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Microbiological Stability of Cosmetics by using Challenge Test Procedure

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A cosmetic product is a substance or mixture intended to be applied on the outer surfaces of the human body or on teeth, on the mucous membranes of the mouth, with the purpose of cleaning, smelling, modifying, protecting, maintaining them in good condition. In order to prevent microbial proliferation in cosmetics, substances with antimicrobial activity are used, to inhibit the development of microorganisms. Among the most commonly used cosmetic contaminants, there are spore-forming bacteria, molds, yeasts and bacteria. In the following study through the challenge test, four cosmetics products were analyzed, including an ultra-moisturizing anti-aging facial cream, a biphasic tonic, an aqueous tanning gel and a hair wax. The main goal was to evaluate the conservative properties of products in catching any microbial contamination, that may occur as a result of use. The Challenge Test has proved to be useful and appropriate to predict the behavior of cosmetics in the event of bacterial contamination. Starting from a high microbial charge for all products, microbial growth after 7 days is stopped, thus demonstrating the good conservative properties of the analyzed products.

Keywords: Cosmetics; Microbiological Risk; Challenge test; Preservatives.

According to the EC Regulation 1223/2009 of the European Parliament and of the Council of 30th of November 2009, a cosmetic product is *"any substance or mixture intended to be applied on the outer surfaces of the human body (epidermis, hair, nails, lips) or on teeth, on the mucous membranes of the mouth, with the purpose of cleaning, smelling, modifying, protecting, maintaining them in good condition or correcting bodily odors"* (Regulation EC No 1223/2009). Substances or mixtures applied on the surface of the body with other purpose than those indicated by the Regulation, including all drugs and top medical devices (e.g. disinfectants, insect repellents) are not cosmetics. Cosmetics can be classified as creams, emulsions, lotions, gels and oils, soaps, prepared for baths and showers, deodorants and antiperspirants, tooth and mouth products, depending on the function, and the area where they are applied. The commitment of the cosmetic industry and competent authorities is aimed at ensuring the safety of cosmetics and at protecting consumers' health (Scientific Committee on Consumer Products, 2006). The current Regulation (1223/2009) stipulates that

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all cosmetics must be manufactured, handled, packaged and sold under such conditions as to avoid damaging human health. The same regulation stipulates that the manufacturer must assess marketed product safety. In this regard, cosmetic company must evaluate the safety of the cosmetics, using a qualified expert, internal or external to the company (Jimenez, 2004). Evaluation procedures must consider both the intrinsic properties of each used component and the amount to which the consumer is exposed in his actual use of the product, to obtain an estimate of the risk associated with the use of the product.

One of the possible risks associated with the use of cosmetics is the microbiological one (Behravan *et al*., 2005; Campana *et al*., 2006; Mugoyela and Mwambete, 2010; Abdelaziz *et al*., 2008). The growth of bacteria in cosmetics can alter the product physical properties (color, odor, consistency) and can also cause serious harm to consumer health, because of the production of endotoxins or harmful metabolites. The Scientific Committee on Consumer Product (SCCP) reports in Notes Guidance the microbiological requirements to be met for the marketing of cosmetics. Microbiological contamination can occur at any stage of life of the product, from the production to the consumer use (Dashen *et al*., 2011; Brannan and Dille, 1990). One of the possible contaminants is the presence of impurities in the raw materials and the use of the most vulnerable compounds to bacterial proliferation (such as water-based, high-protein or plant-based). Containers, packaging, storage, transportation are other critical phases that may affect the quality of cosmetics (Brannan *et al*., 1990; Okeke and Lamikanra, 2001; Ratajczak *et al*., 2015). To prevent microbial proliferation in cosmetics, substances with antimicrobial activity are used, to inhibit the development of microorganisms (Maccioni *et al*., 2002; Hugbo *et al*., 2003; Pinon *et al*., 2015). However, such substances may cause infections and irritations, especially if the product comes into contact with mucous membranes (Isaksson *et al*., 2004; Thyssen *et al*., 2006; Jong *et al*., 2007;). For this reason, the possible preservatives that can be used in cosmetic products are listed in Annex V-List of Preservatives Allowed (EU No 1223/2009). Among these, there are Parabens (e.g. Methylparaben, Ethylparaben), Acidic (eg Sorbic Acid, Benzoic Acid), FormaldehydeDonors (Imidazolidinyl urea, DMDM Hydantoin), Halogenated Compounds (e.g. Chlorphenesin), Isothiazolinones (e.g. MCT / MI), Alcohols (e.g. EthylAlcohol) PhenolicTypes (e.g. Phenoxyethanol, BenzylAlcohol) and Quaternia (eg Benzalkoniumchloride).

