The Comparison of Microbial Challenge Test and In-Use Test Method on Preservative Efficacy Testing in Skincare Products

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Abstract

Background: This study aims to confirm the adequate concentration of preservatives in the skincare product resulting from PET by comparing the microbial challenge test and in-use test methods.

Methods: Four types of skincare products consisting of day cream, face mask, gel, and face mist were tested in this study, and they were packaged in tubes and pots while face mist was in spray bottles. Preservation efficacy testing (PET) is carried out to demonstrate the efficacy of the antimicrobial activity and here was used method of PET is the microbial challenge test based on the Ph. Eur. (European Pharmacopoeia). The skincare products were distributed to 90 volunteers to be applied for 8 weeks during the in-use testing program. Each returned skincare product was then microbiologically examined and classified.

Results: The result suggests that passed-B or even failed criteria with lower concentration of preservatives could be considered adequate preservatives since their performance on in-use tests showed that the product could avoid the growth of microbial contaminants. However, if a lower concentration of preservatives were applied, the potential risk of contamination is still available. In addition, 21 types of microbial isolates were obtained from in-use tests consisting of bacteria, molds, and yeast.

Conclusion: The acceptance criteria of Passed B based on Ph. Eur. microbial challenge test could be considered an adequate preservative and it indicates that a lower concentration of

preservatives could be applied to the skincare resulting in a more economically and relatively safe product.

Keywords: Challenge test, Efficacy, In-use test, Preservatives, Skincare

Introduction.

The size of the cosmetic market in the world and Indonesia, which will reach $463.5 billion by 2027, proves that in the current situation of the Covid-19 pandemic, there is still the possibility of growth in the cosmetics industry.[1] There are three fundamental things that drive the growth of the cosmetic industry in Indonesia, consisted increasing public awareness of maintaining skin health, the average age of Indonesian people among Gen-Z and Millennials who have a high awareness of skin care, and social media that has contributed greatly.[2][3] With the increase in the cosmetic industry sector, the control of cosmetic products must always be carried out, including the four products studied in this study encompassed day cream, face mask, gel, and face mist. These skincare product formulations are mostly susceptible to microbial growth due to their high water content and the source of their ingredients.[4]

Preservatives are usually added to avoid the growth of microbial contaminants in skincare products. The type of preservative used in cosmetic products and also used in this study is an aldehyde-formaldehyde compound in the form of DMDM Hydantoin and also phenolic compound in the form of phenoxyethanol and chlorphenesin. This compound has antimicrobial activity that has a fairly broad spectrum of the mechanism of action and meets the characteristics of preservative compounds used in cosmetic products.[5]

In addition to preservative compounds, the process of evaluating the safety of new cosmetic product formulas is generally carried out as a way to determine the stability of the formula that has an impact on product quality or safety. Three steps that are commonly carried out, especially in preservation efficacy testing (PET) used are (1) physical, chemical, and microbiological testing; (2) microbial challenge test; and (3) in-use testing. The steps taken in this study are steps (2) and (3).[5]

In simple terms, the challenge test is carried out by inoculating the test microorganisms into cosmetic products with known formulation and concentration of preservatives to ensure the stability of the formula. In this test, the test microorganisms will be counted at certain time intervals and the results will be matched with the acceptance criteria, in the form of a decrease in

the number of microorganisms that have been set in the reference.[6] The microorganisms used in the challenge test represent species that cause skin infections, product degradation, and spoilage. The microbial challenge test also has criteria and has been regulated both nationally and internationally, namely by using the European Pharmacopoeia (Ph. Eur.) and Indonesian Pharmacopoeia (FI).[7] Ph. Eur. acceptance criteria are defined according to three groups: passed-A (recommended efficacy), passed-B (minimum efficacy), and failed.[8]

Based on the background, this study aims to confirm the adequate concentration of preservatives in the skincare product resulting from PET by comparing the microbial challenge test and the in-use test method. This confirmation is necessary to ensure the concentration of optimum preservatives in the skincare products resulting in a more cost-effective and safer product.[10]

Material and Methods.

