

# HUMAN ORAL LACTOBACILLI CELL FREE SUPERNATANT FOR COSMETIC CREAM

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## Abstract

The *Lactobacillus paracasei* SD1 and *Lactobacillus rhamnosus* SD11 are commensal microbes found in the oral cavity of caries-free human. They are widely used in oral health care, by incorporating them into fermented milk or fruit juice, to prevent dental caries and to promote the homeostasis of oral microbiota. However, the cell free supernatant (CFS) of *Lactobacilli*, which consists of various beneficial substances for dermal health, is wasted as a by-product. Therefore, it was interesting to investigate the activities and the usefulness of CFS of the *L. paracasei* SD1 and *L. rhamnosus* SD11 for cosmetic applications. Liposomal technology was used to improve the appearance and skin permeation of CFS. The optimal CFS liposome cream containing 2.5% w/w CFS showed good stability and high antioxidant activity. Moreover, the antimicrobial activity of the CFS liposome cream was the same as commercial products. In addition to that, the result of *in vitro* release study demonstrated that the CFS liposome cream could maintain and control the release of active compounds over 24 h. Thus, this study highlights the advantages of the CFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 for dermal applications and not wasting them as by-products. The study also suggested that the CFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 could be good bio-natural sources for cosmetic ingredients, and the CFS liposome cream could be promising for use as a cosmetic product.

**Keywords:** Antimicrobial, antioxidant, cell free supernatant, cosmetic, lactobacilli, liposome cream

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## Introduction

Oxidative stress is one of the causative factors of various chronic disorders such as cancer, obesity, and aging. Oxidative stress is a result of an imbalance between production and elimination of reactive oxygen species or free radicals. Both endogenous and exogenous factors stimulate the production of free radicals. Free radicals cause oxidative damages to DNA molecules, proteins and lipids. Skin is also affected by those oxidative damages leading to skin aging. The common signs of skin aging are increased wrinkles, decreased firmness and reduced elasticity of skin (Mishra *et al.*, 2015, Wang *et al.*, 2017). Antioxidants are substances which acts as a defence against free radicals and terminate the chain reaction before it causes damage to the vital molecules. However, synthetic antioxidants have safety concerns which is why it is more interesting to study natural and antioxidants from bio-resources (Wang *et al.*, 2017).

The skin is a dwelling place for over 100 distinct species of commensal microorganisms (Huang and Tang, 2015). The skin microflora maintains the acidic pH of the skin and prevent the colonization of harmful pathogens in the skin (Ansari, 2014). Most skin disorders such as acne and dermatitis are the result of an imbalance of skin flora (Cinque *et al.*, 2011). For example, hormonal changes in puberty increase the production of lipids, which leads to the proliferation of lipophilic microorganisms such as *Propionibacterium acne* (Grice and Segre, 2011). Therefore, homeostasis of skin microbiome is essential for skin health (Grice and Segre, 2011; Huang and Tang, 2015).

Probiotics, also known as beneficial microorganisms, are found in skin, oral cavity, nares, gastro-intestinal tract and vagina of human body. Recent studies reported that probiotics confer valuable benefits to skin such as antiaging, restoring skin microbiota, decreasing skin inflammation and preventing or treating acne and dermatitis. In topical applications, probiotics can be used in living form as well as in inactivated forms. Previous studies have reported that the cell free supernatant (CFS) of *Lactobacilli* contains

various bio-active compounds which are beneficial for dermal applications (Lew and Liong, 2013; Lew *et al.*, 2013). Furthermore, supernatant of lactic acid bacteria consists of antimicrobial substances such as bacteriocins, bacteriocin-like substance, organic acids and hydrogen peroxides (Wannan *et al.*, 2013). However, the CFS of *Lactobacilli* is usually removed as waste in many pharmaceutical and food industries.

The *Lactobacillus paracasei* SD1 and *Lactobacillus rhamnosus* SD11 are found in carries-free subjects. The previous study proved the cosmeceutical potential of lyophilized CFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 such as activity studies, synergistic response studies and cytotoxicity studies. Moreover, encapsulation of lyophilized CFS mixture of *L. paracasei* SD1 and *L. rhamnosus* SD11 by using liposomal technology have already been reported (Htwe *et al.*, 2019). 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.

