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Development of Bacterial Cocktail of *Staphylococcus hominis*, *Staphylococcus warneri*, *Bacillus subtilis*, and *Micrococcus luteus* strains as Active Ingredients for Skincare Formula

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ABSTRACT

The use of microbial skin health care products that balance one's skin microbiome mimicking healthy skin is rapidly increasing. Postbiotics have more advantages in terms of safety, shelf life, active peptides, and other active compounds, such as GABA, which benefit human skin. In our previous study, we isolated skin commensal bacteria, i.e., *Staphylococcus hominis* MBF12-19J, *Staphylococcus warneri* MBF02-19J, *Micrococcus luteus* MBF05-19J, and *Bacillus subtilis* MBF10-19J. This study aimed to produce a bacterial cocktail (BC) formula of those strains, elevate the BC formula into a ferment lysate (FL), and characterize the activities and test for skin sensitivity. The antibacterial activity was carried out by conducting a competition test using *Propionibacterium acnes* as an indicator bacterium, whereas the antiradical scavenging activity assay was performed using the DPPH method. Skin sensitivity was tested using the patch test method. The bacterial cocktail was lysed by optimizing the enzymatic and ultrasonication methods, whereas sucrose was added as lyoprotectant to obtain a stable powder FL. Potential activity to inhibit the growth of *P. acnes* was achieved using a formula of FL consisting of *M. luteus* MBF05-19J, *B. subtilis* MBF10-19J, *S. warneri* MBF02-19J, and *S. hominis* MBF12-19J at a ratio of 1.5:1.5:0.5:0.5 or equivalent to DNA copy number/mL 1.01E + 16:1.14E + 24:1.96E + 22:1.50E + 18. The skin sensitivity test results showed no sensitivity reaction, guaranteeing that CFS and FL are safe to use for the skin. Sucrose-FL at 12% sucrose formula exhibited higher physical stability than those without. However, the result of potential antiradical scavenging showed mild and very mild activities compared with the use of standard ascorbic acid.

Keywords: Bacterial Cocktail, Skin Commensal, Antibacterial, Antiradical Scavenging Activity, Skin Sensitivity Test

INTRODUCTION

Skin is an ecosystem consisting of 1.8 m^2 of unique and diverse habitats that support a variety of microorganisms (Grice & Segre, 2011). Many of these microorganisms are harmless and, in some cases, have vital functions for humans. Symbiotic microorganisms occupy various skin niches and protect the skin against invasion by more pathogenic or dangerous organisms (Grice & Segre, 2011). The symbiotic relationship between the skin microbiota and the host is an aspect used as a basis for skin microbial exploration as a potential source for the development of active pharmaceutical ingredients (API) for cosmetics or skin health (Beri, 2018).

Several probiotic bacteria originating from the facial skin of young Indonesian adults that are beneficial to human skin were isolated, as reported (Khayyira *et al*., 2020). Among them was *Staphylococcus hominis* MBF12-19J, a commensal bacteria that can protect the skin from the colonization of pathogenic bacteria by producing antibacterial molecules, namely, hominicin (Pauer *et al*., 2019). *Staphylococcus warneri* MBF02-19J was also isolated. This bacterium was reported to produce the bacteriocin nukacin ISK-1 and warnericin RK, the first antimicrobial peptides with activity against *Legionella* (Azevedo *et al*., 2015). The next bacterium isolated was *Bacillus subtilis* MBF10-19J. This bacterium can produce bacitracin on the surface of the skin, a toxin that helps fight pathogens, as reported (Oruko *et al*., 2019). The last bacterium reported was *Micrococcus luteus* MBF05-19J. This bacterium was reported to produce DNA-repair enzymes used to prevent *photo neoantigens* in the skin due to UV rays (Hofer *et al*., 2011).

