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Original article

Microbiological Quality Assessment of Skin and Body care Cosmetics by using Challenge test

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ABSTRACT

Cosmetic products may be exposed to microbial contamination during storage or transport, and to avoid the risk of microbial growth, manufacturers add preservative compounds as a protection for the product from spoilage. The Microbial Challenge test is a procedure to evaluate the preservative efficacy by challenging the product with testing microorganisms to determine the quality of preservation. In this study, thirty-two cosmetics products used for body and skin care were collected from markets and pharmacies in Mecca region, these products are subjected to microbiological analysis, results show that most samples are contaminated except six samples. Non contaminated samples were subjected to European Pharmacopeia 7.0 standards. Results show that two samples, foaming gel and body and face cream are failed to demonstrate the required microbiocidal effect against the *S. aureus* test species, results recorded 1.21 x 10^5 and 6.80 x 10^5 (CFU/ml) respectively at the second day of incubation, other products: shower gel, hand wash, body lotion and shampoo demonstrate that required microbiocidal effect against the test species during day 2, 7, 14 until day 28th. The microbial count number is less than 10 during all incubation periods. To prevent contamination in cosmetics, manufacturers are required to add a good preservative system to the products and examine them before sale. Due to the high percentage of microbial contamination in cosmetics in Mecca region and for consumers safety, this study is prepared.

1. Introduction

The US Food and Drug Administration (FDA, 1995) defines cosmetics as "substances used for beautification, cleansing, promotion, attractiveness, or alteration of appearance.".

Cosmetics include some nutrient ingredients such as polysaccharides, proteins, glycosides, vitamins alcohol, lipids, amino acids, water, and peptides, which support a lot of microorganisms to survive and grow (Neza and Centini, 2016). High percentages of natural components are more susceptible to microbial growth (Stoffels, 2012).

According to the EU Cosmetics Directive 76/768/EEC, cosmetics shouldn't contain more than 10^2 CFUs of aerobic mesophilic microorganisms / ml or g. For category products, *E. coli, P. aeruginosa, S. aureus, and C. albicans* must not be detectable in 0.1 or 0.5 ml/or g/ml of the product (Neza and Cenini, 2016). Microbial contamination could happen during storage or use by the consumer, for that the manufacturers add preservatives to their products to reduce microbial risk (Brannan and Dille, 1990).

The main purpose of Preservatives is to inhibit the growth of microorganisms inside cosmetic products (Baqer et al., 2014). They also have different benefits such as extending product shelf life, ensuring product safety for consumers, maintaining product quality, compliance with regulatory guidelines, and cost-effectiveness (Ruohonen, 2022). An effective preservative must have Minimum Inhibitory Concentration (MIC) to kill most bacteria associated with cosmetics. (Giorgio et al., 2018).

Preservatives include different types of chemicals such as formaldehyde, parabens, triclosan, alcohols, QACs, sorbic, benzoic, and dehydrated acids. (Hill, 1995 and Scott, 2020). In most cosmetics, more than one preservative is used in the product, the most known compound that use to preserve is parabens and its derivates which have broad spectrum against microorganisms (Onurdağ et al., 2010), in body care products, the most frequently preservatives that also used are Methylisothiazolinone and Methylchloroisothiazolinone, they are used together as a biocide (Collier et al., 1990).

The preservative needs to be affordable, colorless, odorless, controllable, inert, active at various pH levels, stable to UV light, and neither irritating nor sensitizing (Ming et al., 2022).

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In manufacturers, cosmetic regulations now require providing a safety data sheet include details about ingredients and report of preservative assessment which include results of challenge tests (Skowron et al., 2017). To evaluate the quality of preservation system, a challenge test or (preservatives effectiveness test) is used as a procedure to determine if the cosmetics have a good preservative system to prevent microbial contamination from primary ingredients or while being used by consumers (Hodges and Hanlon, 2000).

This test is designed by using specific bacterial strains, fungal strains, and yeasts to challenge them against preservative substances (Chapman et al., 1997). If the preservative substance fails to prevent microbial growth, it means it isn't a good preservative system and it needs to be reassessed, while if the organisms show resistance to preservative, it would be considered (Chapman, 1998). It is important to improve the preservative system to prevent microbial growth during manufacturing, storage or use by consumers.

