.

The vesicle sizes of L-12 did not change significantly when stored at 4±1°C and 30±1°C, however, it increased significantly when stored at 45±1°C for 3 months (Figure 4.8). The increase in the sizes of lipid vesicles when stored at 45±1°C for long terms were also observed in previous studies as well (Amnuaikit et al., 2018; Limsuwan et al., 2017). The vesicle sizes of L-15 also increased but the sizes remained ≤ 500 nm after keeping them at different temperatures over 3 months (Figure 4.9). Although the particle sizes of all formulations were increased when time increased, they showed narrow size distribution (PDI ≤ 0.3). The increase in the sizes of liposomes during storage is common (Amnuaikit et al., 2018; Chorachoo et al., 2013). It may be due to aggregation or fusion of liposome vesicles.

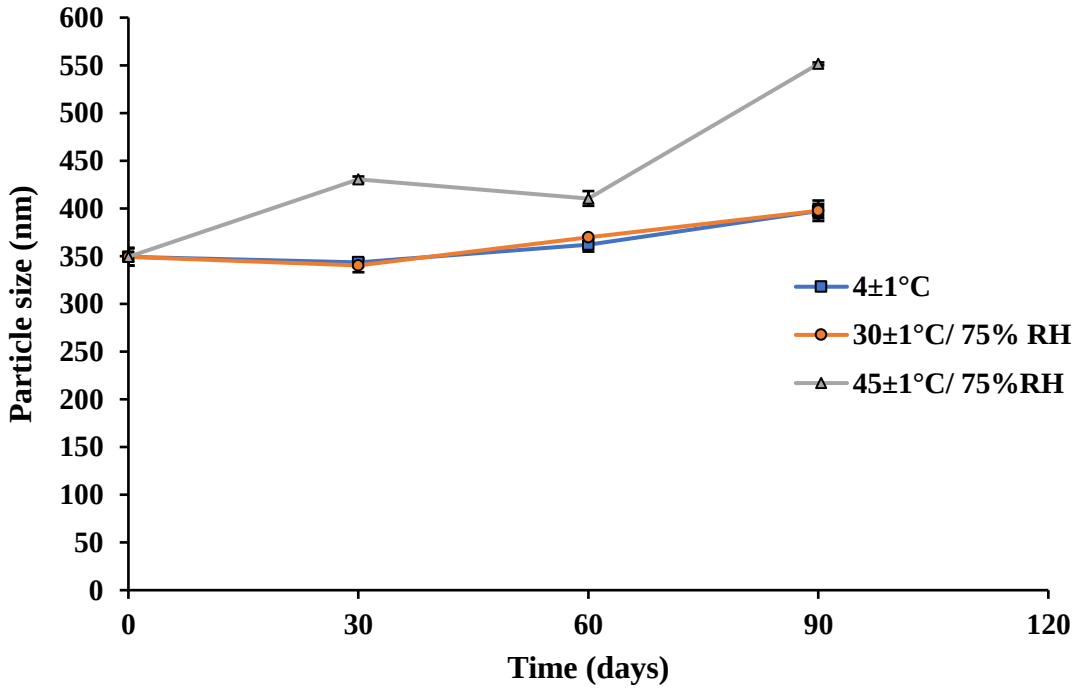

Figure 4.8 Stability profiles in terms of particle sizes of L-12 kept at 4±1°C, 30±1°C/ 75% RH and 45±1°C/ 75%RH

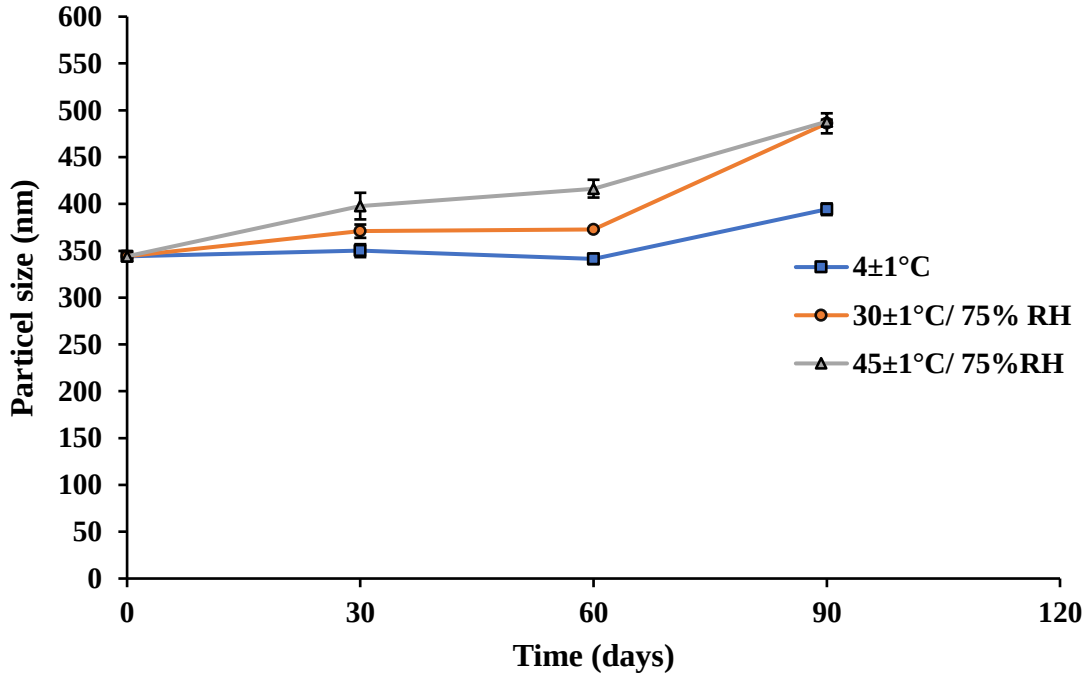

Figure 4.9 Stability profiles in terms of particle sizes of L-15 kept at 4±1°C, 30±1°C/ 75% RH and 45±1°C/ 75%RH

The zeta potentials of all formulations were not change significantly and remained within the ranges from -40 to -60 mV before and after stability (Figure 4.10 and 4.11). Zeta potential values indicate the repulsion forces between the vesicles. The high zeta potential values cause strong repulsion forces between vesicles and hence prevent the agglomeration of liposomes. High zeta potential values of L-12 and L-15 after stability test indicating that both formulations were stable over 90 days storage at different temperatures.