To this day, a cosmetic formulation of probiotic is yet to be fully developed. Therefore, this study aims to develop cosmetic formulations by using lyophilized CFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 liposomes and investigate their physical properties, antioxidant activities, antimicrobial activities and *in vitro* release.

## Materials and Methods

### Materials and Reagents

The materials and reagents for present study were: absolute ethanol (Labscan Asia Co., Ltd, Bangkok, Thailand), ascorbic acid (Sigma-Aldrich, China), cetearyl hexanoate or Lanol 1688, and polyacrylamide and C13-14 Isoparaffin and Laureth-7 or sepigel 305 (ADINOP Co., Ltd., Bangkok, Thailand), disodium hydrogen orthophosphate anhydrous and sodium

dihydrogen orthophosphate (Univar<sup>®</sup>, New South Wales, Australia), glycerine, phenoxyethanol, and propylene glycol (P.C. Drug Center Co., Ltd, Thailand), mineral oil light (Namsiang International Co., Ltd, Bangkok, Thailand), 1,1-Diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich, Germany), sodium chloride (Carlo Erba, Milan, Italy), Spectra/Pro<sup>®</sup> 3 Dialysis membrane, MWCO 3500 Dalton (Spectro Laboratories, Inc., CA, USA), and tocopherol acetate (Namsiang International Co., Ltd, Bangkok, Thailand).

The liposomes of lyophilized CFS (LCFS) of *L. paracasei* SD1 and *L. rhamnosus* SD11 were obtained from the previous study (Liposome composition: 1  $\alpha$ -phosphatidylcholine from soybean, cholesterol from lanolin and tween 80) (Htwe *et al.*, 2019).

### Preparation of Cosmetic Formulations containing CFS Liposomes

The cream base was prepared by cold process emulsification method. The ingredients of the cream base are presented in Table 1. Briefly, the oil phase and water phase were weighted in separate beakers, and then the water phase was gradually added into the oil phase with constant stirring until congealed.

**Table 1. Ingredients of cream base**

| Phase | Ingredients        | %w/w |
|-------|--------------------|------|
| Oil   | Lanol 1688         | 12.0 |
|       | Mineral oil        | 5.0  |
| Water | Glycerin           | 8.0  |
|       | Propylene glycol   | 2.0  |
|       | Sepigel 305        | 10.0 |
|       | Tocopherol acetate | 2.0  |
|       | Phenoxyethanol     | 0.5  |
|       | Water              | 70.5 |

The liposome containing 5% w/w of LCFS mixture of *L. paracasei* SD1 and *L. rhamnosus* SD11 were prepared according to Htwe *et al.* (2019). This LCFS concentration was selected based on EC<sub>50</sub> values of antioxidant activity and MIC values of antimicrobial activity (at least 100×EC<sub>50</sub> values and 10 MIC values), because it could cover all bioactivities and dilution processes. CFS liposome was prepared by the modified

ethanol injection method (Htwe *et al.*, 2019) and this method is briefly explained here. The water phase consisted of 5% w/v of LCFS mixture, tween 80 and water. The oil phase contained lipid and cholesterol in absolute ethanol. The oil phase was homogenised by sonicating at 50°C for 30 min. Then, the 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. The active was incorporated into the cream base either in the form of LCFS liposome (20% and 50% w/w), or original LCFS powder form (1% and 2.5% w/w) in order to obtain the final active concentrations of 1% and 2.5% w/w. The above mentioned creams are referred to as liposome and non-liposome cream respectively. The LCFS liposomes were added into the cream base with constant stirring. The mixture was stirred until homogenous.

### Characterization of Cosmetic Formulations

The physical appearance of the formulations was visually observed including colour, texture and phase separation. The pH of formulations was measured with pH meter (Mettler-Toledo, Switzerland). The viscosity of each formulation was measured with Brookfield viscometer (LVT Brookfield dial reading, 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. All the measurements were done in triplicate.