In recent decades, microbial therapeutics, including postbiotics, have been widely studied and developed as a new class of API (Jimenez *et al*., 2019). Postbiotics have more advantages: they are safe because they use bacterial postbiotic results, making them easier to control (Aguilar-Toalá *et al*., 2018); they have less side effects because they use Generally Recognized as Safe (GRAS) bacteria isolated from the skin microbiome (Choi *et al*., 2019); and they produce active compounds such as GABA because they are lactic acid bacteria, microorganisms capable of producing GABA (Tanamool *et al*., 2020). These GRAS bacteria can be developed to produce a microbial cocktail postbiotic preparation, which is a mixture of two or more bacteria that provides benefits for facial skin health (Kato *et al*., 2008). This preparation is more efficient than single bacteria because interactions between bacteria can affect biotherapeutic potential such as antioxidant, antibacterial and antiinflamatory (Vázquez-Castellanos *et al*., 2019) and expand the antibacterial activity of the preparation as it consists various bacterial postbiotics.

The advantages of microbial cocktail prompted the idea to develop API by exploring microbial strains isolated from Indonesian skin commensal bacteria in the form of a microbial cocktail as a skin health care product. To evaluate potential source for the development of API for cosmetics or skin health care, it is essential to conduct early pharmacological screening study of the microbial cocktail postbiotic on antibacterial activity toward acne vulgaris-causing bacteria (*Propionibacterium acnes*) as well as antiradical scavenging activity.

MATERIAL AND METHODS

The bacterial strains used in this study were *S. hominis* MBF12-19J, *S. warneri* MBF02-19J, *M. luteus* MBF05-19J, and *B. subtilis* MBF10-19J, a collection of cultures from the Laboratory of Microbiology and Pharmaceutical Biotechnology, Faculty of Pharmacy, University of Indonesia. *Propionibacterium acnes* was obtained from the Clinical Microbiology Laboratory, Faculty of Medicine, University of Indonesia. The chemicals and medium used in this study were lysozyme (Sigma-Aldrich, Singapore), tryptic soy broth (Difco, France), phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, Singapore), bacto agar (Difco, France), nutrient agar (Merck, Germany), blood agar (Microbiology FK University of Indonesia), glycerol (Merck, Germany), Tris-HCl buffer, DPPH (Merck, Germany), sucrose (Wako, Japan), aquadest (Indonesia), aquabidest (Indonesia), 0.22 µm filter membrane (Sartorius, Germany), and waterproof patch (Indonesia). The equipment and instrument used in this study were analytical balance (OHAUS & Acculab); autoclave (Tomy); incubator (Memmert); refrigerator (Sansio and Toshiba); laminar air flow cabinet (Esco, Faster Bio48); freeze dryer (Buchi); fume hood (ChemFast); ultralow temperature freezer at 80°C (U101 Innova); freezer at 4°C (GEA); refrigerator at −20°C (GEA); disposable petri dishes (Biologix); microtips 10, 200, and 1000 µl (Biologix); microcentrifuge (Eppendorf); centrifuge (Gyrozen); vortex mixer (Health, Digisystem); inoculating loop; glassware (Pyrex); microplate reader (Shimadzu); 96-well microplate (Biologix); spectrophotometer (GeneQuant); centrifuge tube (SDL); test tube (Iwaki Pyrex); measuring flasks (Iwaki Pyrex); round flasks (Iwaki Pyrex); Erlenmeyer flask (Schott Duran); volumetric pipettes of various sizes (Iwaki Pyrex); micropipettes of various sizes (Gilson); incubator at 37°C (Merck); orbital incubator shaker; ultrasonicator (LABSONIC); moisture balance analyzer (Mettler Toledo); particle-size analyzer (Malvern); and 96 well-plate.

Identification of bacteria

All bacteria were grown on specific media, such as *B. subtilis* on nutrient agar; *S. warneri* and *S. huminis* on tryptic soy agar (TSA) ; and *P. acne* and

M. luteus on blood agar. The cultures were incubated at 37°C for 24-48 h. Different colonies growing on agar were purified. The colonies were identified for colony morphology visual observations (shape and color) and microscopic observations with Gram staining according to the the American Society for Microbiology protocol.

Preparation of commensal strains

The commensal probiotic strains used in this study originated from the facial skin of young Indonesian adults. The bacteria were verified via real-time polymerase chain reaction and checked using BLAST provided by the NCBI. Subsequently, the commensal probiotic strains were streaked on a specific medium, i.e., *S. hominis* MBF12-19J and *S. warneri* MBF02-19J were grown on TSA, *B. subtilis* MBF10-19J was grown on nutrient agar, and *M. luteus* MBF05-19J was grown on blood agar. All were incubated at 37°C aerobically for 24–48 h. The results were observed for a single pure colony that continued to propagate in a subculture medium.