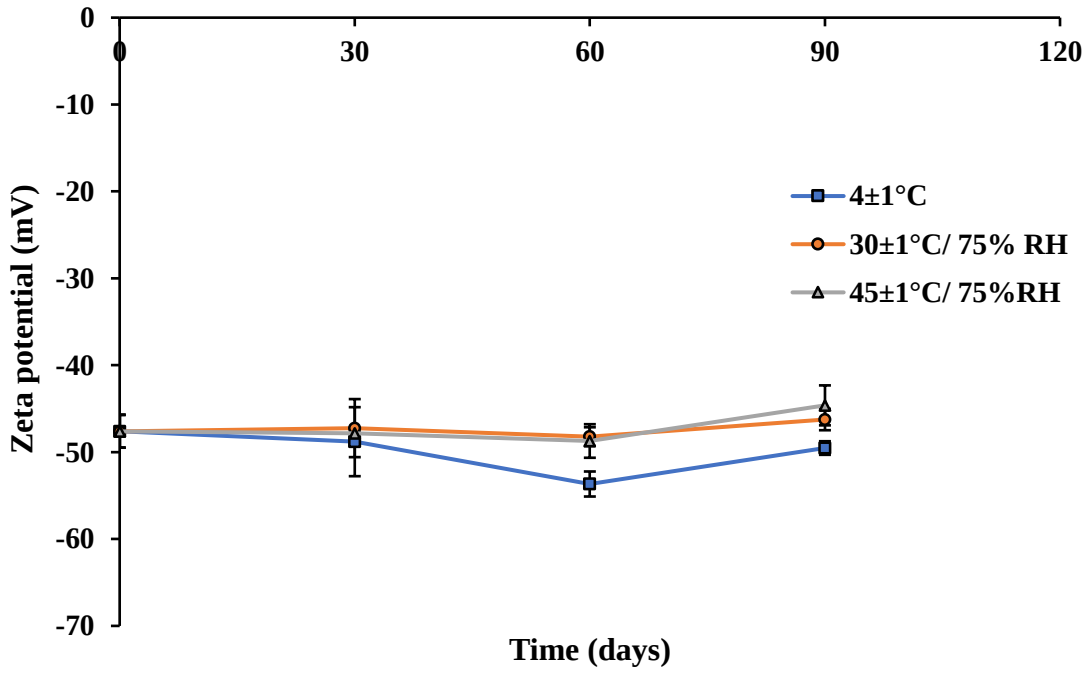

Figure 4.10 Stability profiles in terms of zeta potential of L-12 kept at 4±1°C, 30±1°C/ 75% RH and 45±1°C/ 75%RH

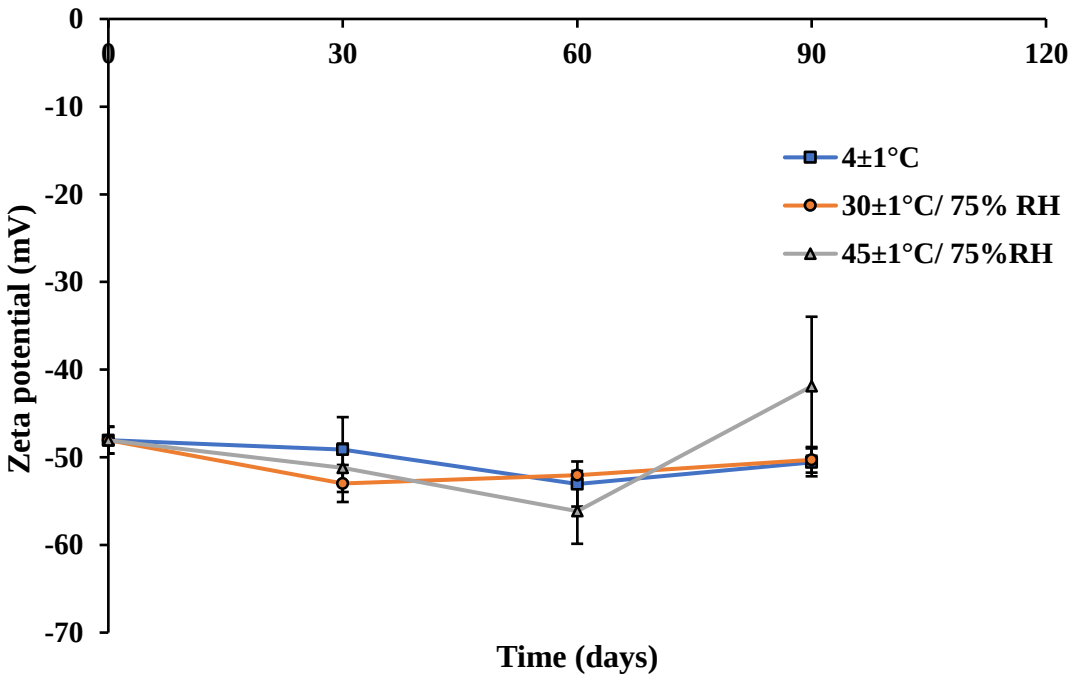

Figure 4.11 Stability profiles in terms of zeta potential of L-15 kept at 4±1°C, 30±1°C/ 75% RH and 45±1°C/ 75%RH

The entrapment efficiency of all formulations decreased gradually with time; however, the changes are not significant ($P < 0.05$) as demonstrated in Figure 4.12 and 4.13. The results indicated that the leakage of actives from the lipid vesicles was not significant over long term of storage at different temperatures ($P < 0.05$).

