Development of a standardized method for the evaluation of the microbial protective efficiency of cosmetic packaging

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Abstract
The doubt regarding adverse effects of antimicrobial preservative, has modified the demand by the consumers which impose more and more the production of low-level and even preservative free cosmetics. In these products, protection against microbial contamination is therefore devoted to the packaging. This have prompted the emergence of a great diversity of so called “protective”, “overprotective” and “barrier” packaging, but this designation is not normalized and the choice of the right packaging adapted to each cosmetic product remains essentially empirical, hazardous and time consuming. The cluster Cosmetic Valleys decided to launch a commission aimed at defining a complete and experimentally validated method allowing to classify the microbiological protection level of cosmetic packaging. As reported herein, this was requiring the development a specific bacteriostatic medium usable over 7 days and an in vitro procedure reproducing in-use contamination and consumer gesture. On the basis of tests realized over 800 packs of different origins and performances, a classification in six grades was proposed in order to distinguish the protective efficiency of cosmetic packaging. This work can be considered is a first step towards a regulatory text.

Introduction
Demonstration of the health and environmental risks associated to the presence of preservatives in personal care and cosmetics has prompted the emergence of low level and even preservative free products (Halla et al., 2018). These formulations being particularly sensitive to the microbial contamination, the protection of the product was devoted to the packaging. The role of the packaging in the preservation of cosmetics is well known and in regulatory texts the pack is considered as an essential part of the final product (Lundov et al., 2009; Regulation EC 1223/2009). Consequently, a large range of high technology primary packaging (airless, air filter, shutter equipped devices...) susceptible to provide both physical and microbial protection to the product was developed (Crozier, 2018a). These packaging have essentially two targets, i.e. protecting the product into the device by limiting all potential retro-contamination, but also ensuring the delivery of low contaminated and safe doses all over the shelf life of the product. These objectives are somehow contradictory as, except for single use devices which are faced to other problems, such as increased contain-container
interactions (Feuilloley & Orange, 2018) and ecological compatibility (Thompson et al., 2009), it is particularly difficult to associate increased hermetic closure and regular delivery of the content over the whole life of the product. In addition, delivering a dose implies the potential accumulation of a remaining amount of product inside (dead space zones) and/or even outside of the device with a high risk of contamination when the product is not self-protected.

The packaging being an intrinsic element of low-level and preservative free cosmetic products, it is essential to adapt its performances to the self-protection of the formulation in order to insure the sanitary safety. On another side, as some active cosmetics ingredients also have antimicrobial activity, even in the absence of added preservatives (Papageorgiou, 2010; Herman et al., 2013), the formula can have a certain microbial protection and the pack should be adapted to this situation to avoid over-quality costs. Protocols have been proposed to evaluate the microbial protection provided by the cosmetic packaging (Devlieghere et al., 2015; Briasco et al., 2016). However, packaging and formulation are generally made by different partners and, in the absence of regulatory texts and even technical tools allowing to define a clear hierarchy from low to overprotective and barrier packaging, selecting the right association is essentially empirical and potentially hazardous.

The cluster Cosmetic Valley, world’s leading center for resources in perfumery and cosmetics, offered the possibility to associate professionals of packaging and cosmetic industry with academic investigators in order to define for the first time a technically functional harmonized procedure that should be applicable by all industrial partners and potentially translated in the future into a harmonized regulatory standard. In the present manuscript, we expose the logical and technical approach employed to define this procedure, experimental studies realized to overcome fundamental technical obstacles and the tests realized for its validation.

Material And Methods

Definition of the target and regulatory context

A “Guideline for evaluation of physical antimicrobial protection provided by packaging” was developed for comparison of the performances of commercially available cosmetic packaging, taking into account the physical properties (fluidity) of the potential final formulation but independently of its
composition. This procedure can be also employed for comparison of devices under development, although in this case the real protection level should remain relative. The results should be integrated into a microbial risk assessment procedure as described by ISO 11930 (2019) and ISO 29621 (2017). It appeared essential that the tests were possible to realize in packaging and cosmetic industries. Then, as the staff is normally trained to work in sterile conditions and has access to the necessary equipment, all microbial strains had to be level two or under regarding their biological risks (European Community classification, 2005). One of the target was also to take into account the real gesture of the consumer, as it is the principal source of contamination. For that reason, a specific in vitro contamination procedure was developed to mimic normal use.

