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Liposomes of probiotic's lyophilized cell free supernatant; a potential cosmeceutical product

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The potential uses of lyophilized cell free supernatant (CFS) of human oral derived *Lactobacillus paracasei* SD1 and *Lactobacillus rhamnosus* SD11 as cosmeceutical ingredients were investigated in the present study. Lyophilized CFS of both strains showed the antioxidant activity in concentration dependent manner. They also exhibited antimicrobial activity against *P. acne*, *S. aureus* and *S. epidermidis*. In combination, these two strains produced synergistic responses, not only on antioxidant activity but also on antimicrobial activity. A liposomal delivery system was employed to mask the unpleasant colour and odour of CFS. The optimal liposome formulation was characterized by a particle size of 344 nm, PDI of 0.19, zeta value of -48.05 mV and %EE of 69.45. The cytotoxicity results showed that the lyophilized CFS, which was toxic, became non-toxic after encapsulating into liposomes. Altogether, current findings demonstrate the worthiness of development of liposomes of probiotic's lyophilized CFS for cosmeceutical applications.

1. Introduction

The skin is the largest organ in the human body covering an area ranking from 1.5 to 3 square meters in an adults. The skin's primary function is to protect the body from invasion of foreign materials, to regulate body temperature, to prevent water loss and to store lipid contents (Grice and Segre 2011; Huang and Tang 2015). Skin is also an ecosystem which is inhabited by different species of microorganisms including bacteria, viruses, fungi and mites, and most of them are beneficial to the host (Grice and Segre 2011; Chen and Tsao 2013). The compositions of skin microflora can vary by host factors and environmental factors. The host factors are age, sex, gene, immune system, anatomical site and pathobiology. The environmental factors are UV light, temperature, humidity, 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 which in turn affect the homeostasis of the skin flora (Grice and Segre 2011; Cinque et al. 2011; Huang and Tang 2015).

The skin microflora fulfils an essential role in skin health by protecting against invasion by harmful microorganisms, restoring the equilibrium of skin microbiota and balancing pH of the skin (Chen and Tsao 2013). Most of the skin disorders such as acne, dermatitis, atopic dermatitis, sensitive skin, eczema or psoriasis are the results of unbalanced levels of skin flora. Therefore, homeostasis of skin microbiome is essential for skin health (Grice and Segre 2011; Cinque et al. 2011; Huang and Tang 2015).

Probiotics, which are live micro-organisms, when administered in adequate amount confer health benefit the host (Lee 2009). In the past, most of the studies related to the beneficial effects of probiotics were focused on the gastro intestinal tract (GIT). Beyond GIT, probiotics provide profound advantages to skin such as balancing skin microflora, increasing skin barrier function, reducing skin inflammation and preventing or treating skin diseases associated with altered microflora such as acne, dermatitis, psoriasis. Moreover, lactic acid bacteria (LAB) are capable of protecting the skin from UV radiation, repair skin damages, improve the radiance of the skin's complexion, prevent age related signs of skin and retard skin aging (Cinque et al. 2011).

Although LAB have been widely used in different fields due to their various advantages to the host, the CFS was wasted as a by-product. The CFS of lactobacilli contains many bioactive compounds essential for skin health such as lactic acid, acetic acid, diacetyl, hyaluronic acid, sphingomyelinase, lipoteichoic acid and peptidoglycan. The CFS, cell lysate or metabolites are more stable than living form, and hence more suitable for development of topical application (Lew et al. 2013). Several studies reported the antioxidant property of probiotics both *in vitro* and *in vivo* (Nyanzi et al. 2015; Xing et al. 2015). Furthermore, the supernatant of LAB consists of antimicrobial substances such as bacteriocins, bacteriocin-like substance, organic acids and hydrogen peroxides (Wannun et al. 2016).

Probiotics can be obtained from various sources such as fermented food, milk, as well as from human GI, faeces, vagina, and oral. Even today, there are limited studies of human oral origin LAB compared to the other sources. *L. paracasei* SD1 and *L. rhamnosus* SD11, found in human oral cavity, are facultative anaerobic, gram positive, rod-shaped bacteria, which are belonging to the *Lactobacillus* genus. One of the important factors to consider while selecting the probiotic strain is the production of bacteriocin. Previous studies reported in detail concerning purification and characterization of bacteriocins, which were found in supernatants of *L. paracasei* SD1 and *L. rhamnosus* SD11 (Teanpaisan et al. 2015; Wannun et al. 2016). The probiotic potential and safe use of *L. paracasei* SD1 and *L. rhamnosus* SD11 for both short term and long term, as well as both *in vitro* and *in vivo* have already been investigated (Wannun et al. 2014; Teanpaisan and Piwat 2014; Teanpaisan et al. 2015; Wannun et al. 2016; Rungsri et al. 2017).