Preparation of bacterial cocktail and lysate fractions

The bacterial strains were cultured into tryptic soy broth medium in glass tubes for aerobic enrichment for 24 hours at 37℃. They were then subcultured in a 100-mL broth medium to reach the concentration equal to standard *Mc Farland* 0.5, with measured OD_{600} absorbance 0.1±0.05, equivalent to cell density $1.5x10^8$ CFU/mL. After incubation, the cultures were arranged in compositions (Table IV supplemental materials) and mixed thoroughly using a magnetic stirrer for 4 h at 37°C (Ardiansyah, 2020). Then the bacterial cocktail (BC) was separated via centrifugation.

Each cocktail fraction was subjected to cell disruption using combined enzymatic and ultrasonicator methods. Cocktail cell pellets were resuspended in Tris-HCl buffer solution (pH 8.0) and enzyme (lysozyme). The lysed mixture was incubated on ice for 30 min, and then PMSF was added prior to the ultrasonication process. Ultrasonication was conducted at a cycle duration ratio of 0.5 s, 40 cycles, amplitude of 75%, and duration of 75 s with a pause of 15 s/cycle. Lysate was obtained via centrifugation at 10,000 rpm for 20 min. Moreover, the lysate was placed in a vial at −20°C (Musnandi *et al*., 2021). Then, the lyoprotectant (sucrose) was added to each cleared lysate fractions and CFS, followed by freeze-drying.

Competition test using cross streak method

A bacterial cocktail composition was arranged as presented in supplemental materials Table 4. The bacterial cocktail K1 and K2 composition were calculated according to cell viability measurement, as reported (Baikuni *et al*., 2022). In addition, composition modifications of K3 and K4 were arranged according to the competition test assay of each commensal bacteria against *P. acnes* using the cross-streak method. All four bacterial cocktails were cultured in blood agar medium according to the arrangement with a single stroke using a sterile cotton swab at the center of the petri dish and then incubated for 48 hours. *P. acnes* culture suspension was then grown with a streak perpendicular to the initial bacterial streak. The culture on a solid medium was then incubated for 4 days anaerobically at 37°C, and then the zone formed was observed visually and measured using a caliper (Powthong & Suntornthiticharoen, 2017).

Antiradical Scavenging Activity using the DPPH method

Using the DPPH method, the antiradical scavenging activity of the lysate fractions of the bacterial cocktail was tested using a 96-well microplate. Each lysate fraction were resuspended in pure water to obtain a concentration of 20,000 ppm. Solutions with this concentration were produced in a series of dilutions with concentrations of 10,000, 12,000, 14,000, 16,000, and 18,000 ppm. Every 100 µL of serial dilution solution was added 0.5-mM DPPH 100 µL and made three times of replication. The mixture was homogenized and incubated for 30 min in a dark room at 37°C. Absorbance measurements were performed using a microplate reader at an optimized wavelength of 517 nm (Wahid *et al*., 2017). The antiradical scavenging activity was measured by calculating the IC_{50} value. IC_{50} is a quantitative measure that indicates the amount of substance or the effective concentration for the substance needed to inhibit DPPH radical activity by 50% (Sri Palupi & Widyanto, 2020).

Skin sensitivity test

A patch test was conducted to test the sensitivity of the skin. It is carried out *in vivo* by attaching chemicals (allergens) to sensitive skin in certain forms and concentrations in an occlusive manner. This test is an *in vivo* visualization of the elicitation phase of a delayed-type hypersensitivity reaction (type IV) (Bruno, 2019).