The stable formulation was selected based on the criteria of particle sizes ≤ 500 nm, PDI ≤ 0.3, high zeta potential values and relatively high %EE (Amnuaikit et al., 2018; Limsuwan et al., 2017). Therefore, L-15 was selected as a stable formulation for further studies.

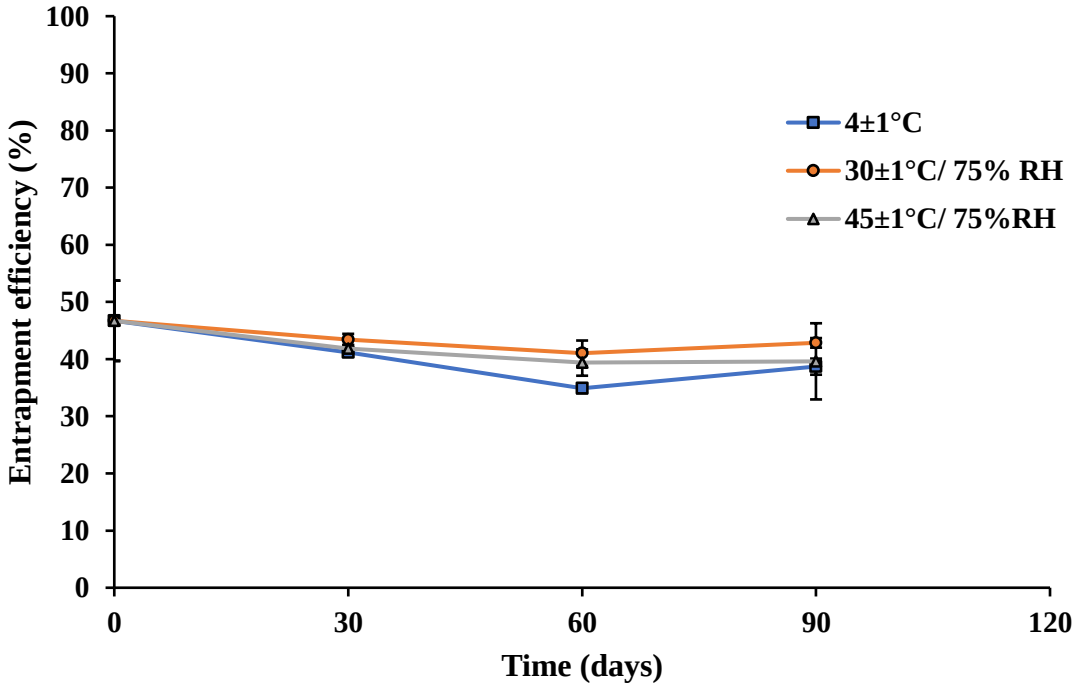

Figure 4.12 Stability profiles in terms of Entrapment efficiency (%) of L-12 kept at 4±1°C, 30±1°C/ 75% RH and 45±1°C/ 75%RH

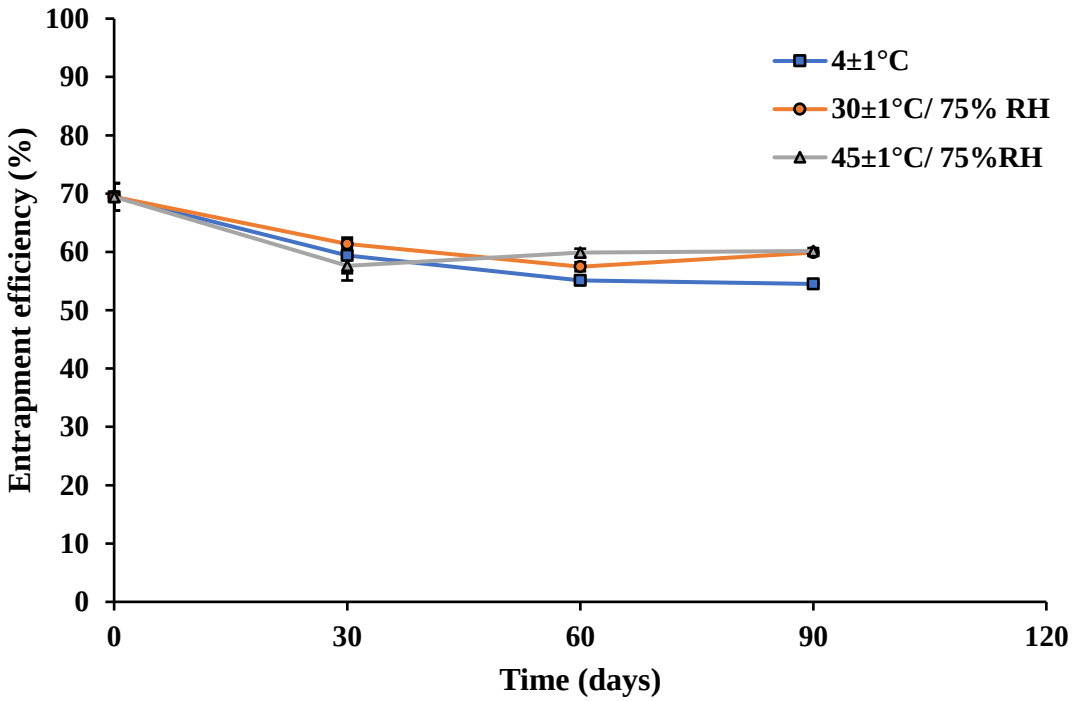

Figure 4.13 Stability profiles in terms of Entrapment efficiency (%) of L-15 kept at 4±1°C, 30±1°C/ 75% RH and 45±1°C/ 75%RH

4.6 Antioxidant activity of LCFS liposome after *in vitro* release test

The liposome with good stability (L-15) was evaluated for the *in vitro* release compared with LCFS solution at the same concentration (5% w/v actives). The *in vitro* release study was evaluated by determining the antioxidant activity of the fluid from the receptor chamber at different time intervals. The results are displayed in Figure 4.14 and Table 4.6.