In this study, the potential uses of CFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 for cosmeceutical applications will be investigated. While using CFS as cosmeceutical ingredient, some drawbacks have to be considered such as short shelf life, unpleasant odour and colour that is consequence of bacteria culture media. The CFS was therefore lyophilized to preserve and to maintain long shelf life. Liposomal encapsulation technology was used to mask or improve odour and appearance of lyophilized CFS.

Liposomes were first introduced by Bangham, who was a British haematologist, in 1961. Liposomes are synthetic vesicles in which an inner aqueous core is entirely enclosed by lipid bilayers membranes. Liposomes can encapsulate either hydrophilic actives in an inner aqueous core, or lipophilic actives in the lipid bilayers membrane. Since the phospholipid bilayers membranes of liposomes are similar to the biological membranes, they are identical to the human skin, which helps them to penetrate the skin easily. Liposomes have been extensively used for topical applications in cosmetic and pharmaceutical fields as they are non-toxic, biocompatible and biodegradable. Moreover, liposomes can enhance penetration of drugs, control release of the active ingredients, localize the active ingredients at the targeted site and reduce undesirable systemic absorption (Nounou et al. 2008; Akbarzadeh et al. 2013; Aparajita and Ravikumar 2014).

Therefore, we examined the antioxidant activity and the antimicrobial activity of lyophilized CFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 and their synergistic effects, to develop liposomes containing lyophilized CFS, and to evaluate their physicochemical properties.

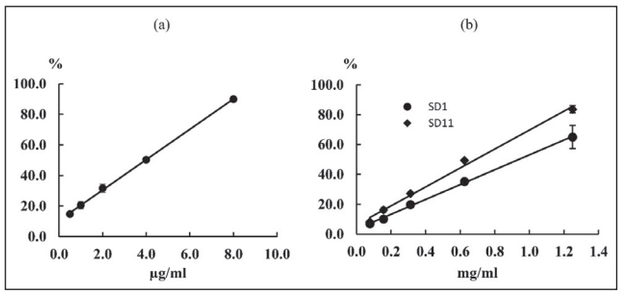

Fig. 1: Linear regression analysis of concentrations against % DPPH radical scavenging activity. (a) Ascorbic acid (y = 9.958x + 10.594, r^2 = 0.9995) (b) *L. paracasei* SD1 (y = 49.756x + 3.2882, r^2 = 0.9988) and *L. rhamnosus* SD11(y = 63.49x + 6.1803, r^2 = 0.993). All values presented in each graph are mean±S.D. (*n*=3).

Table 1: Different concentrations of lyophilized CFS of *Lactobacilli* and their experimental scavenging capacity, theoretical scavenging capacity and synergistic effect

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

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

2. Investigations, results and discussion

2.1. DPPH radical scavenging activity assay

The linear relationship curve for standard ascorbic acid (Fig. 1a) was plotted with a good correlation coefficient (r^2 = 0.9995). The DPPH quenching capacity of lyophilized CFS of both *L. paracasei* SD1 and *L. rhamnosus* SD11 increased with increasing concentration as shown in Fig. 1b. In DPPH scavenging assay: a low EC_{50} value indicates potent antioxidant activity. The EC_{50} of lyophilized CFS of *L. paracasei* SD1 was 0.94±0.1 mg/ml and *L. rhamnosus* SD11 was 0.69±0.02 mg/mL; ascorbic acid was 3.97±0.16 µg/ml. Based on EC_{50} values, both *Lactobacillus* strains possessed significantly lower antioxidant capacity than ascorbic acid. When comparing two strains, the antioxidant activity of *L. rhamnosus* SD11 was higher than that of *L. paracasei* SD1. The previous studies also reported that the antioxidant activities of *Lactobacillus* strains were dependent on concentration and strain specific (Tsai, et al. 2013; Chooruk et al. 2017).