Preservative efficacy test is applied in different countries such as (USP method, 2000), (EP, 2002), and universal ISO 11930. (EU, 2019). According to the EU Regulations of cosmetic products, it is a legal requirement to offer the result of challenge test in the Cosmetic Product Safety Report (CPSR) before selling them to stores (Russell, 2003).

Challenge tests are important for evaluating the safety of cosmetic products susceptible to spoilage, especially in products containing water. European Pharmacopeia has two sets of acceptance standards: criteria A and criteria B. For criteria A, bacteria must have a 2 log reduction at 2 day log 3 at 7 day and 14 days and no increase in microbial growth at 28 day and for fungi no increase at day 2 and 7, the log reduction at 14 day and in 28 day no growth increase. Criteria A is always accepted as a primary method, and in justified cases criteria B may be used (Siegert, 2010 & British Pharmacopoeia. 2002).

According to FDA reports, most cases of microbial contamination are due to use ineffective preservative systems and not inspecting the quality of products before use.

The aim of this study is to assess the microbiological quality of some skin and body care cosmetics which are available in a lot of markets and pharmacies in Mecca region. The microbiological stability of these products is examined by using the European Pharmacopoeia challenge test or preservative efficacy test.

2. Material and methods

2.1. Cosmetic products

A total of thirty-two cosmetic products used for body and skin care collected from different brands and purchased from pharmacies and outlets in Mecca region in the western part of KSA, the latitude of Mecca is 21.422510 and the longitude is 39.826168. These products are (4 foaming gels, 5 shower gels, 5 handwashes, 3 body lotions, 5 hair conditioners, 3 hand creams and 4 shampoos). Samples sent to the lab in sterilized condition. Cosmetics are labeled with description on each product such as brand, weight,physical form, container type and country of origin.

2.2. Microbiological analysis

In the beginning, 10 g of each sample was diluted with the 10 ml of phosphate buffer at pH 7and mixed very well to do serial dilution. 1 ml of sample solution added to 9 ml of saline solution in a sterilize test tube to make 10^{-1} dilution. Dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} are created. Each dilution transferred to two different plates, one includes Tryptone Soy Agar (TSA) incubated for 48 h at 37 °C for growing bacteria, and the other one is incubated on Sabouraud Dextrose Agar (SDA) at 25 °C for 5–7 days for growing fungi. All Collected samples were analyzed to detect the (CFU) on incubated plates.

2.3. Identification of microorganisms

2.3.1. Bacteria

Bacterial strains were identified by describing the colonies, interaction with gram strain and appearance under microscope according to Bergey's manual (Bergey and Holt, 2000). Biochemicals tests are used to identify bacteria by API-20E kit which contains (urea test, indole test, gelatinase, citrate, H_2S production, sugars fermentations and other tests, additionally, to catalase and oxidase (Barrow and Feltham, 1993).

2.3.2. Fungi

For fungal stains, identification is done based on their cultural and microscopic characteristics, according to manual (Barnett and Hunter, 1972).

2.4. Efficacy of antimicrobial preservation (European Pharmacopeia 7.0)

In this experiment, 250 g or ml is taken from each product to conduct a full challenge test. Four portions including twenty g from each sample were transferred to sterile containers, samples inoculated separately with 0.2 ml tested strain as shown in Table 1. The inoculated sample portions were mixed using sterile tools and incubated at temperature room. The challenge test protocol of the European Pharmacopoeia is followed in exact detail (European Pharmacopoeia, 2010).

Cosmetic samples were inoculated with different test microorganisms. At set intervals of 2nd, 7th, 14th, and 28th days microbial counts are taken. The reduction of Log number in each sample should comply with criteria A in the European Pharmacopeia. This method determines the effectiveness of preservative system based on decreasing microbial growth during incubation periods.