It was clearly observed that the release rate of the actives from the liposomes was slower than from the aqueous solution. It was because the encapsulated active molecules needed to diffuse through the lipid bilayer to the external aqueous media, then through the membrane to be finally released into the receptor compartment. The rapid release of the actives from aqueous solution has also been reported in many studies (Hussain et al., 2014; Nava et al., 2011). From the release profile, it was found out that the actives from the liposomes released gradually over 24 hours. Therefore, the *in vitro* release study indicated that the liposome delayed the release of actives, leading to a controlled release of the actives over long term.

Table 4.6 *In vitro* release of LCFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 at various time (h) by using cellulose acetate membrane

Formulations	Antioxidant activity at various time (h)							
	0.5	**1**	**2**	**4**	**6**	**8**	**12**	**24**
Active solution	25.37 ± 0.95	30.53 ± 0.68	33.42 ± 1.67	38.11 ± 0.78	46.36 ± 0.98	61.42 ± 0.46	69.76 ± 1.29	91.51 ± 1.79
L-15	15.17 ± 0.08	22.63 ± 0.32	28.76 ± 0.38	35.45 ± 1.67	43.16 ± 0.33	42.30 ± 1.69	46.78 ± 2.03	68.07 ± 2.37

Data are mean ± S.D. (n=3).

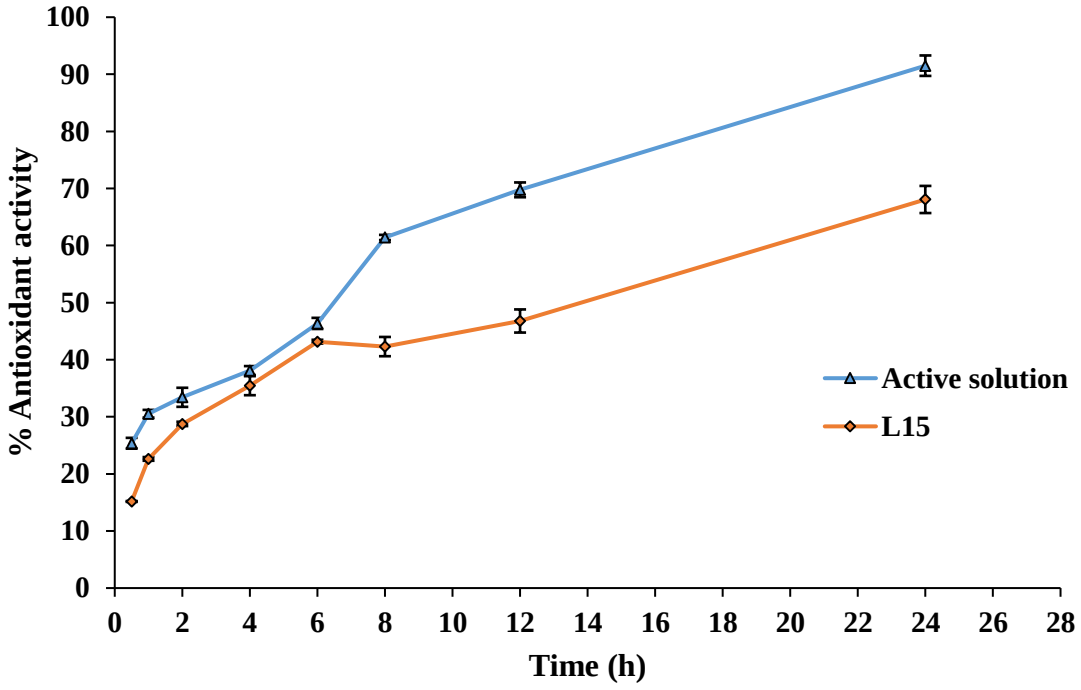

Figure 4.14 *In vitro* release studies of LCFS solution and LCFS liposome

4.7 Cosmetic formulation

4.7.1 Cream base formulation

Cream base formulations were prepared and their stability investigated by freeze thaw cycle for 5 cycles, and kept at room temperature for 30 days. The physical appearance, pH and viscosity were evaluated before and after the stability testing. The results were presented in Figure 4.15 and Table 4.7. The physical appearance of all cream base formulations remained unchanged after the stability test. However, a slight change occurred in pH and viscosity of all formulations. Therefore, the cream base formulation with better appearance, suitable pH and high viscosity was selected to develop LCFS liposome cream.