Selected bacterial model
In order to be economically realistic, it appeared rapidly that the procedure should provide results using a single model microorganism. In addition to be level two or under, the following key criteria were retained for its selection:

- Presence in the microbial library of the majority of industrial sites
- Aerobic and easily growing
- Mean size in order to avoid potential under estimation of the protective performances of the packaging
- Frequent in the environment and potentially on the human skin
- Mobile in order to can diffuse in dead spaces and be detectable

Considering all these elements, the species Pseudomonas aeruginosa was selected. All the tests were realized using the strain ATCC® CRM-9027. Equivalent strains CIP® 82.118, NCIMB® 8626, NBRC® 13275 or KCTC® 2513 should be also usable.

Type of packaging and sampling procedure
Predictable low-level non-protective packaging against microbial contamination, such as pots or open tubes, providing no protection to the content were excluded from this study. At the other extremity of the protection spectrum, hermetic sterile single use packaging and corresponding to pharmaceutic quality products, were also considered out of the scope of the present study.

It was then decided to develop the present procedure for two types of devices:

- Overprotective packaging
- Absolute barrier packaging

Considering their production mode, in order to reach reliable statistical values it was considered that
the tests had to be realized over a minimum of 50 randomly selected devices. Before entering into the testing procedure all devices were submitted to decontamination using the technique adapted to their composition (ionization, autoclaving...). All devices were filled in sterile conditions, and control sterility tests were realized.

Testing media
Overprotective packaging are not meant to be totally hermetic to contamination. As one of the target of the protocol was to take into consideration the real “in use” gesture of the consumer, it was decided that the contamination procedure should be renewed over at least one week. The contamination level of distributed doses can be measured immediately, but that of the formulation present into the reservoir of the device can be only measured after opening. Considering that bacteria can multiply very rapidly it should be impossible to determine at which time of the test the initial contamination occurred. Then, it was necessary to develop a bacteriostatic medium for \textit{P. aeruginosa}.

In addition, as the performances of the packaging are adapted to the mean fluidity of cosmetic creams, this bacteriostatic medium had to be supplemented with a reticulation agent to adapt the viscosity.

Barrier packaging should normally block all bacterial contamination although they can deliver repeated doses. In this regard, perfectly safe absolute barrier packaging remain to be created, but very high efficiency barrier devices, adapted to low protection formula, have been yet marketed. Therefore, it was proposed to test it using a classical fertile medium for \textit{P. aeruginosa}. However, as these packaging are also designed to be used with cosmetic creams, classical fertile media are too fluid and needed to be also formulated using a reticulation agent.

Bacteriostatic and fertile media developed in the present study are detailed in the results chapter.

Contamination procedure
In order to reproduce a normal use of the device, it was decided to expose the packaging to contamination by wiping the nozzle while dispensing a dose on a sterile compress (30 g/m², NF-EN 29073-1) impregnated with bacteria at a concentration of $10^6$ CFU/cm² (Fig. 1). This bacterial concentration was selected to be equivalent to the mean bacterial charge of the human skin (Wilson,
In order to ensure a homogeneous contamination, the compress was open to form a 100 × 200 mm rectangle and inserted into a sterile stomacher bag. The bacteria solution (5 mL, $10^8$ CFU/mL) was distributed over the whole surface of the pad and after closure of the bag, the liquid was spread by exerting a gentle pressure from the center to the periphery. This procedure was validated by testing using colored media supplemented with phenol red in order to control the homogeneous distribution of the solution.

The contamination simulation was realized over 8 days at room temperature as presented in Table 1. Considering that days 1 and 2 were used for realization of the controls, filling of the packaging and sub-culturing the bacteria, the real simulation procedure started on day 3 by controlling the sterility of the pack and exposition to the contaminated pad in the morning and afternoon at 6 h interval (minimum). Thereafter, from Day 4 to 7, the packaging was exposed twice a day at 6 h interval to the contamination as shown in Fig. 1, and 2 doses were collected. A first dose was also collected on Day 3 before the first daily contamination and was used as a control. On Day 8, a numeration was realized on 1 g of delivered medium. Then, the packaging was open in sterile condition and an aliquot of the formulation remaining in the container was collected and plated on Petri dishes in order control the potential contamination developing into the container.

For determination of the contamination level of delivered doses, each dose was weight and diluted 1/10 and 1/100 (w/v) in Eugon LT100 medium (Fisher Scientific) before plating on trycase soya broth (TSA)-agar solid medium. Eugon LT100 was necessary to neutralize the potential remaining bacteriostatic activity of the medium used to test overprotective packaging. CFU were counted after 48 to 72 h of incubation at 32.5 ± 2.5 °C.