2.5. Tested microorganisms

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3. Results

A total of 32 cosmetic products were examined to detect the purity of these samples from microorganisms. Reslut in Table 3 shows twenty-six samples found contaminated with bacteria and fungi. These products are contaminated with pathogenic bacteria and fungi. The bacterial isolates identified by API-20E kit Table 2. The majority of bacteria are related to gram positive bacteria such as *Staphylococcus* spp, *Micrococcus* spp, *Streptococcus* spp, *Bacillus* spp and also gram-negative bacteria such as *E. coli* and *Psudeomonas* spp.

Some products are found contaminated with fungi. Fungal isolates were identified based on cultural and microscopic properties. Mycelium and spores 'arrangement on sporophores or vesicles are observed. fungal isolates were found related to *Aspergillus* and *penicillium* genera.

The highest percentage of contamination was found in hair conditioner (sample 23) with 48 x 10^5 CFU/g, the sample is commentated with *Staphylococcus* spp, *Bacillius* spp, *Aspergillus* spp, *Penicillium* spp, and the lowest percentage of contamination found in foaming gel (sample 1) and shower gel (sample 7) with 17x10^5 CFU/g for both with *Staphylococcus* spp. Fungi recorded a few occurrences in most cosmetic products, in samples (5) shower gel, (23) hair conditioner and (29) shampoo.

Table 1
Initial Inoculum Level (CFU/g) for each tested strain.

Microorganisms	Initial Inoculum Level (CFU/g)
Staphylococcus aureus NCTC10788	9.60 x 10^6
Pseudomonas aeruginosa NCTC12924	2.20 x 10^6
Candida albicans NCPF3179	1.40 x 10^6
Aspergillus brasiliensis NCPF 2275	4.00 x 10^5

Table 2
Identification of bacteria by using biochemical test with API-20E kit (+) positive, (-) negative.

Bacterial Isolate	Gram stain	Catalase test	Oxidase test	Citrate test	Indole test	H_2S production	Urea test	Sugar Fermentation test	Gelatin hydrolysis test
Bacillus spp	Gram positive	+	+	–	–	–	+	–	+
Escherichia spp	Gram Negative	+	–	–	+	–	–	+	–
Micrococcus spp	Gram positive	+	+	–	–	–	+	+	+
Staphylococcus spp	Gram positive	+	–	+	–	+	+	+	–
Streptococcus spp	Gram positive	–	–	+	–	+	–	+	+
pseudomonas spp	Gram Negative	+	+	+	–	–	–	–	+

Six samples were shown to be in good microbiological condition during maintain them on TSA and SDA plates containing no detectable contamination as shown in Table 3, the samples number are (3) foaming gel, (6) shower gel, (12) face and body cream, (14) hands wash, (18) body lotion and (31) shampoo). Non contaminated cosmetic products are sent to ISCA cosmetics testing labs, NewPort.UK. https://iscacosmetictesting.com/ to evaluate the efficacy of preservation. Information about the products is summaries in Table 4.

3.1. *Efficacy of antimicrobial preservation (European Pharmacopeia 7.0)*

Six samples were examined for their anti-microbial efficacy by the European Pharmacopeia 7.0 test. Table 5 and chart1 clarify the log reduction of account of bacteria and fungi during day 2, 7, 14 until day 28th which show no increase in number of microorganisms.

Results in Table 6 Shows that foaming gel is failed to demonstrate the required microbiocidal effect against the *S.aureus* test species, it recorded 1.21 x 10^5 CFU at the second day of incubation, and based on that, the sample is not effectively preserved according to the European Pharmacopoeia test method.

In Table 8, face and body cream is also failed to demonstrate the required microbiocidal effect against the *S.aureus* test species, the growth of *S.aureus* was recorded 6.80 x 10^5 CFU from second day of test when inoculated with product.

Tables 7,9,10,11 show that samples 2,4,5,6 which include shower gel, hand wash, body lotion and shampoo demonstrate the required microbiocidal effect against the test species during day 2, 7, 14 until day 28th, it should be effectively preserved according to the European Pharmacopoeia for topical preparations.