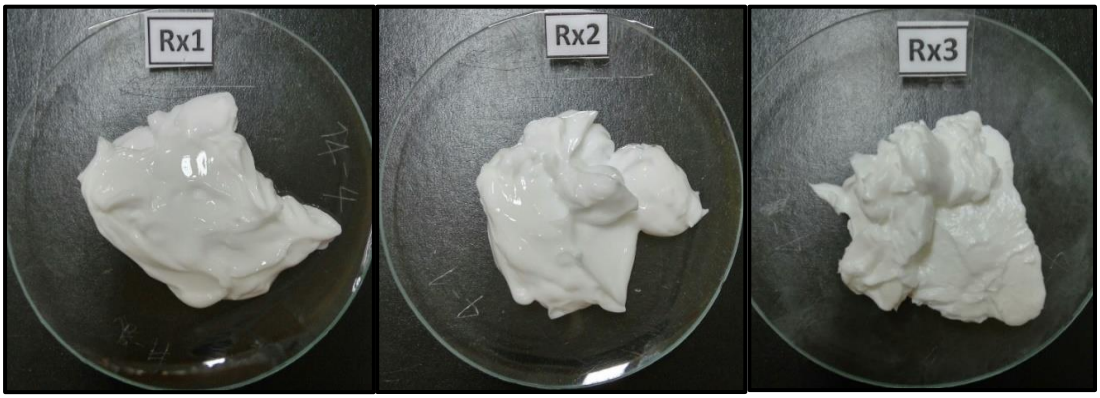

Figure 4.15 Physical appearance of cream base formulations

Table 4.7 Physical properties of cream base before and after stability test

No	Physical appearance	pH			Viscosity (x10^3 cP)		
		Before FT	**After FT**	**After 30days**	**Before FT**	**After FT**	**After 30days**
Rx1	White viscous cream	6.94 ± 0.01	6.50 ± 0.01	6.94 ± 0.01	303.16 ± 0.90	273.00 ± 2.21	271.44 ± 0.00
Rx2	White viscous cream	6.92 ± 0.01	6.74 ± 0.02	6.92 ± 0.01	287.82 ± 3.31	271.44 ± 4.41	268.84 ± 0.90
Rx3	White low viscous cream	8.62 ± 0.01	8.42 ± 0.04	8.62 ± 0.01	277.68 ± 0.00	252.72 ± 4.42	253.76 ± 3.60

Data are mean ± S.D. (n=3).

4.7.2 Formulation of LCFS liposome cream

The LCFS liposome creams were prepared by incorporating 20% and 50% w/w of liposomes with 1% and 2.5% w/w LCFS respectively, into the selected cream base (Rx1). The LCFS creams were also prepared in which they contained the same concentrations as liposome creams. It was noticed that the colour intensity of liposome cream was lighter than the LCFS cream since the LCFS was encapsulated in the lipid vesicles of liposome (Figure 4.16). The pH values of the formulations decreased after incorporating the actives into the cream base since the LCFS of *Lactobacilli* contained lactic acid and other short chain fatty acids. The viscosity of liposome cream was significantly lower than LCFS cream ($P < 0.01$). It was observed that the percentage of liposome was inversely proportional to the viscosity. The reason may be related to the higher water content of liposomes. The results of pH and viscosity were summarized in Table 4.7.

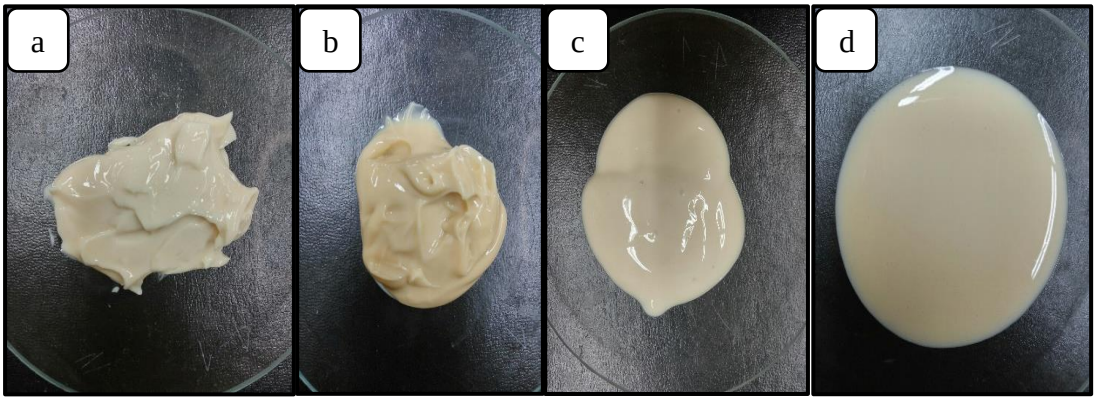

Figure 4.16 Physical appearance of cream formulations containing LCFS mixture of *L. paracasei* SD1 and *L. rhamnosus* SD11 (a) 1% w/w solution (b) 2.5% w/w solution (c) 20% w/w L-15 and (d) 50% w/w L-15

4.7.3 Stability of LCFS liposome cream

The stability of LCFS liposome cream and LCFS cream were determined by their physical appearance, pH, viscosity and antioxidant activity. The results are summarized in Figure 4.17, Table 4.8 and 4.9. The physical appearances of all formulations had not changed both after freeze thaw cycle, and after storing for 90 days at room temperature. As shown in Table 4.8 and Figure 4.18, the pH of all formulations was remained within pH 5 to 5.5, which is similar to the pH of the skin. The viscosity of LCFS liposome cream formulations were more stable than the LCFS cream (Table 4.8 and Figure 4.19).