Table I. Characterization of bacterial colony

Name of bacteria	Colony shape	Colony color	gram
S. warneri	Circular	White	+
S. hominis	Circular	White	+
B. subtilis	Rod-like	Brown	+
M. luteus	Circular	Yellow	+
P. acnes	Rod-like	White	+

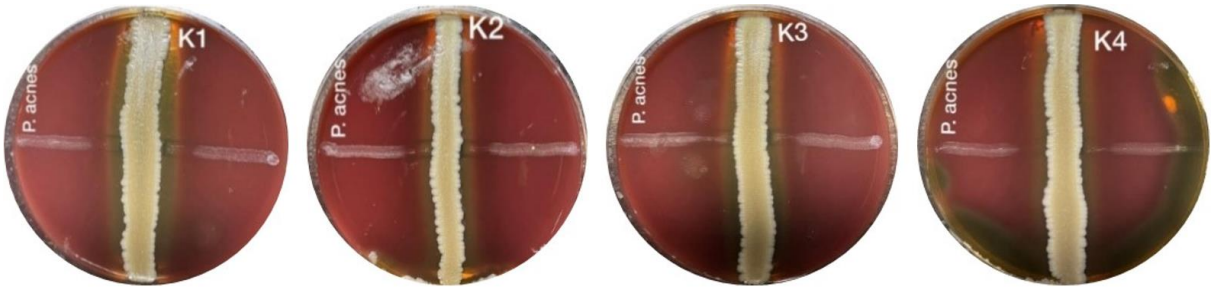

Figure 1. Bacterial cocktail culture fractions showing inhibition zone against *Propionibacterium acnes.* Cocktail K4 shows the strongest growth inhibition at 6.25 mm, followed by the K3 composition with an average inhibition zone of 5.25 mm. The results prove that when bacteria are formulated into cocktails, they can suppress the growth of the pathogenic microorganism *P. acnes*.

This method is employed to identify allergens, the cause of contact dermatitis (DKA). DKA occurs as a result of exposure to haptenic or antigenic external materials with a chemical structure similar to that of a previously sensitized person's skin. The subjects that met the inclusion and exclusion criteria under ethical clearance no. KET-331/UN2.F1/ETIK/PPM.00.02/2021 were treated by applying limited samples of only lysate and CFS fractions, which have been diluted with pure water on the skin of the upper arm and covered with a waterproof patch. The attachment was left to stand for 48 h. After 48 h, the patch was removed, and the skin changes and abnormalities were observed. The reactions were assessed according to the International Contact Dermatitis Research Group (ICDRG).

Stability test

A stability test was conducted for all fractions at room temperature (25°C ± 2°C). The stability parameters observed were visual observation, moisture content, and particle-size distribution. Evaluations were carried out for 1 month and again after 1 month. Visual observations included form, color, and odor all of fractions. The next parameter was moisture content; the water content was determined using a moisture analyzer, samples were taken (±1 g) and spread on an aluminum plate, and the temperature was set to 105°C. The value listed on the instrument was recorded (Srifiana *et al*., 2014). The last parameter was particle size. The particle-size distribution was performed using a particle-size analyzer. The sample was dispersed in aquadest. The results of the measurements indicated the range of particle sizes based on the volume and surface area of the particles (Srifiana, 2014).

RESULTS AND DISCUSSION

Identification of bacteria

Each bacterial culture in its specific media obtained pure colonies with different characteristics (Table I).

The competition test showed inhibition zone against *P. acnes*

The composition of K4 showed the largest average inhibition zone against *P. acnes*, which was 6.25 mm, followed by the composition of K3 with an average inhibition zone of 5.25 mm (Figure 1). The compositions of K3 and K4 produced the largest inhibition zones because both contained a higher composition of *B. subtilis* MBF10-19J than K1 and K2. These results are consistent with those of the competition test on each skin commensal bacteria against *P. acnes* which showed that there was competition for *B. subtilis* MBF10-19J against the growth of *P. acnes* (Baikuni *et al*., 2022).