4. Discussion

In this study, 32 unused skin and body care products were investigated to detect their microbiological stability, 26 samples were found contaminated with bacteria and fungi, these samples record unacceptable microbial count number according to FDA. The most frequent microorganisms are pathogenic related to gram positive cocci and bacilli, gram negative bacilli and *Aspergillus* and *Penicillium* genera which is consistent with a lot of studies **(**Ravita et al., 2009; Abdelaziz et al., 1989**).**

One of the important reasons is the efficacy of preservative of product because if the preservation system is not good, the possibility of cosmetic spoilage is certain (Kavitha, 2018), in addtion of that loss economic and health issues if the product contact with the skin **(**Birteksoz Tan et al., 2013**).**

Six samples show good condition and there is no detectable contamination, their anti-microbial efficacy is examined according to European Pharmacopeia 7.0 test. samples are inoculated with tested organisms and incubated for intervals of 2nd, 7th, 14th, and 28th days. Two samples, foaming gel and body and face cream are failed and found that preservative systems are not effective against *S. aureus* at the second day of incubation, although these products are very expensive, but it doesn't mean there is no mistakes in manufacturing or selection the raw materials (Bresciani and Poulet, 2014). According to INCI website https://incidecoder.com/ we got the information about the body and skin care cosmetics to recognize the ingredients for each product that collected. Foaming gel products include sodium propylparaben and sodium methylparaben as a preservative compound. Parabens are chemicals used for preserving cosmetics and pharmaceutical products. They have several derivates such as methylparaben, ethylparaben, propylparaben, and butylparaben (Crovetto et al., 2017). They are effective against bacteria and fungi, have a low sensitization potential, and are generally considered safe for use in cosmetics (Sandle, 2016). In the study of **(**Bargiota et al., 1987**),** they mentioned that some strains of *S. aureus* are resistance to methyl and propyl parabens while other strains are sensitive to parabens and this may related to membrane lipid composition. Study of **(**Ferrarese et al., 2003**)** isolated *E. gergovia* and *P. aeruginosa* from a cosmetic manufacturer, they noticed bacterial strains have high resistance against formaldehyde-releasing preservatives and parabens and. On the other hand, a lot of studies demonstrated that parabens could inhibit the growth of microorganisms. (Zadan and Serry, 1984) demonstrate that *P.aeruginosa* was unable to grow on methyl paraben, (Accot and Labuza, 1975) reported that methyl and propyl parabens inhibited growth of *A.brasilliensis* at 0.05 and 0.1 % concentrations during the period of incubation for nine months. in the concentration arrange (0.015–0.3) %, parabens can block the transport of electron through membrane systems **(**Denyer, 1995**).**

In Face and body cream, the preservative compounds in this product are phenoxyethanol and DMDM Hydantoin. Phenoxyethanol is used as an antibacterial ingredient or preservative to prevent cosmetic and skin care products from spoiling **(**Dreno et al., 2019**).** DMDM Hydantoin is a formaldehyde releaser, manufacturers put it in cosmetic products in very low concentrations to inhibit microbial growth, preservative is very effective against bacteria, fungi, and yeast.

Our results show that other products shower gel, hand wash, body lotion, and shampoo have an effective preservative system and count number is less than 10 during all incubation periods.

In shower gel, preservatives are Phenoxyethanol, Methylisothiazolinone Methylchloroisothiazolinone, and Potassium Sorbate. These compounds are derivates of Isothiazolinones, they are heterocyclic organic compounds. most often used as a preservative in the shampoos; skin creams, and lotions **(**Yim et al., 2014**).** Phenoxyethanol (PE), has a wide spectrum against different microorganisms such as yeast and bacteria, but it has low inhibitory effect on skin mycoflora **(**Dreno et al., 2019**).** They exhibit inhibitory action, when the proteins are oxidating, the thiol (-SH) groups of the cysteine residues. This oxidation inhibits the proper functioning of the structural proteins present in the cell wall and cell membrane of the microbial contaminants, and may also inhibit the enzyme metabolism **(**Lambert, 2012**).** A lot of studies confirmed its efficacy against bacteria and fungi **(**Głaz

Table 3
Colony form unit of each sample incubated on TSA and SDA and isolated microorganisms.