The preservative must be stable, non-volatile, active at different pH, manageable, inert, stable to UV, colorless, odorless, inexpensive and not irritating and/or sensitizing. To be effective against a microorganism, a preservative must have a Minimum Inhibitory Concentration (MIC), higher than the maximum permissible concentration. Usually, multiple substances are used to exploit any synergistic phenomena and increase the spectrum of action. According to United States Pharmacopia (2000, 2007), among the most commonly used cosmetic contaminants, there are spore-forming bacteria (*Bacillus subtilis*, *Bacillus megatherium*), moulds (*Aspergillus* sp., *Rhizopus* sp., *Penicillium* sp.), yeasts (*Candida* sp., *Saccharomyces* sp.) and pathogenic bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*) (Muhammed *et al*., 2011; Shittu *et al*., 2006; Becks and Lorenzoni, 1995). According to SCCP's Notes of Guidance, pathogenic microorganisms such as *Pseudomonas aeruginosa*, *Candida albicans* and *Staphylococcus aureus* must be absent in 0.5 grams of cosmetics intended for children under three years old, or for specific areas, such as mucous membranes. The EC Regulation no. 1993/2009 establishes that product safety assessment shall be carried out considering cosmetic storage capacity and its microbiological stability over time. The most known and most widely used method of cosmetic industry is the Challenge Test (Brannan *et al*., 1997; UNI EN ISO 11930:2012; Siegert, 2010, 2013). This method reproduces at laboratory scale the microbial aggression that a product can undergo during manufacture, storage and use allowing to evaluate cosmetic antimicrobial protection. The aim of the work was to compare the antimicrobial properties of four cosmetics in compliance with the requirements of current legislation.

MATERIAL AND METHODS

Tested cosmetics

Four commercial cosmetic products were used. These included an ultra-hydrating

facial cream, a tonic biphasic, a watery sunscreen and a hair wax. Cosmetics were preserved with phenoxyethanol and ethylhexyglycerin having a broad-spectrum activity against bacteria, fungi and yeast.

The unused products were obtained from a local supermarket, transferred to laboratory intact and analyzed as soon as possible.

Each cosmetic was analyzed for the determination of total bacteria count, in order to establish possible initial contamination. One gram of samples was aseptically placed into a 9 mL sterile diluent (NaCl 0.9%). A volume of 100 µL of samples and its decimal dilution were spread on Triptone Soya Agar plate and incubated at 37 °C for 24-48 h. After incubation, results were reported as number of colony forming unit for mL (CFU/mL).

Test Microorganism

The following bacterial strains, recognized as pathogen species for human, were employed in the challenge test: *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538) and *Candida albicans* (ATCC 10231). Inoculum were prepared from each strains according to manufacturer's instructions. Broth suspension were maintained in specific growth medium until reaching a concentration about 10^8 CFU/mL. Bacteria concentration was determined spectrophotometrically.

Single Challenge Test

Twenty grams of samples were prepared from each cosmetic product. Liquid products were used as they were, while cream and solid were diluted with sterile solution (containing 0.9% of sodium chloride), warmed at 45 °C for 1 h and mixed by mechanical agitation. Samples were then spiked with test microorganism to obtain a concentration of 10^6 CFU/g for bacteria and 10^5 CFU/g for yeast.

Preparation were stored at room temperature for 30 days. After 2, 7, 14 and 30 days one gram products were aseptically sampled and analyzed for enumeration of viable microorganism and pathogen bacteria. Microbiological standard methods used for determination of *P. aeruginosa*, *E. coli*, *S. aureus* and *C. albicans* are listed in Table 1.

Table 1. Microbiological parameters analyzed and standard methods used.