Microbial Preparation

Referring to European Pharmacopoeia, the microbes used in this study were *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, *and Aspergillus niger* ATCC 16404. All cultures were activated on TSA (Tryptone Soy Agar) medium for bacteria and SDA (Saboraud Dextrose Agar) for yeast and mold. They were inoculated onto a solid inclined medium according to the type of microbe using the streak method. Bacterial cultures were incubated for 24 hours at 35℃, *C.albicans* for 48 hours at 25℃, and *A.niger* for 7 days at 20-25℃ or until sporulation.[7]

Cosmetic Sample Preparation

Liquid preparations in the form of solutions that are water-based (face mist products; labelled as sampel D) can be directly treated. Meanwhile, liquid preparations in the form of emulsions, suspensions, and semisolids that are water-based (day cream, face mask, and gel; labelled as sample A, B and C respectively) must be pre-treated. Those sample are dissolved in NaCl 0.9% solution in a ratio of 1: 1 into a sterile falcon. Furthermore, the mixture will be heated at a temperature of 40-45^{o}C for 15-20 minutes and homogenized using a vortex.[7]

Challenge Test

The amount of 0.1 mL of each microbial culture suspension (≤1% v/v product) was added to the previously prepared sample formula until the number of cells in the product 10^5 – 10^6 CFU, cells, or spores/mL. After that, homogenization is carried out until it is evenly distributed. Samples without preservatives or those that have been neutralized using a neutralizer are also used as validation.

The mixture of cultures and samples of the products than will be tested on days 0, -2, -4, -7. The samples that have been inoculated with microbes are stored at 25°C (room temperature) for 7 days and were kept away from direct sunlight. At each sampling point, the number of viable cells was counted using the total plate count (TPC) method. The results are used to determine whether the product is passed/not based on the European Pharmacopoeia reference and grouping the products based on their efficacy criteria [7]

In-Use Test

The in-use test was carried out on 90 participants for 2 months of use and each participant got a sample of day cream, face mask, gel, and face mist. After 2 months of use, those samples were returned and evaluated. Then, the products were composited from 5 samples. One mL of each composite was taken and diluted with NaCl 09% that had been added with a neutralizing solution. Last, the solution mixture was homogenized and 0.1 mL of the product was inoculated into a TSA plate using spread method. The samples were incubated at room temperature and tested on days 0, 2, 4, 7 by calculating the number of cells. The results are used to determine the grouping of the products based on their numbers of microbial contaminants according to their criteria.[9]

Isolation and Identification of Microorganisms

Colonies that are obtained from in-use testing will be observed macroscopically and microscopically. In addition, genetic analysis was carried out using 16S rRNA and make a phylogenetic tree of all the contaminant microbe isolates.[9]

Results.

Based on the data in **Table 1**, it can be stated that almost all formulas in each cosmetic product passed the challenge test according to the international standard European

Pharmacopoeia 10th Edition in 2019 and the national standard of the Indonesian Pharmacopoeia 6th edition in 2020. In addition, it can also be seen that there is a trend of the decline in the acceptance status of the challenge test according to the EP standard for each product as the concentration of preservatives in the product decreases. The majority of variation 01 (with the highest concentration of preservatives) has an acceptability status value of "passed A" according to the EP standard, except for product C which has a "passed B" status in its variation 01. Day cream and face mist products have the same results in variations 02 and 03 with the status "passed B" according to the EP standard, while the face mask products still have the status of "passed A" for variation 02 and "passed B" for variation 03 according to the EP standard. Meanwhile gel products have a "failed" status in variations 02 and 03. All products and variations have "passed" status according to IP standards, except for gel products variations 02 and 03 which have "failed" status and have results that are in accordance with the results obtained according to EP standards.

Table 1. Interpretation of Challenge Test Results on All Types of Products

Produk	**Variasi**	**Challenge Test Results**	
		EP	**IP**
Day Cream (A)	A-01	Passed A	Passed
	A-02	Passed B	Passed
	A-03	Passed B	Passed
Face Mask (B)	B-01	Passed A	Passed
	B-02	Passed A	Passed
	B-03	Passed B	Passed
Gel (C)	C-01	Passed B	Passed
	C-02	Failed	Failed
	C-03	Failed	Failed
Face Mist (D)	D-01	Passed A	Passed
	D-02	Passed B	Passed
	D-03	Passed B	Passed

Based on **Table 2**, it can be observed that the in-use testing results for day cream, face mask, and gel product samples were in two types of tube and pot packaging, while face mist was in spray packaging. According to the data, it was found that there are sample products without any microbial contamination after daily used by users for two months. In addition, the sample with contamination showed the decrease of contaminant microbial cells number after 7 days.