Basically, the radical scavenging activity is directly proportional to the concentration of antioxidants. However, that basic theory did not correlate to high synergistic response (Table 1, sample 11). It has been reported that, not all combinations were capable to produce a synergistic effect and in fact could result in an antagonistic effect (Peyrat-Maillard et al. 2003). While samples 7, 8, 9 and 10 showed synergistic effects, sample 7 produced the highest synergistic activity ($P < 0.01$). It was suggested that the combined concentration plays an important role in obtaining a synergistic effect (Liu et al. 2008).

2.2. Lactic acid analysis

The amount of lactic acid in lyophilized CFS of *L. paracasei* SD1 was 221.85±1.85 mg/g and that of *L. rhamnosus* SD11 was 288.86±0.63 mg/g. Lactic acid can diffuse easily in non-dissociated form through the bacterial cell membrane, lowering the pH and disturbing cellular enzymatic activities of the bacteria.

2.3. Antimicrobial activity

Inhibition zones, MIC, MBC, MIC in combination and FICI values of lyophilized CFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 against skin pathogens are summarized in Table 2. The results of agar well diffusion assay and broth microdilution assay indicated that lyophilized CFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 possessed similar inhibitory efficacy on those skin pathogens. This might be because they belong to the same *Lactobacillus* species, or were possibly isolated from the same origin. Previous studies reported that in CFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 contain paracasei SD1 and fermencin SD11 respectively, which are broad spectrum antimicrobial bacteriocins, active against gram positive and negative bacteria, as well as yeast (Wannun et al. 2014; Teanpaisan and Piwat 2014; Teanpaisan et al. 2015).

In addition, the combination of the two strains showed synergistic effects (Table 2). It is known that the CFS of *Lactobacillus* contains many bioactive compounds which contribute to antimicrobial activity such as bacteriocin, proteins, organic acids, hydrogen peroxides and other cell wall fragments. Therefore, the synergistic effect seems to be the outcome of summation of the activities of these antimicrobial compounds.

2.4. Characterization of CFS loaded liposomes

After incorporating the lyophilized CFS into liposomes, the brown colour and unpleasant cultural media odour of CFS had faded since it was encapsulated inside the liposomal vesicles (Fig. 2). The physicochemical properties of formulations are summarized in Table 3. It was observed that the particle sizes were decreased when total lipid contents were increased from 20 to 80 µmol. Moreover, smaller particle sizes were obtained in the formulations containing surfactant although they contained the same total lipid contents. It was noticed that the concentration of surfactant was inversely proportional to the particle sizes. In addition, higher surfactant concentration leads to consistency of size distribution, reduction of interfacial tensions

Table 2: Antimicrobial activity of lyophilized CFS 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

Table 3: Compositions, physicochemical properties and entrapment efficacy lyophilized probiotic CFS loaded liposomes

Code	Compositions	Ratio	Total lipid (µmol/ml)	Particle size (nm)	PDI	Zeta potential (mV)	%EE	Appearance
L-1	SPC:CHOL	4:1[a]	20	ND	ND	ND	ND	ppt
L-2			40	ND	ND	ND	ND	ppt
L-3			60	629.7 ± 9.4	0.35 ± 0.01	-38.73 ± 0.12	24.90 ± 2.26	ppt after one week
L-4			80	442.6 ± 16.4	0.21 ± 0.01	-45.81 ± 3.30	26.52 ± 1.94	ppt after one week
L-5	SPC:TW80	84:16[b]	20	ND	ND	ND	ND	ppt
L-6			40	475.9 ± 4.2	0.31 ± 0.01	-37.95 ±3.12	14.32 ± 1.71	ppt after one week
L-7			60	367.1 ± 22.2	0.12 ± 0.08	-44.83 ± 0.99	35.61 ± 1.46	Pale yellow
L-8			80	344.8 ± 3.4	0.19 ± 0.06	-45.26 ± 2.38	43.37 ± 2.19	Pale yellow
L-9	SPC:CHOL:TW80	4:1:1[a]	20	ND	ND	ND	ND	ppt
L-10			40	405.9 ± 13.3	0.17 ± 0.08	-34.46 ± 2.99	33.40 ± 1.20	ppt after one week
L-11			60	362.1 ± 6.7	0.18 ± 0.01	-47.80 ± 2.33	36.14 ± 0.33	Pale straw colour
L-12			80	349.3 ± 9.2	0.03 ± 0.02	-47.60 ± 1.89	46.71 ± 7.02	Pale straw colour
L-13	SPC:CHOL:TW80	4:1:1[a]	40	ND	ND	ND	ND	ppt
L-14			60	333.6 ± 2.5	0.12 ± 0.02	-46.94 ± 3.58	46.28 ± 0.96	Straw colour
L-15			80	344.1 ± 1.1	0.19 ± 0.03	-48.05 ± 1.53	69.45 ± 2.34	Straw colour