Microbiological parameter	Standard Methods
Pseudomonas aeruginosa (ATCC 9027)	UNI EN ISO 22717:2009
Escherichia coli (ATCC 8739)	UNI EN ISO 21150:2009
Staphylococcus aureus (ATCC 6538)	UNI EN ISO 22718:2009
Candida albicans (ATCC 10231)	UNI EN ISO 18416:2009
Total bacteria count	UNI EN ISO 21149:2009

RESULTS AND DISCUSSION

Table 2 summarized results of single challenge test obtained from the four test microorganisms and the four cosmetics products.

Figure 1 (a,b,c, d and e) report reduction rate of microorganism during test period.

As for ultra-hydrating facial cream, the total bacterial count is reduced by 75% after 2 days and by about 99% after 7 days. For *E. coli* the reduction rate is 95% after 2 days and 100% after 7 days. Similarly, to other pathogens: the growth of *P. aeruginosa*, *S. aureus* and *C. albicans* decreased respectively by 96%, 98% and 97%, respectively after two days, and 100% after 7 days. For biphasic tonic, the total bacterial count is reduced by 11%, after 2 days, while for *E. coli*, *P. aeruginosa*, *S. aureus* and *C. albicans*, it is reduced by 68%, 18%, 48% and 79%. After 7 days growth is reduced by 100% for all tested organisms. Watery Sunscreen shows a percentage reduction like that of ultra-hydrating facial cream. Total bacterial counts are reduced by 54% and 100% after 2 and 7 days. For E. *coli* the reduction rate ranges from 93% to 100% from 2 to 7 days. *P. aeruginosa* growth is reduced by 98% after 2 days. For *S. aureus* and *C. albicans* reduction rate is 99% and 95% after 2 days and 100% after 7.

Table 2. Challenge test results.

		Ultra hydrating cream	Tonic biphasic	Watery sunscreen	Hair wax
Total Bacteria	t=0	1*106	1*106	1*106	1*106
Count CFU/g	t=2	2.5*105	8.9*105	4.6*105	7.2*105
	t=7	7,3*103	6.0*103	3.4*103	5.9*103
	t=14	<10	<10	<10	<10
	t=30	<10	<10	<10	<10
Escherichia coli	t=0	1*106	1*106	1*106	1*106
CFU/g	t=2	4.7*104	3.2*105	6.7*104	5.6*105
	t=7	4.2*102	2.2*102	3.3*102	2.4*102
	t=14	<10	<10	<10	<10
	t=30	<10	<10	<10	<10
Pseudomonas	t=0	1*106	1*106	1*106	1*106
aeruginosa CFU/g	t=2	3.6*104	8.2*105	2.4*104	6.4*105
	t=7	2.6*102	1.6*102	1.2*102	1.8*102
	t=14	<10	<10	<10	<10
	t=30	<10	<10	<10	<10
Staphylococcus	t=0	1*106	1*106	1*106	1*106
aureus CFU/g	t=2	2.1*104	5.2*105	1.2*104	4.7*105
	t=7	9.5*102	8.9*102	8.5*102	7.0*102
	t=14	<10	<10	<10	<10
	t=30	<10	<10	<10	<10
Candida albicans CFU/g	t=0	1*105	1*105	1*105	1*105
	t=2	3.2*103	2.1*104	5.2*103	2.1*104
	t=7	3*102	2.3*102	1.9*102	3*102
	t=14	<10	<10	<10	<10
	t=30	<10	<10	<10	<10

After 2 days hair wax shows a decrease in growth of 28%, 44%, 36%, 53% and 79%, respectively for total bacterial count, *E. coli*, *P. aeruginosa*, *S. aureus* and *C. albicans*. After 7 days growth is reduced by 100%. No increase in bacterial growth was observed in tested cosmetics and for all species analyzed until the end of the test.

In the following study through the challenge test, four cosmetics products were analyzed, including an ultra-moisturizing anti-aging facial cream, a biphasic tonic, an aqueous tanning gel and a hair wax. The main goal was to evaluate the conservative properties of products in catching any microbial contamination, that may occur because of use.