Table 2. Average Number of Viable Contaminant Microbial Cells in each Type of Cosmetic Product

Variation	Packaging	Number of viable contaminant cells (log CFU/mL)		
		Day 0[a]	Day 4	Day 7
A-01	*Tube*	1.643(1.1)[b]	0.832(0.8)	0.000(0.0)
	Pot	4.121(0.5)	1.080(1.1)	0.000(0.0)
A-02	*Tube*	2.001(0.9)	1.207(2.1)	1.125(1.9)
	Pot	4.010(0.6)	3.194(0.6)	1.384(1.2)
A-03	*Tube*	2.100(0.1)	1.000(1.0)	0.413(0.7)
	Pot	3.788(0.4)	1.463(1.3)	1.633(1.1)
B-01	*Tube*	0.000(0.0)	0.000(0.0)	0.000(0.0)
	Pot	0.863(1.5)	0.847(1.5)	0.000(0.0)
B-02	*Tube*	0.000(0.0)	0.000(0.0)	0.000(0.0)
	Pot	1.834(1.6)	0.847(1.5)	0.000(0.0)
B-03	*Tube*	1.563(1.4)	1.434(1.3)	1.050(1.1)
	Pot	1.890(1.6)	0.867(1.5)	0.000(0.0)
C-01	*Tube*	1.760(1.6)	0.717(1.3)	0.333(0.6)
	Pot	0.918(1.6)	0.333(0.6)	0.000(0.0)
C-02	*Tube*	0.000(0.0)	0.000(0.0)	0.000(0.0)
	Pot	0.796(1.4)	0.000(0.0)	0.000(0.0)
C-03	*Tube*	0.863(1.5)	0.667(1.2)	0.000(0.0)
	Pot	0.934(1.6)	0.000(0.0)	0.000(0.0)
D-01	*Spray*	0.979(1.0)	0.881(1.2)	0.333(0.5)
D-02	*Spray*	1.202(1.1)	0.550(0.6)	0.217(0.5)
D-03	*Spray*	0.934(0.9)	1.264(1.4)	0.167(0.4)

[a] The time after (delay time) used by the user for 8 weeks
[b] Standar deviation
A = day cream; B = face mask; C = gel; D = face mist

For more details, it can be observed that there are microbial contaminants in all day-cream product formulas (A-01, A-02, and A-03), both in tube and pot packaging. In the face mask product formula 1 (B-01), it can be seen that there are microbial contaminants only in the type of pot packaging and no microbial contaminants in the type of tube packaging. Then it can be seen in formula 2 (B-02) that there are microbial contaminants also only in the type of pot packaging and no microbial contaminants in the type of tube packaging. While it can be seen in

formula 3 (B-03) that there are microbial contaminants in both types of packaging. Furthermore, microbial contaminants were also found in all gel product formulas in tube and pot packaging, except for tube packaging in variation C-02. Lastly, similar to day cream products, microbial contaminants were found in all face mist product formulas (D-01, D-02, and D-03).

Table 3. In-Use Testing Acceptance Status Results in each Type of Cosmetic Product

Variations & Packaging	In-Use Testing Status	Variations & Packaging	In-Use Testing Status
A-01 (tube)	M (*passed*)	A-01 (pot)	P (*failed*)
A-02 (tube)	M (*passed*)	A-02 (pot)	P (*failed*)
A-03 (tube)	M (*passed*)	A-03 (pot)	P (*failed*)
B-01 (tube)	W (*passed*)	B-01 (pot)	M (*passed*)
B-02 (tube)	W (*passed*)	B-02 (pot)	M (*passed*)
B-03 (tube)	G- (*failed*)	B-03 (pot)	M (*passed*)
C-01 (tube)	M (*passed*)	C-01 (pot)	M (*passed*)
D-01 (spray)	M (*passed*)		
D-02 (spray)	M (*passed*)		
D-03 (spray)	M (*passed*)		