[a] molar ratio, [b] weigh ratio
L-1 to L-12 containing 2% w/v actives and L-13 to L-15 containing 5% w/v actives. Data are mean ± S.D.(*n*=3). ND means not determined. ppt means precipitate.

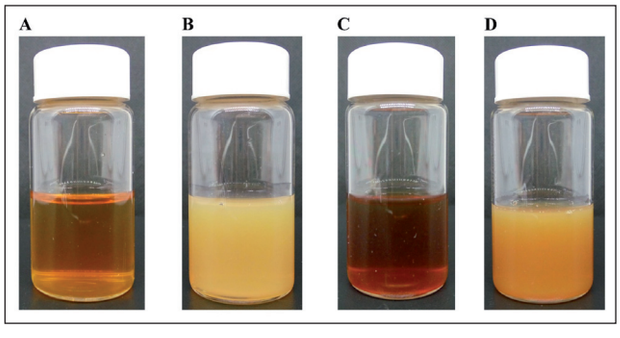

Fig. 2: Physical appearance of solutions and liposomes containing lyophilized CFS mixture of *L. paracasei* SD1 and *L. rhamnosus* SD11 (A) 2 % w/v solution (B) 2 % w/v liposome (C) 5 % w/v solution (D) 5 % w/v liposome.

thereby improving the stability of liposomes (Bnyan et al. 2018). Zeta potential values of the formulations were negatively charged and were generally between -30 to -50 mV. The net negative charge of liposomes might be due to the orientation of the phospholipid carboxyl head group. High values of negative charges residing on the surface of the vesicles inhibit the agglomeration of liposomes by creating repulsive forces between vesicles (Bnyan et al. 2018). The entrapment efficiency (EE) was influenced by the compositions of liposome formulations, total lipid contents and the percentage of active incorporated into liposome. It is commonly reported that inclusion of cholesterol into liposome systems increases the rigidity of membrane and hence prevents leakage of drugs, stabilizes the liposome and increases %EE. Conversely, L-3 and L-4 which were composed of soybean phosphatidyl choline (SPC) and cholesterol (CHOL) showed low %EE and precipitated after one week. The reason was that CHOL reduces the internal aqueous volume of liposomes while %EE of hydrophilic drugs depend on the internal aqueous volume and the concentration of drugs inside the aqueous core (Eloy et al. 2014). It was observed that the surfactant concentration was directly proportional to %EE (Table 3). However, liposomes with high surfactant concentration (L-8) could not load high concentrations of active (5% w/v) (data not shown). This might be due to an unstable vesicular system since unsaturated double bonds interaction of surfactant on the lipid bilayers can cause pores within the membranes. It can be overcome by the addition of CHOL into the vesicle system. CHOL can fill the pores formed by the interaction of surfactant and *vice versa*, surfactant can reduce the rigidity of cholesterol and provide flexibility to the membrane (Glavas-Dodov et al. 2005; Bnyan et al. 2018). Regardless of compositions, %EE increased as the total lipid contents increased. Moreover, the formulations with the high total lipid content were capable of successfully encapsulating 5 % w/v active since the high lipid concentration created large internal aqueous volume for the encapsulation of the active. The optimal formulations, L-12 and L-15, were selected based on the criteria of particle sizes ≤ 500 nm, PDI ≤ 0.3, high zeta potential values and relatively high %EE.

2.5. Cytotoxicity of lyophilized CFS loaded liposomes on HaCaT

The results of cytotoxicity test are illustrated in Fig. 3. The % cell viability of 5% w/v free active was 47 % which means it can exert moderate cytotoxicity potential according to ISO 10993-5. This might be due to acidic pH since *Lactobacilli* produce short chain fatty acids during fermentation process. However, after incorporating actives into liposomes, the cytotoxicity was significantly decreased with % cell viability of 76 % for L-15 and 82 % for L-12. It was noted that the % cell viability was not significantly different between actives loaded liposomes and blank liposome. Therefore, it was clearly observed that the liposomes remarkably reduced cytotoxicity of the actives.