Table II. Antiradical scavenging activity of bacterial cocktails using the DPPH method

Sample	IC50 (µg/mL)	% Inhibition
Bacterial cocktails without lyoprotectant	31.287	23.15%
Bacterial cocktails + 8% lyoprotectant	30.626	18.17%
Bacterial cocktails + 10% lyoprotectant	45.214	25.47%
Bacterial cocktails + 12% lyoprotectant	48.046	17.77%
CFS without lyoprotectant	13.141	47.54%
CFS + 8% lyoprotectant	22.875	44.30%
CFS +10% lyoprotectant	10.519	25.19%
CFS + 12% lyoprotectant	12.578	42.41%
Ascorbic acid	15.25	75.6%

Table III. Skin sensitivity test results

Sample	Volunteer	Allergy (%)	Irritation (%)
CFS	30	0	0
Lysate	30	0	0
Control	30	0	0

The combination of the four bacteria in the BC formulation will produce more varied antimicrobial peptides. The results of this study are similar to those of the research conducted by Hamad *et al*. (2017), which proved that the combination of a probiotic filtrate mixture containing the strains of *Bifidobacterium bifidum*, *Lactobacillus plantarum*, and *Lactobacillus acidophilus* exerted a more significant inhibitory effect as antibacterial than when probiotics were not combined, or antibacterial assays were performed alone. The competition generated by the BC postbiotics is probably due to the bacteria producing antimicrobial compounds, such as bacteriocins, enzymes, or organic acids, which can have bacteriostatic or bactericidal properties (Vieco-Saiz *et al*., 2019).

Antiradical scavenging activity using the DPPH method

On the classification of Molyneux (2004) (Table II), the results did not show antiradical scavenging activity using the DPPH method with an IC_{50} value in the range of more than 1000 µg/mL and categorized as absence of antiradical scavenging activity. The explanation is probably due to the preparation of lysate fractions; the lysate fraction should be prepared so that all active components are kept in the lysate ferment form without separation between the filtrate and the debris. The CFS fractions of BC on the other hand, do not contain active cells or peptides that demonstrate antiradical scavenging activity. The BC lysates in this study do not entirely contain a single compound that is bioactive, apparently, although there is still a mixture of various compounds (Khayyira *et al*., 2020).

From the results of the percentage of inhibition, the average value of the percentage of inhibition of CFS was greater than that of lysate at a concentration of 1000 µg/mL. The results of this study were in agreement with those of the research conducted by Shen *et al*. (2011). CFS has a higher percentage of inhibition than cell lysate because the absorbance obtained is high. The higher the absorbance, the lower the free radical scavenging activity of DPPH. In this study, the IC_{50} value was unknown (Shen *et al*., 2011). However, the ability of CFS and BC lysate to scavenge free radicals was still lower than that of standard ascorbic acid, which was 75.91% at a concentration of 30.5 µg/mL.

Skin sensitivity test

The interpretation of the patch test results was performed at 48 h. It was done together with the help of a dermatologist-accompanying doctor using a score from the ICDRG. The results obtained from 30 volunteers were negative; there were no sensitivity reactions, either allergies or irritation (Table III). The skin of the volunteers did not exhibit erythema, infiltration, papules, or vesicles. It was confirmed that all the fractions tested are safe to use topically on human skin.