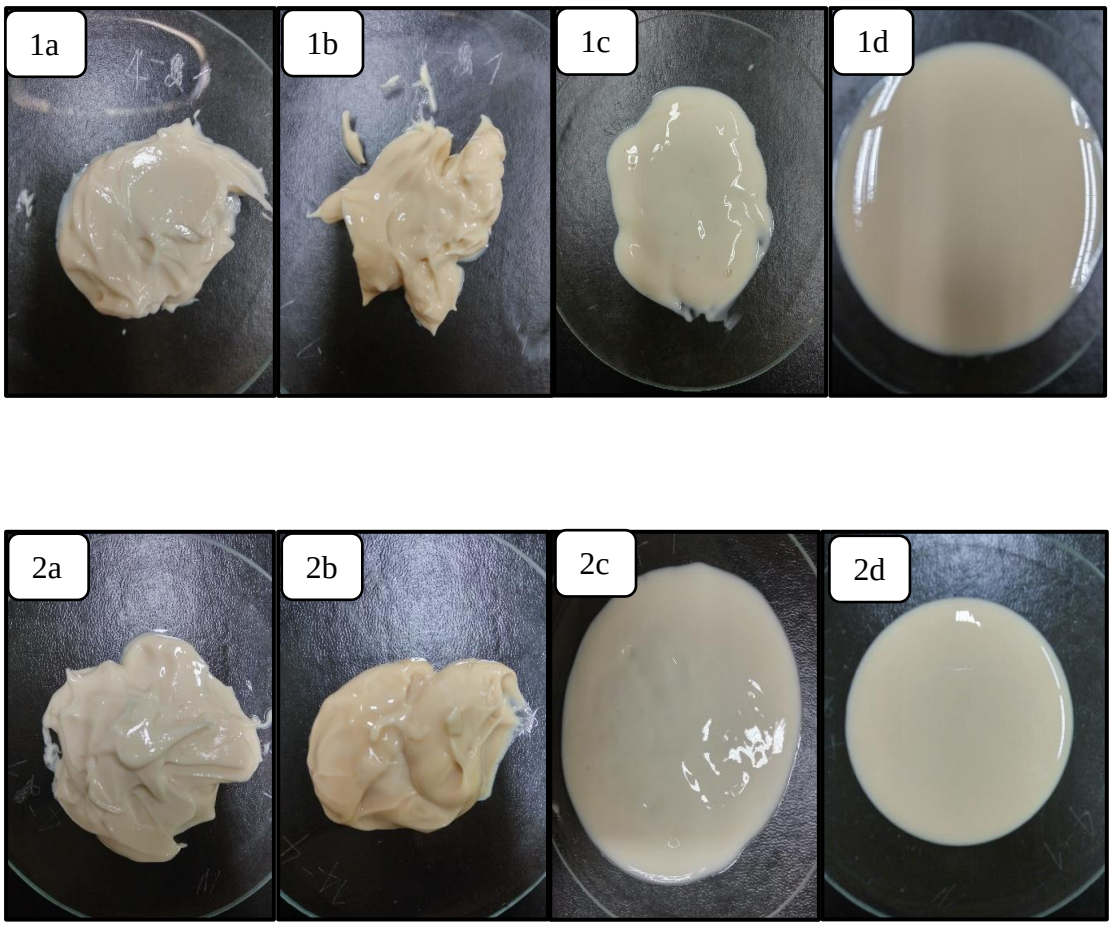

Figure 4.17 Physical appearance of cream formulations containing LCFS mixture of *L. paracasei* SD1 and *L. rhamnosus* SD11 (1a - 1d) after freeze thaw and (2a - 2d) kept 90 days at room temperature where (a) 1% w/w solution (b) 2.5% w/w solution (c) 20% w/w L-15 and (d) 50% w/w L-15

Table 4.8 pH and viscosity of LCFS cream and LCFS liposome cream before and after freeze thaw

Formulation	Physical appearance	pH		Viscosity (x10^3 cP)	
		Before stability	**After FT**	**Before stability**	**After FT**
1% LCFS	Yellowish white cream	5.22 ± 0.02	5.21 ± 0.05	300.56 ± 1.80	228.28 ± 0.90
2.5% LCFS	Yellowish brown cream	5.01 ± 0.04	5.07 ± 0.08	255.84 ± 2.70	179.4 ± 0.00
20% L-15	Yellowish white lotion	5.36 ± 0.02	5.34 ± 0.02	25.22 ± 1.13	24.18 ± 0.68
50% L-15	Yellowish brown lotion	5.26 ± 0.04	5.20 ± 0.02	9.49 ± 0.23	9.88 ± 0.23

Data are means ± S.D. (n=3).

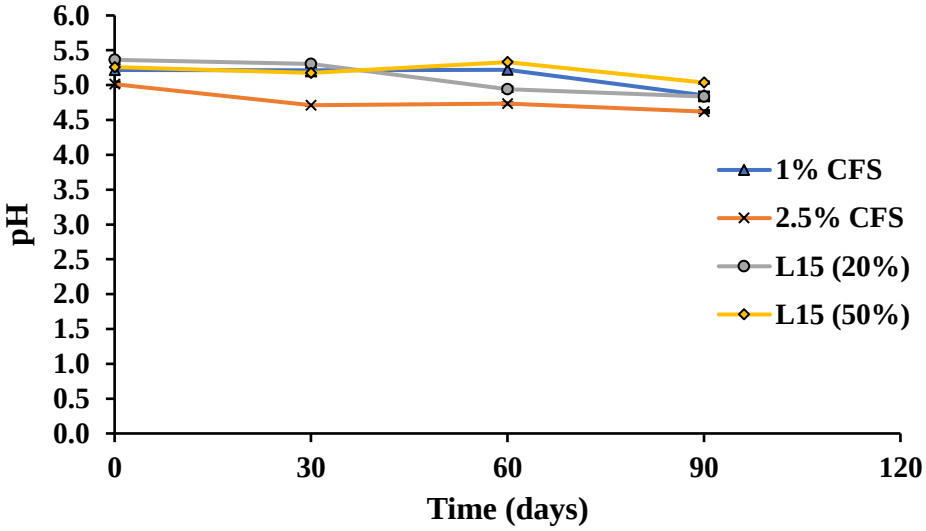

Figure 4.18 Stability profiles in terms of pH of each formulation kept at room temperature for 90 days