*A = day cream; B = face mask; C = gel; D = face mist
*W = well-preserved (passed); M = marginally-preserved (passed); P = failed; G (Gram Negative)

Based on the results of in-use testing in **Table 3**, it was found that the majority of cosmetic product formulas in tube packaging had a better acceptance status than pot packaging. Day cream products in tube packaging have marginally preserved status, which is better than pot packaging that has failed status. Furthermore, the face mask products in tube packaging have a well-preserved status, which is better than pot packaging has a marginally preserved status. But variation B-03 tube packaging has failed status because there are Gram-negative bacteria in the microbial contaminants. Furthermore, the gel product was found to be marginally preserved for both types of packaging. Finally, all the face mist product was found to be marginally preserved for all variations of preservative concentration. Comparative data on the results of the challenge test and in-use test can be seen in **Table 4.**

Table 4. Comparative Data on the Results of Challenge Test and In-Use Test

Products	Variation	Challenge Test		In-Use Test
		EP	IP	
Day Cream (A)	A-01	Passed A	Passed	M (passed) - tube
				P (failed) - pot
	A-02	Passed B	Passed	M (passed) – tube
				P (failed) – pot
	A-03	Passed B	Passed	M (passed) - tube
				P (failed) - pot
Face Mask (B)	B-01	Passed A	Passed	W (passed) - tube
				M (passed) - pot
	B-02	Passed A	Passed	W (passed) - tube
				M (passed) - pot
	B-03	Passed B	Passed	G- (failed) - tube
				M (passed) - pot
Gel (C)	C-01	Passed B	Passed	M (passed) - tube
				M (passed) - pot
Face Mist (D)	D-01	Passed A	Passed	M (passed)
	D-02	Passed B	Passed	M (passed)
	D-03	Passed B	Passed	M (passed)

Table 5 shows the results of the identification of microbial contaminants found in each cosmetic product. In total, there were 21 types of isolates found as contaminants. The majority of the contaminant isolates came from a group of Gram-positive bacteria, especially those in the form of cocci and staphylococci (grape-shaped cocci), but there were also isolates of fungi and yeasts. Meanwhile, it can be seen also from the table below, that the isolates that most often appeared to be contaminants came from Gram-positive staphylococci, especially isolate G followed by isolate N, then Gram-positive bacillus isolate J and coccibacil (short bacillus) Gram-

positive isolate L. In addition, the genus that is often found in all isolates is *Staphylococcus* sp. followed by *Pseudomonas* sp.

Table 5. Microbial Contaminant Identification Results

Isolate	Type of Isolate	Microscopic Form	Gram stain	Number of Occurrences of Isolates				Total	%	Genus/Species
				Day cream (A)	Face Mask (B)	Gel (C)	Face Mist (D)			
A	Fungi	Nonsepta with conidiospores					1	1	2,17%	*Aspergillus sydowii*
B	Bacteria	Staphylococci	+	1			1	2	4,35%	*Staphylococcus* sp.
C	Bacteria	Cocci	+	1			1	2	4,35%	*Pseudomonas* sp.
E	Bacteria	Bacil	-		1			1	2,17%	*Pseudomonas* sp.
F	Bacteria	Coccibacil	+				1	1	2,17%	*Kytococcus sedentarius*
G	Bacteria	Staphylococci	+	2	2		3	7	15,22%	*Micrococcus* sp.
H	Yeast	Round					2	2	4,35%	-
I	Bacteria	Cocci	+				1	1	2,17%	*Bacillus* sp.
J	Bacteria	Bacil	+	2			3	5	10,87%	*Priestia flexa*
K	Bacteria	Staphylococci	+	3		1		4	8,70%	*Staphylococcus* sp.
L	Bacteria	Coccibacil	+	3		2		5	10,87%	*Stenotrophomonas* sp.
M	Fungi	Nonsepta with conidiospores		2				2	4,35%	*Aspergillus sydowii*
N	Bacteria	Staphylococci	+	4			1	5	10,87%	*Staphylococcus* sp.
O	Bacteria	Bacil	+		1			1	2,17%	*Pseudomonas* sp.
P	Bacteria	Cocci	+		1			1	2,17%	*Macrococcus* sp.
Q	Bacteria	Cocci	+		2			2	4,35%	*Staphylococcus* sp.
R	Yeast	Round				1		1	2,17%	*Rhodotorula* sp.
S	Bacteria	Cocci	+			1		1	2,17%	*Staphylococcus* sp.
T	Bacteria	Bacil	+			1		1	2,17%	*Acinetobacter* sp.
								Total	100,00%	