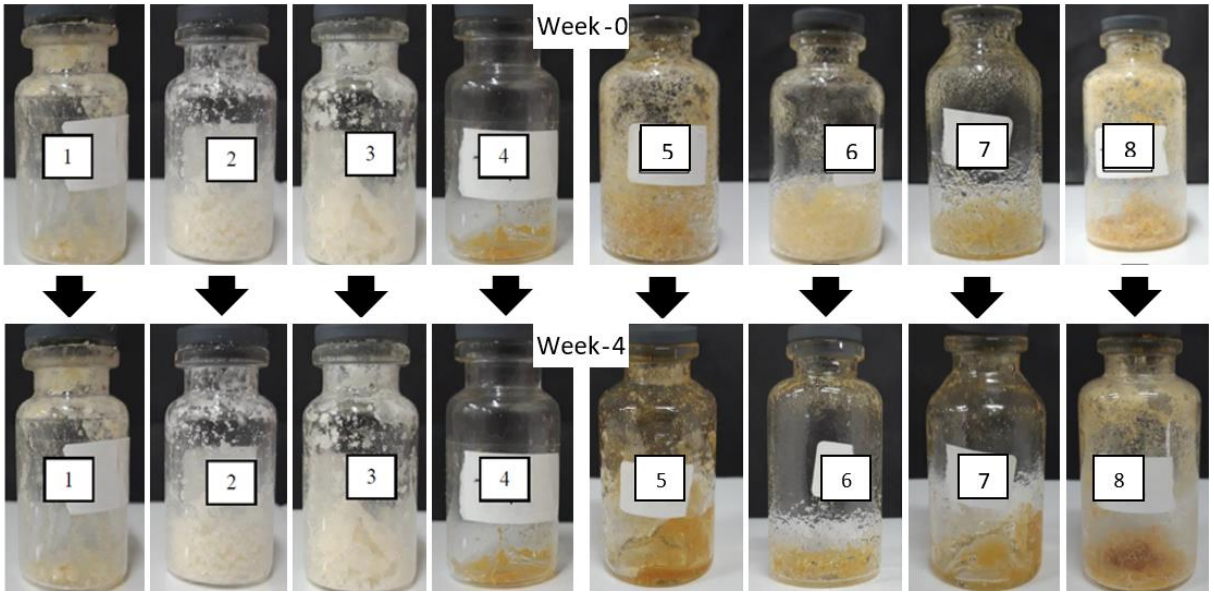

Figure 2. Result after the stability study of bacterial cocktail lysates via visual observation; 1. lysate + 8% lyoprotectant; 2. lysate + 10% lyoprotectant; 3. lysate + 12% lyoprotectant; 4. lysate without lyoprotectant; 5. CFS + 8% lyoprotectant; 6. CFS + 10% lyoprotectant; 7. CFS + 12% lyoprotectant; 8. CFS without lyoprotectant

The sensitivity test in this study was conducted on volunteers with different ages, gender, and ethnic characteristics. This test is carried out to see a type-IV hypersensitivity reaction that may occur due to a substance, the cause of DKA (allergic contact dermatitis), on different individual factors. The skin sensitivity reaction of individuals varies depending on the influencing factors, such as age, gender, ethnicity, skin anatomy, environmental factors, cosmetic products, and sociocultural factors (Duarte *et al*., 2017).

Young people are more likely to have sensitive skin than adults. Epidemiological studies also demonstrated that women's skin is more sensitive than men's about 50%–61% of the population, whereas men only ranging from 30% to 44%. This is because women's skin thickness is lower than men's, and women's hormonal factors can interfere with skin moisture. In addition, ethnic differences in skin structure can be attributed to sensitive skin. One study explained that light-skinned people experience erythema more often than dark-skinned people (Duarte *et al*., 2017). However, in this study, there was no evidence that the differences in the above factors could affect the sensitivity reaction of human skin.

Stability of lysate bacterial cocktail
Visual observation

Based on visual observations on the BC lysate fraction and CFS after freeze-drying, it was found that the lysate fraction with lyoprotectant had a yellowish white color and odorless. On the other hand, the lysate without lyoprotectant had a yellow color and was also odorless. CFS with and without lyoprotectant had a brownish–yellow color and a characteristic peptone odor (Figure 2).

The results of the observation of BC lysates on the 4th week at room temperature (25°C ± 2°C) indicated no change in the form, color, or odor of each formulation. However, in the visual observation of CFS bacterial cocktail at room temperature (25°C ± 2°C) on the 4th week, there was a change in shape and color: CFS added with lyoprotectants had a lumpier shape, and its color changed from yellow brown to light brown; moreover, in CFS without lyoprotectant, there was no change in shape, color, or odor.

Moisture content

The moisture content of CFS and bacterial cocktail lysate was tested using a moisture analyzer. The difference in the value of water content was influenced by the difference in the concentration of lyoprotectant (sucrose) from each formula.