Number of Sample	Cosmetic category	Country of region	Microorganisms	CFU/(10^5/ g) or	
				Bacteria (TSA)	Fungi (SDA)
1	Foaming gel	France	*Staphylococcus* sp.	17	ND
2		Korea	*Staphylococcus* sp, *E.coli*	31	ND
3		France	–	ND	ND
4		USA	*Staphylococcus* sp	28	ND
5	Shower gel	China	*Staphylococcus* sp. *Bacillus* sp, *Aspergillus*	36	22
6		UK	———	ND	ND
7		Korea	*Staphylococcus* sp.	17	ND
8		USA	*Bacillus* sp, *Staphylococcus* sp.	21	ND
9		Germany	*Pseudomonas* sp, *E.coli*	27	ND
10	Face and body cream	USA	*Bacillus sp, Streptococcus sp*	34	ND
11		Germany	*Staphylococcus* sp., *Bacillus* sp	45	ND
12		UK	———	ND	ND
13	Hands wash	USA	*E.coli,Penicillium* sp	33	< 10
14		UK	———	ND	ND
15		UK	*Staphylococcus* sp	29	ND
16		India	*Micrococcus* spp, *Streptococcus sp, Aspergillus*	39	< 10
17		Koea	*Staphylococcus* sp, *Streptococcus* sp	45	ND
18	Body lotion	UK	———	ND	ND
19		France	*Staphylococcus* sp., *Bacillus* sp	26	ND
20		USA	*Staphylococcus* sp	37	ND
21	Hair conditioner	UK	*Bacillus* sp, *Staphylococcus* sp	46	ND
22		Germany	*Pseudomonas* sp, *Aspergillus* sp	19	< 10
23		Korea	*Staphylococcus* sp, *Bacillus* sp, *Aspergillus* spp, *Penicillium* spp	48	23
24		USA	*Staphylococcus* sp	27	< 10
25		France	*Staphylococcus* spp., Micrococcus spp.	34	ND
26	Hand cream	UK	*Bacillus* spp	21	ND
27		Korea	*Staphylococcus* sp	39	ND
28			*Pseudomonas* sp, *Aspergillus* sp	22	ND
29	Shampoo	China	*Staphylococcus* sp, *Aspergillus* sp, *Penicllium* sp	31	27
30		USA	*Streptococcus sp*	28	ND
31		France	———	ND	ND
32		China	*Bacillus* sp, *Aspergillus* sp	27	13

*ND (not detect).

et al., 2023). In the study of (Nasrollahi et al., 2022), seven preservatives are commonly used in face care cosmetics, these compounds are butylparaben. benzyl alcohol, phenoxyethanol, methylparaben, propylparaben, alcohol 70 %, benzalkonium chloride, and results show that used preservatives can inhibit the growth of fungi but can't prevent the growth of bacterial microflora.

In hand wash, preservatives compounds are Iodopropynyl butyl carbamate and Phenoxyethanol. Iodopropane Butyl carbamate or (IPBC) is a substance that prevents growth of bacteria and preserve personal-care cosmetics from degradation (Lanigan, 1998).

In Body lotion, the preservative compound is Benzyl Alcohol. It is an aromatic alcohol, it has a strong effect against bacteria and fungi, it is common added as ingredient in the industry facial care such as cleansers, soaps, skin lotions, shampoos, and creams. This compound and its derivatives are generally potent against *P. aeruginosa* and *S. aureus* (Sulaiman et al., 2020).

In shampoo, Pyrithione Zinc (ZPT) is a perseverative chemical has

Table 4
Information about the selected cosmetics and preservative chemicals.

Sample no.	Product Type	Preservative Information	Quantity
3	Foaming gel	Sodium Propylparaben, Sodium Methylparaben	200 ml
6	Shower gel	Phenoxyethanol, Potassium Sorbate, Methylchloroisothiazolinone, Methylisothiazolinone	200 ml
12	Face and body cream	Phenoxyethanol, DMDM Hydantoin	300 ml
14	Hands wash	Phenoxyethanol, Iodopropynyl Butylcarbamate.	200 ml
18	Body lotion	Benzyl Alcohol	200 ml
31	Shampoo	Pyrithione Zinc (1 %), Methylchloroisothiazolinone, Methylisothiazolinone	200 ml

Table 5
Log reduction in number of bacteria and fungi during incubation period.