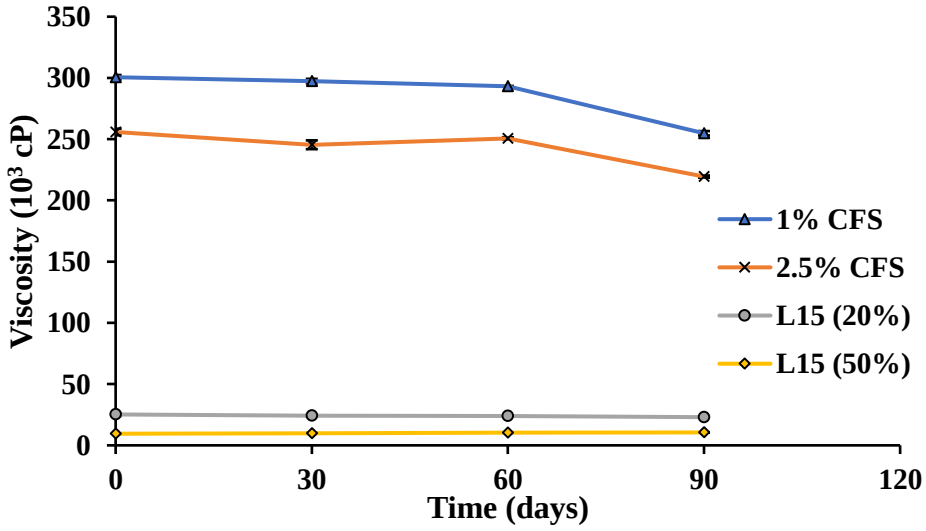

Figure 4.19 Stability profiles in terms of viscosity of each formulation kept at room temperature for 90 days

In terms of antioxidant activity, the activity increased with increase in concentration of actives as shown in Table 4.9. It was clearly observed that the antioxidant activity of LCFS liposome cream was higher than that of LCFS cream which consisted of the same concentrations. After freeze thaw cycles, only minor changes of antioxidant activity were occurred in all formulations except 20% w/w L-15 formulation. It was noticed that approximately half of the antioxidant activity of 20% w/w L-15 formulation was decreased after it was kept for 90 days at room temperature. Nevertheless, the antioxidant activity of all formulations decreased after 90 days storage at room temperature. It may be due to the release of actives from liposome vesicles over long time storage. The 50% w/w liposome cream was selected for further investigations, because of its consistency of appearance, good physical properties and high antioxidant activity after stability testing.

Table 4.9 Antioxidant activity of LCFS cream and LCFS liposome cream before and after stability tests

Formulation	**%antioxidant activity ± SD**		
	Before stability	**After FT**	**After 90 days at RT**
1% active	52.44 ± 1.16	57.23 ± 0.69	41.75 ± 0.64
2.5% active	72.69 ± 1.16	77.25 ± 0.36	73.57 ± 0.65
20% L-15	61.01 ± 0.53	52.04 ± 0.60	39.18 ± 0.84
50% L-15	84.40 ± 1.61	83.25 ± 0.36	75.65 ± 0.57

Data are means ± S.D. (n=3).

4.8 Antimicrobial evaluation of LCFS liposome cream

The antimicrobial evaluation of LCFS liposome cream and LCFS cream (both containing 2.5% w/w LCFS) was carried out and compared with three commercial products: commercial 1 (Tomei Anti-acne cream); Commercial 2 (Laurence acknew cream); and commercial 3 (Vitara antiacne moisturizing cream). The antimicrobial evaluation was carried out by using agar well diffusion assay. The three common skin pathogens, namely, *P. acne*, *S. aureus* and *S. epidermidis* were used in this study. The results are summarized in Table 4.10.

As shown in Table 4.10, non-liposome cream has no inhibitory activity while liposome cream and commercial creams inhibited against *S. aureus* and *S. epidermidis*. It was noted that commercial 1 has the strongest inhibitory against *S. aureus*. The inhibitory effect of commercial products may be related to the presence of preservatives in the formulations. Nevertheless, all formulations possessed low inhibitory potency against *S. aureus* and *S. epidermidis* since they showed narrow inhibition zones. It was expected because the formulation was not intended to be used as a drug, but to be used as a cosmetic product. Although the formulations had low inhibitory potency, the cosmetics are usually applied repeatedly and daily for long term care. Therefore, the actives will cumulate in the skin and prevent the invasion of pathogens to the skin.

In addition to that, all formulations showed no inhibition against *P. acne*. It may be related to the mechanism of agar well diffusion assay. The agar well diffusion assay depends on the diffusion of antimicrobial substances of actives into the agar. On the other side, dysbiosis of skin microflora is one of the causative factors of acne. Furthermore, probiotics use different mechanisms to inhibit the pathogens such as balancing skin pH, restoring homeostasis of skin microflora, promoting the production of ceramides and increasing the skin barrier functions (Cinque et al., 2011; Huang and Tang, 2015; Krutmann, 2012). Many studies have also suggested that probiotics need to be used long term in order to achieve the inhibitory effects against pathogens (Baquerizo Nole et al., 2014; Lew and Liong, 2013).

Table 4.10 Inhibitory activity of different formulations on gram positive pathogens

Pathogens	**Relative inhibitory activity**				
	LCFS cream	**Liposome cream**	**Commercial 1**	**Commercial 2**	**Commercial 3**
P.acne ATCC 66991	-	-	-	-	-
S.aureus ATCC 29213	-	+	+	+	+
S.epidermidis ATCC 12228	-	+	+	+	+

(-) no inhibition; (+) presence of inhibition

4.9 Antioxidant activity LCFS liposome cream after *in vitro* release test

The *in vitro* release of LCFS liposome cream was evaluated and compared with LCFS cream of the same concentration (2.5% w/w LCFS). The *in vitro* release study was evaluated by determining the antioxidant activity of the fluid from receptor chamber at different time intervals. The results are showed in Figure 4.20 and Table 4.11.

The antioxidant activity of LCFS cream increased dramatically until 8 hours and then decreased over 24 hours. The highest antioxidant activity of LCFS cream, which was observed at 8 hours, was lower than 50% which implied that more than 50% of actives remained in the formulation. In contrast, the antioxidant activity of LCFS liposome cream increased steadily over 24 hours. Moreover, the antioxidant activity of liposome cream was significantly higher than non-liposome cream ($P < 0.05$), except between 2 and 4 hours. The results of *in vitro* release study indicated that liposomal carrier system could improve and extend the release of actives over 24 hours.