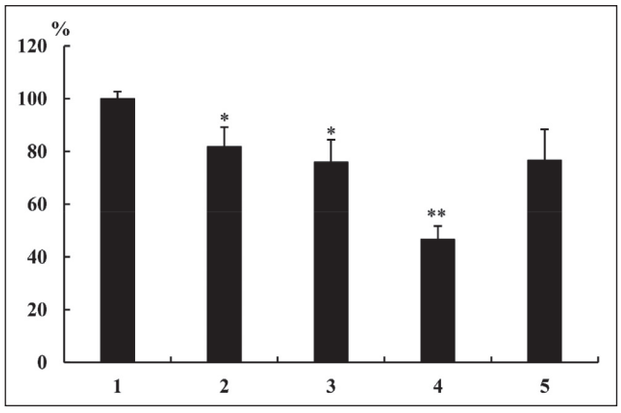

Fig. 3: Percentage of cell viability (1) Control (2) L-12 (3) L-15 (4) 5% w/v active solution (5) Blank liposome. Data are mean±S.D. (n=3). *P < 0.05 and ** P < 0.01 compared to the control.

2.6. Potential of liposomes of probiotic's lyophilized CFS as cosmeceutical product

Lyophilized CFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 have antioxidant activity, and antimicrobial activity against skin pathogens. Moreover, they produced synergistic responses when used in combination. The optimal liposome formulation was composed of SPC:CHOL:TW80 of 4:1:1 in molar ratio with 80 µmol total lipid. The obtained liposomes were able to improve the appearance and odour of lyophilized CFS. In addition, the liposomal formulations significantly reduced cytotoxicity compared to free active. Therefore, current findings revealed the potential benefits of using CFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 in cosmeceutical applications. Advantages of utilization of liposomes as a carrier system are also highlighted.

3. Experimental

3.1. Materials

The reagents and chemicals used in this study were 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Germany), ascorbic acid (Sigma-Aldrich, China), absolute ethanol and H_3PO_4 (85%) (RCI Labscan, Thailand), l-α-phosphatidylcholine from soybean (SPC) (Sigma-Aldrich, USA), Cholesterol from lanolin (CHOL) (Sigma-Aldrich, Singapore), Tween 80 (TW80) (Sigma-Aldrich, Switzerland), Lactic acid (L 6661) (Sigma-Aldrich, USA) and Triton X 100 (Loba chemie, India).

3.2. Probiotic strains

Human oral origin *Lactobacilli*, namely, *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.

3.3. Preparation and lyophilization of CFS

The strains were cultured on de Man Rogosa Sharpe agar (Difco™, USA) at 37 °C for 24 h under anaerobic conditions (80 % N_2, 10 % H_2, and 10 % CO_2). A single colony of each strain was cultured separately in MRS broth and incubated anaerobically for 24 h. The cultures were centrifuged at 8,000 x g for 10 min 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 h at -110 °C. The lyophilized samples were kept at -20 °C until further use.

3.4. Determination of antioxidant activity of each strain

Five concentrations of each strain, i.e., 0.08, 0.16, 0.42, 0.63 and 1.25 mg/ml, were prepared by dissolving lyophilized CFS in deionized water. Each of these sample solutions (100 µl) were mixed separately with 100 µl of freshly prepared DPPH solution (6 x 10^{-5} M in ethanol) in 96 wells-plate. The reaction mixtures were shaken and incubated for 30 min at room temperature protected from light. Ascorbic acid was used as standard positive control. The reduction of DPPH by the antioxidant was measured at 517 nm by using SPECTROstar^{Nano} microplate reader (BMG LABTECH Gmbh, Germany). The percentage of scavenging activity was calculated as followed:

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

where A is the absorbance at 517 nm. The antioxidant activities of both strains were reported in terms of EC_{50} which means the concentration of antioxidants required to scavenge 50% of DPPH radical.