### Stability Study

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

### Antioxidant Evaluation of Cosmetic Formulations

Cosmetic formulation (1 g) was mixed with absolute ethanol (10 ml). The mixture was sonicated for 45 min and centrifuged again for

15 min. The supernatant was then evaluated for antioxidant activity using DPPH assay. Briefly, a sample solution (100  $\mu$ l) was mixed with an equal volume of DPPH solution ( $6 \times 10^{-5}$  M in absolute ethanol) in a 96 wells-plate. The mixture was shaken and incubated for 30 min at room temperature with light protection. The absorbance of the reaction mixture was measured at 517 nm by using SPECTRO starNano microplate reader (BMG LABTECH GmbH, Germany). The percentage of antioxidant activity was calculated as followed:

$$\text{Antioxidant activity (\%)} = \frac{1 - (A_{\text{sample}}/A_{\text{control}})}{1} \times 100 \quad (1)$$

where A is the absorbance at 517 nm.

### Antibacterial Activity Evaluation of Cosmetic Formulations

The following gram-positive bacteria were used in this study: *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, and *Propionibacterium acne* ATCC 6691. The indicator strains were cultured on blood agar (Difco™, USA) with 5% v/v blood, and incubated for 24 h at 37°C under anaerobic conditions (80% N<sub>2</sub>, 10% H<sub>2</sub>, and 10% CO<sub>2</sub>). The antibacterial activity of the formulations was screened by agar well diffusion method. One millilitre of the broth culture of each pathogen, equivalent to 10<sup>8</sup> CFU/ml, was mixed with 20 ml of warmed BHI (Difco™, USA). The suspension mixture was poured into a petri dish with 6 mm diameter metal cups. After solidifying of the agar, the cosmetic formulations were loaded into the well to the brimful volume capacity by using a syringe. Blank cream and blank liposome were used as blank, distilled water was used as negative control and the three commercial cosmetic products (TOMEI antiacne cream, acnew cream @LAURENCE and VITARA antiacne moisturizing cream) were used as positive control. The plate was then incubated at 37°C under appropriate conditions. The antibacterial activity was evaluated by observing the presence of inhibition zone. Each experiment was done in triplicate.

### In Vitro Release Study

The *in vitro* release of the lyophilized CFS from the formulation was studied using modified Franz-diffusion cell (Hanson 57-6 M, USA). The cellulose acetate membrane was boiled two times with distilled water to remove coating wax. 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 and stirred with a magnetic stirrer at 300 rpm. The circulating water bath was set at 37±1°C. The hydrated cellulose acetate membrane was placed between donor and receptor compartments. After equilibrium for 30 min, 1 g of each formulation was put on to the donor compartment with an effective diffusion area of 1.77 cm<sup>2</sup>. At 0.5, 1, 2, 4, 6, 8, 12, and 24 h, the receptor fluid was withdrawn and immediately replaced with an equal volume of the pre-thermoregulated (37°C) fresh receptor fluid. The release of actives from the formulations were evaluated by determining the antioxidant activity of the withdrawn samples at various time intervals using DPPH assay as mentioned above.

### Statistical Analysis

Results are expressed as mean±standard deviation (SD). The data are analysed with one-way analysis of variance (ANOVA).  $P < 0.05$  was considered statistically significant.

## Results and Discussion

### Characterization of Cosmetic Formulations

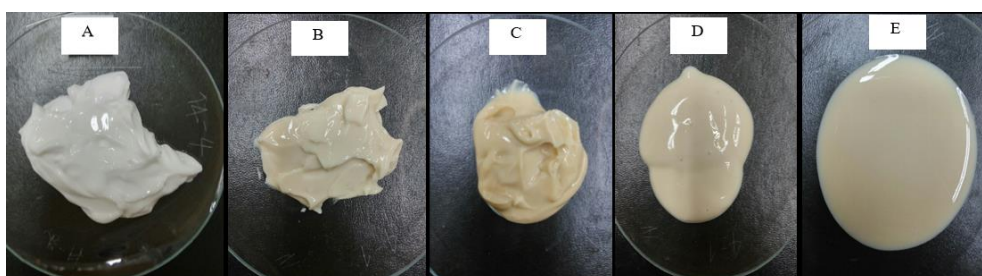
The physical appearance, pH and viscosity of all formulations are presented in Table 2. After incorporating the actives into white colour cream base, it changed into light brown colour. However, it was noticed that the colour intensity of liposome cream was lighter than non-liposome cream since the LCFS was encapsulated in the lipid vesicles of liposome (Figure 1). The pH of the formulations decreased after incorporating the actives into the cream