Discussion.

There are several types of preservatives used in this study, namely a mixture of phenoxyethanol and chlorphenesin for day cream, gel, and face mist cosmetic products, while for face masks using DMDM Hydantoin as the preservative. Phenoxyethanol is one of the most widely used preservatives. This is because it has a broad spectrum, but is slightly weak against Gram-positive bacteria. At low concentrations, phenoxyethanol can lyse bacterial membranes by binding to amino acid residues so that it can change the nature of the protein structure. Phenoxyethanol can also work by releasing oxidative phosphorylation from cellular respiration and competitively inhibiting malate dehydrogenase. Phenoxyethanol plays a role in increasing the permeability of cell membranes to potassium ions and provides a direct inhibitory effect on the synthesis of microbial DNA and RNA.[11] Phenoxyethanol is more often used in conjunction with other preservatives and is rarely used alone. Next is chlorphenesin which has broad antimicrobial activity against bacteria and fungi, but is effective against *Pseudomonas* spp. and Gram-negative bacteria.[12] Chlorphenesin is one of the phenol ether compounds that can disrupt the cytoplasmic membrane and induce potassium ion leakage from the cytosol. Commonly used concentrations are 0.1-0.3%. These two types of preservatives are often used together to provide synergistic effects such as preventing resistance, reducing toxicity, cost, and damage in nature that can occur as an impact in the future.[12] Lastly is DMDM Hydantoin, which is a preservative compound that has a broad spectrum, with better activity against bacteria than against fungi.[13] This preservative is a compound that works by making crosslinking with proteins in the cell. The concentration of commonly used usage is 0.15 - 0.4%.[12]

Based on the results of the challenge test, it was found that for all cosmetic products, variation 01 (which has the highest concentration of preservatives) is the variation that has the highest EP acceptance status, namely “passed A” for day cream, face mask, and face mist products, and “passed B” for gel products. This is in accordance with the literature that higher concentrations of preservatives can better inhibit microbial growth.[12] But based on the data, the results were "failed" for variations 2 and 3 in the gel formula. This might happened because the formulation in gel preparations consists of various plant extracts and high water content, causing the gel preparation to be a good growth medium for microorganisms, and causing the acceptance criteria for product C variations 2 and 3 were failed.[14]

Based on the results of each cosmetic product in the three types of preservative concentration formula variations that have the status of passing the challenge test according to the European Pharmacopoeia and the Indonesian Pharmacopoeia, the next step commonly taken by the beauty industry is an in-use test. The test is used for confirmation of product stability and compatibility tests to users directly. This follow-up test is also needed to determine the minimum shelf life of the product and its period after opening, which also has an impact on the quality or safety of the product before it is produced and sold in bulk. The number of microbial contaminants can also later be correlated with the existing challenge test results as a confirmation test result so that a safe and effective preservative concentration can then be determined or considered for each cosmetic product.[9]

On the results of the in-use test, it can be observed that there is a higher number of microbial contaminants in pot packaging than in tube packaging in each type of product which can occur due to tube packaging that supports minimizing attachment to the environment compared to pot packaging. Dermatologist Victor Georgescu also said that tube packaging is also the safest to use for cosmetic products other than single-dose products because contamination with air or surrounding pollution can be minimized thereby reducing the possibility of contaminant microorganisms entering the product.[15] In addition, it can be seen a downward trend in the number of microbial contaminants in all variations and packaging from day 0 to day 7.