The presence of pathogens in cosmetics is a high risk for the consumer health and for this reason it should be monitored (Budecka *et al*., 2014; Hugbo *et al*., 2003; Lundov *et al*., 2008). Among the possible contaminants of cosmetics there are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus* sp, Gram-negative Enterobacteria and *Klebsiella pneumoniae*. Depending on their intrinsic composition, each cosmetic included in this study represents a good substrate for microbial growth. In particular, biphasic tonic and hair wax, as the first one is rich in free water and the second has a high amount of vegetable. None of the products analyzed showed initial microbial contamination. This demonstrates that the preparation, storage and transport procedures are suitable for product preservation. The Challenge Test has proved to be useful and appropriate to predict the behavior of cosmetics in the event of bacterial contamination during use or under other circumstances. Starting from a high microbial charge (about 10^6 CFU / mL) for all products, microbial growth after 7 days is stopped, thus demonstrating the good conservative properties of the analyzed products. The risk of microbial contamination of the products during the phases of use requires special attention from the consumer in respect of good hygiene

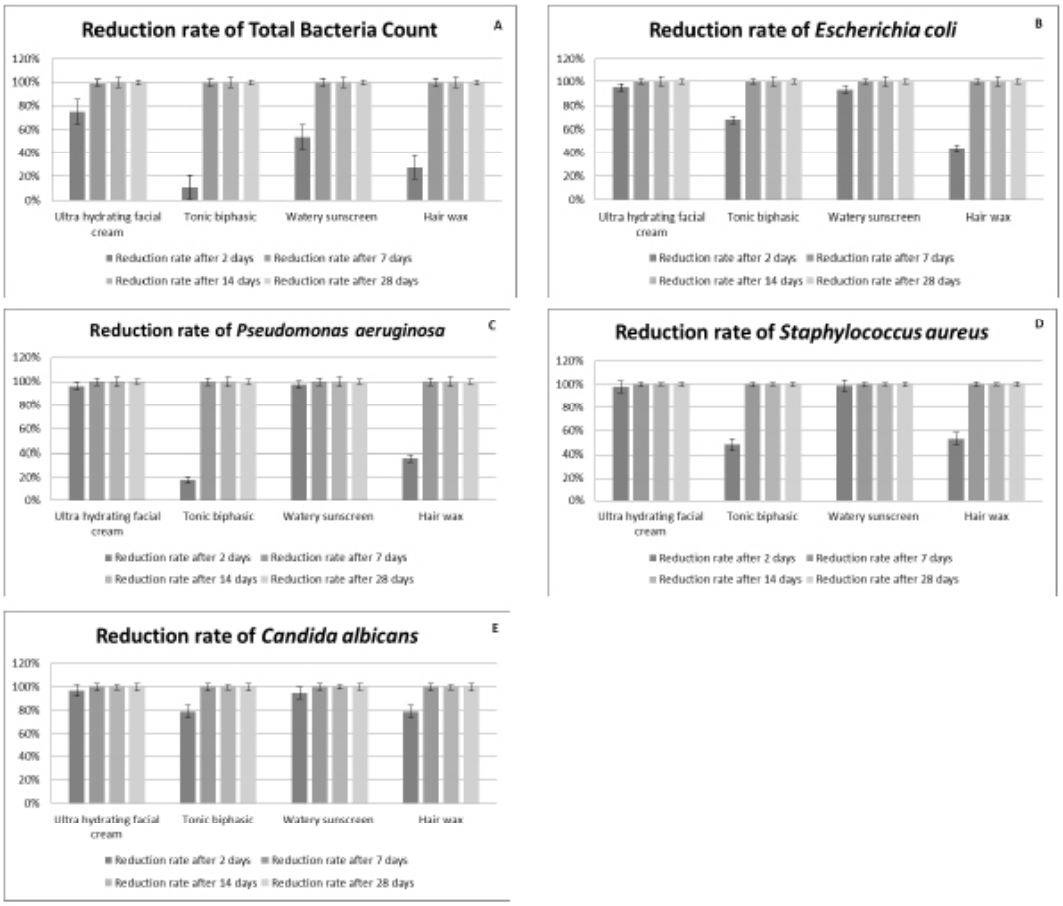

Fig.1. Reduction rate of microorganism used during Challenge test

practices. These include washing your hands before and after the use of cosmetics, whether they are creams, lotions or products for personal use, and to avoid the exchange of products from one person to another, also to avoid cross-contamination phenomena. Furthermore, the products must be kept in suitable locations, no longer contaminated and not used after the expiration date. In fact, after this period, their chemical, physical and microbiological stability is compromised and as a result, the risks to human health increase.

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