3.5. Synergistic effect of combined strains

The synergistic effect of combined strains was determined by following the previous method with slight modification (Liu et al. 2008). Briefly, 100 µl of each combination (50 µl of *L. paracasei* SD1 and 50 µl *L. rhamnosus* SD11) was mixed with 100 µl of freshly prepared DPPH solution in various concentrations (Table 1). The experimental procedures were the same as described in the previous determination of antioxidant activity of each strain.

Synergistic effect (SE) of antioxidant capacity 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 SE was greater than 1.

The experimental scavenging capacity of combined strains was 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) was calculated by:

$$\%TSC = 100-[(100-ESC1) \times (100-ESC2)/100] \quad (4)$$

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

3.6. Analysis of lactic acid

Lactic acid was analysed using HPLC Agilent 1100 series which was equipped with quaternary pump, various wavelength detector (VWD), auto sampler and 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 injection. Lactic acid (L6661) was used as reference standard.

3.7. 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 h.

3.8. Antibacterial assay

3.8.1. Agar well diffusion assay

One millilitre of each tested pathogen (10^8 CFU/ml) was mixed with 20 ml of melted brain heart infusion agar (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 CFS was added into each well. The negative control was distilled water. The plate was then incubated anaerobically at 37°C for 24 h. The antibacterial activity was evaluated by measuring the inhibition zone in millimetres.

3.8.2. Broth microdilution assay

The broth microdilution assay was carried out according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). First, two-fold serial dilutions of CFS with BHI broth (Difco™, USA) were made in 96 wells-plate to obtain the final concentrations ranging from 0.04 to 10 mg/ml. Next, 100 µl of each pathogen (10^8 CFU/ml) was added into each well and incubated at 37 °C under appropriate conditions. The CFS without pathogens was used as sterility control, 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 CFS that completely inhibited the growth of pathogen. 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.

3.8.3. Antibacterial activity of combination

The antibacterial activity after the interactions between *L. paracasei* SD1 and *L. rhamnosus* SD11 were determined by checkerboard method. Briefly, 50 µl of *L. paracasei* SD1 was mixed with an equal volume 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 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 A in combination/MIC of A alone) + (MIC of B in combination/MIC of B alone), (5)

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

3.9. Preparation of liposomes

The lyophilized CFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 were mixed at a ratio of 1:1 for liposome formulation. The lyophilized CFS loaded liposomes were prepared by modified ethanol injection method (Maitani et al. 2001). Briefly, the oil phase which contained SPC and/or CHOL were dissolved in absolute ethanol and sonicated at 50 °C for 30 min until homogeneous. The water phase consisted of 2 % or 5 % w/v of lyophilized CFS mixture and/or TW 80. The temperature of both phases was maintained at 50 °C before mixing. Then, the two phases were mixed in a round bottom flask bottle and the absolute ethanol was removed by Eyela N-1000 series rotary evaporator (Tokyo Rikakikai Co., Ltd., Japan) under reduced pressure at 50±5 °C.

3.10. Characterization of liposomes

Physical appearances of liposomes were visually observed including colloidal appearance, phase separation and precipitation. Physicochemical properties such as size, polydispersity index (PDI) and zeta potential were determined using a ZetaPLAS Zeta Potential Analyzer (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, liposome samples were diluted with milli-Q water.

Entrapment efficiency of liposomes was investigated by the ultracentrifugation method. Free actives from liposomes were separated by ultracentrifugation at 40,000 rpm (SW60 Ti Rotor) for 2 h at 4 °C using Optima™ L-100XP ultracentrifuge (Beckman, USA). The supernatant containing free actives was collected and diluted with absolute ethanol. The total amounts of actives were 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 the previous determination of antioxidant activity of each strain. The drug entrapment efficacy was calculated by the following equation:

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

where T is antioxidant activity of total actives, and F is antioxidant activity of free actives. Since the CFS of LAB contained various bioactive compounds, the DPPH assay was an indirect method to measure the entrapment efficacy of lyophilized CFS loaded liposome.

3.11. 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 assay. The cells ($1x10^5$ cells/well) were seeded in a 96 wells-plate and incubated at 37 °C, 5 % CO_2 for 24 h. The cells were then treated with or without tested liposome formulations for 48 h. The cells were fixed with 40 % w/v trichloroacetic acid and incubated again at 4 °C for 1 h. 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 % active 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.

3.12. Statistical analysis

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

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