**Table 2. Physical properties of formulations before and after freeze thaw cycles**

| Formulations                  | Physical appearance | pH              |                 | Viscosity ( $\times 10^3$ cps) |                   |
|-------------------------------|---------------------|-----------------|-----------------|--------------------------------|-------------------|
|                               |                     | Before FT       | After FT        | Before FT                      | After FT          |
| Cream base                    | White viscous cream | 6.94 $\pm$ 0.01 | 6.50 $\pm$ 0.01 | 303.16 $\pm$ 0.90              | 273.00 $\pm$ 2.21 |
| Non-liposome cream (1% CFS)   | Light brown cream   | 5.22 $\pm$ 0.02 | 5.21 $\pm$ 0.05 | 300.56 $\pm$ 1.80              | 228.28 $\pm$ 0.90 |
| 20% Liposome cream            | Yellowish lotion    | 5.36 $\pm$ 0.02 | 5.34 $\pm$ 0.02 | 25.22 $\pm$ 1.53               | 24.18 $\pm$ 0.68  |
| Non-liposome cream (2.5% CFS) | Light brown cream   | 5.01 $\pm$ 0.04 | 5.07 $\pm$ 0.08 | 255.84 $\pm$ 2.70              | 179.40 $\pm$ 0.00 |
| 50% Liposome cream            | Yellowish lotion    | 5.26 $\pm$ 0.04 | 5.20 $\pm$ 0.02 | 9.49 $\pm$ 0.23                | 9.88 $\pm$ 0.23   |

**Table 3. Antioxidant activity of formulations before and after freeze thaw cycles**

| Formulations                       | Antioxidant activity (%) |                  |
|------------------------------------|--------------------------|------------------|
|                                    | Before FT                | After FT         |
| Non-liposome cream (1% w/w LCFS)   | 52.44 $\pm$ 1.16         | 57.2 $\pm$ 0.69  |
| 20% w/w Liposome cream             | 61.01 $\pm$ 0.53         | 52.04 $\pm$ 0.60 |
| Non-liposome cream (2.5% w/w LCFS) | 72.69 $\pm$ 1.16         | 77.25 $\pm$ 0.36 |
| 50% w/w Liposome cream             | 84.40 $\pm$ 1.61         | 83.25 $\pm$ 0.36 |

**Figure 1. Physical appearance of cream formulations: (A) is the cream base. B, C, D & E are creams containing LCFS mixture of *L. paracasei* SD1 and *L. rhamnosus* SD11. (B) with 1% w/w solution, (C) with 2.5% w/w solution, (D) with 20% w/w Liposomes and (E) with 50% w/w Liposomes**

base since the LCFS of Lactobacilli contained lactic acid and other short chain fatty acids. The viscosity of liposome cream was significantly lower than non-liposome 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.

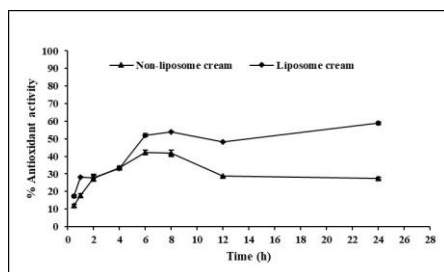
#### Stability of Cosmetic Formulations

The stability of liposome cream and non-liposome cream were determined by their

physical appearance, pH, viscosity and antioxidant activity. The results are summarized in Tables 2 and 3. The physical appearance of all formulations remained unchanged after freeze thaw cycle, however, slight changes occurred in pH and viscosity. Nevertheless, as shown in Table 2, the pH of all formulations remained within pH 5 to 5.5, which is similar to the pH of the skin. Moreover, the viscosity of LCFS liposome creams were more stable than the non-liposome cream.