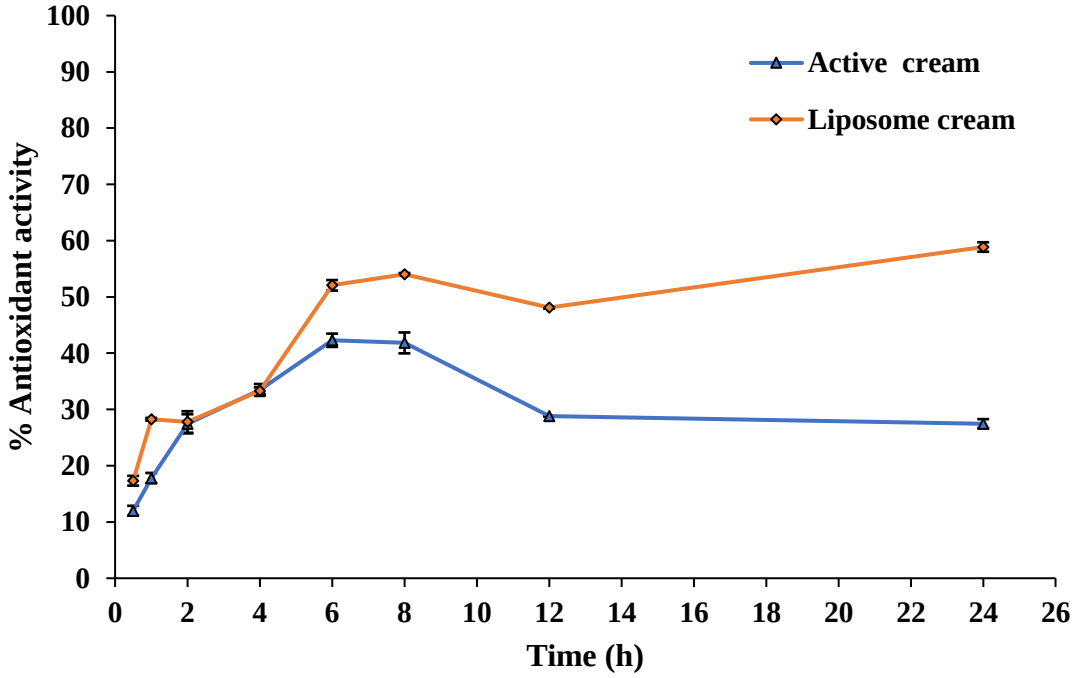

Figure 4.20 *In vitro* release studies of LCFS cream and LCFS liposome cream

Table 4.11 *In vitro* release studies of cream formulations at various time (h)

Formulations	Antioxidant activity at various time (h)							
	0.5	**1**	**2**	**4**	**6**	**8**	**12**	**24**
Active cream	12.00 ± 0.87	17.81 ± 0.91	27.45 ± 1.70	33.47 ± 1.05	42.30 ± 1.18	41.83 ± 1.86	28.82 ± 0.01	27.48 ± 0.80
Liposome cream	17.33 ± 0.87	28.26 ± 0.24	27.77 ± 1.91	33.33 ± 0.59	52.06 ± 0.93	54.00 ± 0.24	48.08 ± 0.17	58.87 ± 0.84

Data are means ± S.D. (n=3).

CHAPTER 5

CONCLUSION

The human oral origin *Lactobacilli*, namely, *L. paracasei* SD1 and *L. rhamnosus* SD11 contain many bio-active compounds which are beneficial to humans. The advantages of these two strains for oral health have been studied recently. In this study, the area of interest was studying the usefulness of LCFS of *L. paracasei* SD1 and *L. rhamnosus* SD11for cosmetic applications. However, there are some limitations in developin a cosmetic product. Therefore, liposomal encapsulation technology was applied to solve the limitations.

The antioxidant activity and antimicrobial activity of LCFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 both individually and in combination were investigated. The results showed that the LCFS of *Lactobacilli* could exhibit not only the antioxidant activity, but also the antimicrobial activity against *P. acne*, *S. aureus* and *S. epidermidis.* In addition to that, these two strains produced synergistic response for antioxidant activity and antimicrobial activity when they were used in combination.

The LCFS mixture loaded liposomes were obtained by modified ethanol injection method. The total fifteen formulations were formulated in this study. Among them, the optimal liposome formulation contained LCFS 5% w/w which was composed of SPC:CHOL:TW80 (4:1:1) in molar ratio with 80 µmol total lipid. Furthermore, the liposomes were able to improve the appearance of LCFS by masking their unpleasant colour and odour. It showed satisfactory stability results in terms of physical appearance, sizes, zeta potential and entrapment efficiency. Moreover, it was found that the liposomal formulations significantly reduced cytotoxicity compared to the free active ($P < 0.05$). The *in vitro* release study showed that although the amount of release of LCFS from the solution was higher than from the liposome, the liposome could extend the release of LCFS over 24 hours.

In the formulation development, LCFS liposome cream containing 20% and 50% w/w liposome and LCFS creams with the same concentrations were formulated. All formulations showed good stability in terms of physical appearance, pH and viscosity. However, the 50% w/w liposome cream showed the best stability with the highest antioxidant activity. Then, the *in vitro* release study was evaluated by comparing liposome cream and non-liposome cream which contained the same concentration of LCFS. It was clearly observed that the liposome cream improves and controls the release of LCFS over a long time. Apart from that, the antimicrobial activity of the liposome cream was also investigated by comparing with three commercial products. Although the liposome cream provided low inhibitory potency, the inhibitory activity was as same as the commercial products.

To conclude this study, the LCFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 could be considered as a good bio-natural sources for dermal health. It also demonstrated the benefits of liposomal delivery system such as masking the unpleasant colour and odour, reducing the cytotoxicity to skin, extending and controlling the release of active compounds. The products of LCFS liposomes are therefore safe to use on human skin, and also looks promising for use as cosmetic and cosmeceutical products.

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