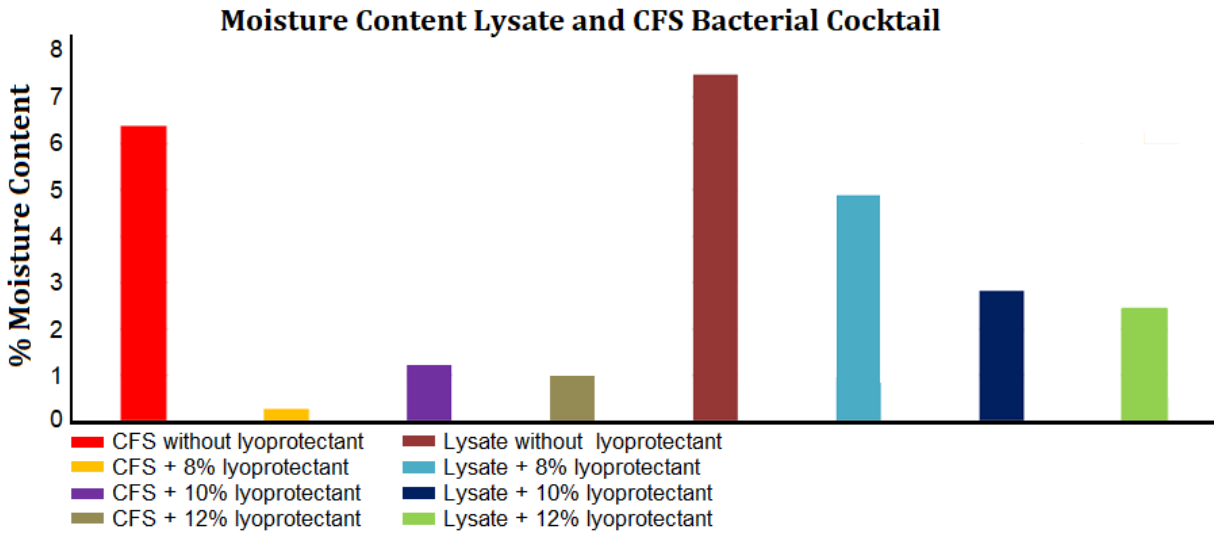

Figure 3. Moisture content bakterial cocktail lysate and CFS

Sucrose has properties that can bind up to 1% water. The ability of sucrose in making the water content values for both CFS and bacterial cocktail lysates with lyoprotectants was lower than those not using lyoprotectant (Figure 3). The high water content in a preparation can cause faster microbial growth (Sulastri *et al*., 2019). The lower the water content, the more effective and stable the process used (Siregar & Kristanti, 2019).

Particle-size distribution

Measurements of the average particle-size distribution of CFS and bacterial cocktail lysates were performed again at week 4 after being stored at room temperature (25°C ± 2°C). The results of the measurement of the average particle-size obtained were 713 nm for CFS without lyoprotectant, 1326 nm for CFS with 8% lyoprotectant, 1387 nm for CFS with 10% lyoprotectant, and 1449 for CFS with 12% lyoprotectant. Meanwhile, the results of the measurement of the average particle size of the bacterial cocktail lysate were 240.2 nm for the lysate without lyoprotectant, 209.7 nm for the lysate with 8% lyoprotectant, 139.6 nm for the lysate with 10% lyoprotectant, and 1069 nm for the lysate with 12% lyoprotectant.

The average particle-size of the BC lysate and CFS decreased at week 4 during storage at room temperature. This is because lyoprotectant (sucrose) can reduce particle size, by preventing molecular diffusion and particle aggregation during the freeze-drying process (Kannan *et al*., 2015). Zeta potential is a measurement of an electric charge on the surface of a particle. The magnitude of the zeta potential can provide information on particle stability (Selvamani, 2018). The zeta potential value of bacterial cocktail lysates with lyoprotectants increased, which was in the range of −26.2 to −41.4 mV. The results of these measurements indicated that the use of sucrose lyoprotectant can prevent particle aggregation and increase stability than when not using it. A zeta potential value that is more positive than +30 mV or more negative than −30 mV is considered good or stable in storage (Clogston & Patri, 2011). A higher zeta potential indicates an increase in electrostatic repulsion and prevents particle aggregation, increasing particle stability. However, if the zeta potential value is less than 5 mV, it can cause aggregation. The zeta potential value is influenced by the nature of the particles and the properties of the solution, such as pH and ionic strength (Gumustas *et al*., 2017).

CONCLUSION

Commensal strains exerted a beneficial effect on human pathogenic skin microbes. A mixture of skin microbiota strains as a cocktail is beneficial to develop as long as they are kept viable and produce active ingredients to the cocktail culture. The future development of bacterial cocktail ferment lysate as a new API has opened an innovation with a promising potential to be developed for skincare formula.

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