**Table 4. Inhibitory activity of formulations on skin pathogens**

| Pathogens                       | Inhibitory activity |              |              |              |
|---------------------------------|---------------------|--------------|--------------|--------------|
|                                 | Liposome cream      | Commercial 1 | Commercial 2 | Commercial 3 |
| <i>S.aureus</i> ATCC 29213      | +                   | +            | +            | +            |
| <i>S.epidermidis</i> ATCC 12228 | +                   | +            | +            | +            |
| <i>P. acne</i> ATCC 66991       | -                   | -            | -            | -            |



**Figure 2.** *In vitro* release profile of CFS, plotted by the percentage of antioxidant activity of the fluid from receptor chamber at different time intervals of liposome cream (♦) and non-liposome cream (▲)

### Antioxidant Activity of Cosmetic Formulations

As shown in Table 3, the antioxidant activity increased with increase in concentration of actives. It was clearly observed that the antioxidant activity of liposome cream was higher than that of non-liposome cream. After 5 freeze thaw cycles, only minor changes of antioxidant activity occurred in all formulations, except 20% w/w liposome cream, which may be due to the release of actives from liposome vesicles under stressful conditions. Thus, 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. The selected formulation had high antioxidant activity of 84%, and ascorbic acid, which was used as a positive control showed 98% of antioxidant activity.

### Antimicrobial Activity of Cosmetic Formulations

The liposome cream and commercial creams inhibited *S. aureus* and *S. epidermidis* (Table 4). However, they showed narrow

inhibition zones which implied low inhibitory potency. Although the formulations had low inhibitory potency, the cosmetics are usually applied repeatedly and daily for long term, as a result, the actives will cumulate in the skin and prevent the invasion of pathogens. 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, Krutmann, 2012; Huang and Tang, 2015). Many studies have also suggested that probiotics need to be used long term in order to achieve the inhibitory effects against pathogens (Lew and Liong, 2012, Baquerizo, 2014).

### Antioxidant Activity of Cosmetic Formulations after *In Vitro* Release Test

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 illustrated in Figure 2. The antioxidant activity of non-liposome cream increased dramatically until 8 h, and then decreased over 24 h. The highest antioxidant activity of non-liposome cream, which was observed at 8 h, was lower than 50% which implied that more than 50% of actives remained in the formulation. In contrast, the antioxidant activity of liposome cream increased steadily over 24 h. Moreover, the antioxidant activity of liposome cream was significantly higher than non-liposome cream ( $P < 0.05$ ), except

between 2 and 4 h. The results of *in vitro* release study indicated that liposomal carrier system could improve and extend the release of actives over 24 h.

### Comparison of Liposome Formulations and Liposome Cream Formulations

When Liposome formulations from previous study (Htwe *et al.*, 2019) was compared to the liposome cream formulation, they were different in terms of physical properties and bioactivities. Liposome formulation had significantly higher antioxidant activity and antimicrobial activity compared to solution of active or cream with the same concentration. Including liposomes can reduce toxicity caused by the active on skin fibroblast cells. However, the disadvantage of using liposome formulation for industrial scale preparation is high price and short stability. Incorporation of liposomes in other base formulations such as cream gel or lotion is one of techniques to utilize the advantages of liposomes with lower price and longer stability of product.

### Conclusions

Concluding this study, it was observed that liposomal delivery system was able to improve the aesthetic of the CFS liposome cream by encapsulating the CFS into lipid vesicles. All cosmetic formulations showed good stability in terms of physical appearance, pH and viscosity. Among them, the liposome cream containing 2.5% w/w CFS showed the best stability with the highest antioxidant activity. 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. In the *in vitro* release study, it was clearly observed that the liposome cream improves and extends the release of lyophilized CFS over a long time. Therefore, the liposome cream containing lyophilized CFS of *L. paracasei* SD1 and *L. rhamnosus* SD11 looks promising and valuable for use as cosmetic products.

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