Furthermore, based on the observation of macroscopic and microscopic characteristics of microbial isolates contaminating cosmetic products, the results of the estimation of the genus/species were obtained with a percent identity value exceeding 95% for all types of isolates based on BLAST results. The isolates that have been identified have habitats on human skin or in environments that have their respective risks to human health. *Aspergillus sydowii* is a saprophytic fungus found in soil that can contaminate food and has been implicated in the pathogenesis of several human diseases, including aspergillosis, onychomycosis, and keratomycosis.[16] *Pseudomonas* sp. is widely found in the environment and is one type of bacteria that acts as a significant nosocomial infection agent. One of the most commonly found species is *Pseudomonas aeruginosa,* which is a normal microflora of the gastrointestinal tract and on human skin.[17] This bacteria is one of the opportunistic pathogens that can cause severe and life-threatening infections.[18] *Kytococcus sedentarius* DSM 20547, the only known producer

of the antibiotics monesin A and B, has been isolated from varying environments, including human skin, groundwater, and even airline cabins. It can be a human opportunistic pathogen.[19]

Macrococcus sp. has a level of homology that is quite close to *Staphylococcus* and is widely found in the skin of animals such as dogs and also in dairy/meat products consumed by humans. Therefore, there is a possibility of these bacteria contaminating existing cosmetic products if users do not wash their hands hygienically after handling pets such as dogs and also after processing or consuming dairy/meat products.[20] *Staphylococcus aureus* is widely distributed in nature and includes normal microflora in humans and is often found in humid areas.[21] *Staphylococcus aureus* species on the skin can cause ulcers, cellulitis, and staphylococcal scalded skin syndrome (SSSS) while *Staphylococcus epidermidis* can cause opportunistic infections when attacking a weakened immune system and in excessive amounts.[22] *Micrococcus* sp. is also a normal bacterial microflora on human skin and is generally not a pathogenic bacterium.[23] Several species can be found in airborne dust (*M. roseus*), in soil (*M. denitrificans*), in water (*M. colpogenes*), and on the skin (*M. flavus*).[24]

Bacillus sp. is amongst the most frequently found microbes in cosmetics raw materials including water, milk, essential oils, and plant tissues.[25] Although anthrax remains the best-known *Bacillus* disease, in recent years other *Bacillus* species have been increasingly implicated in a wide range of infections including abscesses, bacteremia/septicemia, wound and burn infections, ear infections, endocarditis, and meningitis.[26] *Priestia flexa* is a Gram-positive bacterium formerly belonging to the genus *Bacillus*. Research on this bacterium is still limited and suspicions of disease due to bacterial infection are still often associated with diseases caused by bacteria of the genus *Bacillus.*[27] *Stenotrophomonas* sp. are Gram-negative bacteria, with the most common species, *Stenotrophomas maltophilia,* found in food and water sources. *S.maltophilia* is emerging as an important cause of skin infection in immune-responsive patients.[28]

Previously considered nonpathogenic, *Rhodotorula* sp. have emerged as opportunistic pathogens with the ability to colonize and infect susceptible patients. Recent studies have demonstrated that the incidence of fungemia caused by *Rhodotorula* sp. was between 0.5% and 2.3% in the USA and Europe.[29] *Rhodotorula* sp. is widely used in the cosmetic industry for the production of carotenoids. *Acinetobacter* sp. is a type of bacteria that is present in the environment and can live on human skin. If the bacteria enter the body, this can cause infections.

Some types of *Acinetobacter* sp. cause blood, lung, or urinary tract infections.[30] Most healthy people have a low risk of *Acinetobacter* sp. infections.

Conclusion.

The four types of cosmetic products tested in this study have the result that the acceptance criteria of Passed B based on EP and IP microbial challenge test could be considered an adequate preservative since it gives an almost similar result to Passed A criteria during the in-use test. This can be applied to the beauty industry in the manufacture of skincare with several positive benefits such as the costs incurred by the company will certainly be less and the use of fewer preservatives can reduce allergic reactions and is safer for consumers who have allergies to the preservatives compounds. Meanwhile, the recommended and safer packaging to use is tube packaging compared to pot packaging.

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Conflict of Interest Statement.

The authors have declared no conflict of interest.

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