	Log Reduction			
	2nd Day	7th Day	14 th Day	28 th Day
Bacteria	2	3	–	NI*
Fungi	–	–	2	NI*

*NI: no increase in count number compared to the previous findings.

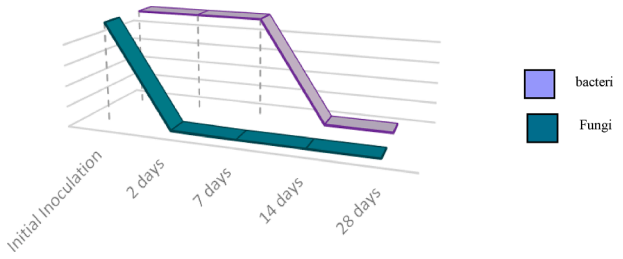

Chart 1. Shows log reduction in number of bacteria and fungi during incubation period.

Table 6
(Foaming gel) inoculated with tested microorganisms during 2,7, 14,28 days.

Test Species	CFU/g after inoculation			
	2nd Day	7th Day	14 th Day	28 th Day
P.aeruginosa	< 10	\		\
S.aureus	1.21 x 10^5	\		\
C.albicans			\	\
A.brasilliensis				\

Table 7
(Shower gel) inoculated with tested microorganisms during 2,7, 14,28 days.

Test Species	CFU/g after inoculation			
	2nd Day	7th Day	14 th Day	28 th Day
P.aeruginosa	< 10	< 10		< 10
S.aureus	< 10	< 10		< 10
C.albicans			< 10	< 10
A.brasilliensis			< 10	< 10

Table 8
(Face and body cream) inoculated with tested microorganisms during 2,7, 14,28 days.

Test Species	CFU/g after inoculation			
	2nd Day	7th Day	14 th Day	28 th Day
P.aeruginosa	< 10	\		\
S.aureus	6.80 x 10^5	\		\
C.albicans			\	\
A.brasilliensis				\

Table 9
(Hand wash) inoculated with tested microorganisms during 2,7,14, 28 days.

Test Species	CFU/g after inoculation			
	2nd Day	7th Day	14 th Day	28 th Day
P.aeruginosa	< 10	< 10		< 10
S.aureus	< 10	< 10		< 10
C.albicans			< 10	< 10
A.brasilliensis			< 10	< 10

Table 10
(Body lotion) inoculated with tested microorganisms during 2,7, 14,28 days.

Test Species	CFU/g after inoculation			
	2nd Day	7th Day	14 th Day	28 th Day
P.aeruginosa	< 10	< 10		< 10
S.aureus	< 10	< 10		< 10
C.albicans			< 10	< 10
A.brasilliensis			< 10	< 10

Table 11
(Shampoo) inoculated with tested microorganisms during 2,7, 14,28 days.

Test Species	CFU/g after inoculation			
	2 days	**7 days**	**14 days**	**28 days**
P.aeruginosa	< 10	< 10		< 10
S.aureus	< 10	< 10		< 10
C.albicans			< 10	< 10
A.brasilliensis			< 10	< 10

fungistatic and bacteriostatic inhibiting bacterial cell division, it is an ionophore plays important role in transporting through the plasma membrane and delivering copper into the cell (Abdali et al., 2021).

Finally, the test method (EP) is the most stringent test method and is the most difficult to pass. The samples that have failed show that they do not have the preservative efficacy to pass the criteria on the second day (Crémieux et al., 2005)The other test methods such as ISO 11930 or USP are more flexible and do not test the product from the second day and start at seventh day, so the preservative system has a chance to work. Sometimes, passing the second day mark can be quite difficult (Siegert, 2014).

5. Conclusion

Using body and skin care products is a part of regular daily lifestyle. A lot of companies produce these products for consumers with different criteria. According to FDA reports, most cases of microbial contamination are due to use ineffective preservative systems and not inspecting the quality of products before use. Our study recommends companies to test cosmetics before selling them to shops and stores. It is very important that cosmetic products comply with regulatory standards and consumer expectations.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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