Results

Formulation of the bacteriostatic and fertile media

The literature is rich in media aimed at increasing the cultivability of bacteria. Conversely, bacteriostatic media are rarely available, particularly for a versatile species such as P. aeruginosa. Moreover, whereas some media are presenting a bacteriostatic activity limited to 48 h or even 72 h (Zwisler laboratorium® medium), in the present situation, in order to avoid any artefact in the
evaluation of the packaging, it was necessary that the medium remained bacteriostatic over a minimum of 7 days. Another source of complexity was the obligation to set the fluidity of the medium compatible with the mean fluidity of cosmetic formulations classically employed in the packaging. All compounds also required to be of limited cost and compatible with the safety rules of industrial companies.

Starting from the composition of the Zwisler laboratorium® medium, in order to develop a medium with bacteriostatic activity on *P. aeruginosa* over one week, a series of tests was realized using media of basic formula:

- **DPBS** (Dulbecco's Phosphate-Buffered Saline) (Thermo Fisher) 900 mL
- Glycerol (Carl Roth) 100 mL
- MgSO4 (anhydrous) (Sigma Aldrich) 4 g
- Phenol red (Merck Millipore) 5 mg
- Low viscosity carboxymethyl cellulose (Sigma Aldrich ) 25.0 g
- Bacteriostatic agent (percentage variable between tested molecules)

The principal bacteriostatic agents tested are presented in Fig. 2. Chlorhydric, citric and sorbic acid were tested considering the hypothesis that a pH decrease can affect the growth of *P. aeruginosa* (Sporer et al., 2017). Boric acid was tested as in addition to its acid effects, borate ions can also affect the bacterial growth (Lum & Meers, 1989). Phenoxyethanol, used in cosmetics for its broad preservative activity (ANSM, 2012) was also included into the tests. Sodium benzoate is a food preservative also used in pharmaceuticals formulations. Methyl isothiazolinone (MIT), a powerful synthetic biocide, was also tested albeit of its known skin sensitization activity. We also decided to try the addition of methyl paraben (Methyl parahydroxybenzoate MPOB), the E218 food preservative.

These compounds were tested over a wide range of doses, although only the more representatives are shown in the figure. Capryl glycol, a skin conditioning agent with antimicrobial activity and nalidixic acid, a DNA gyrase inhibitor known for blocking bacterial division were also tested in preliminary studies (data not shown).

Boric acid showed a very interesting bacteriostatic activity on *P. aeruginosa*, particularly when it was used at 0.26% (pH = 6.00). Chlorhydric acid pH = 5.39 also showed an inhibitory effect, but this activity was lost with very limited increases of the pH values. Decreasing the pH to lower value is
lethal to the bacterium. The response of P. aeruginosa to phenoxyethanol was unexpected. Except at the higher concentration employed (0.35% and above), the bacterium started to grow for 1 or 2 days but finally decayed, probably under a toxic effect of this molecule. Sorbic acid was totally unable to block the growth of P. aeruginosa at any concentration tested. Just to a concentration of 0.045%, sodium benzoate was only capable to delay the onset of P. aeruginosa multiplication, but at higher doses no detectable viable bacterium was detected at day 7. Methyl paraben (POBM) also showed an opposite response ranging from a limited inhibition of bacterial growth at 0.1% to a complete inhibition at 0.15%. At day 7, methyl isothizolinone (MIT) passed from a total absence of inhibition at 0.001% to a total inhibition at 0.002%. Citric acid partly blocked the growth of P. aeruginosa at pH = 4.79 and 4.81 but acted as an inhibitor at pH = 4.94. Then, except boric acid all other compounds were unable to inhibit the development of P. aeruginosa over 7 days or the range of active concentration was so narrow (particularly pH values) that their use in reproducible tests was hardly possible. Then, although boric acid is by itself of restricted use because its classification by UE as carcinogen, mutagen and reprotoxic (CMR) 1B or 2 following its concentration, it was selected for production of the bacteriostatic medium. However, the viscosity of this first bacteriostatic medium was too low, although it included carbomethylcellulose. Then, a second series of tests was realized using different reticulation agents. The viscosity of the formula was estimated visually (Table 2) and that of the more suitable solutions was measured using a Viscotester IQ Haake rheoviscosimeter with coaxial cylinders and a CC25 DIN/Ti gap. Considering a mean fluidity (18000 ± 3000 mPas at 25 °C, shear 15 s$^{-1}$) in the range of most of typical cosmetic lotions, hydroethylcellulose (HEC, 25 g/L) was selected to formulate the bacteriostatic medium. The activity of this medium was controlled using a 100 CFU/mL P. aeruginosa inoculum as any modification of the formula can influence the bacteriostatic properties. It was validated considering acceptance of a maximal variation < 50% (0.3 log) according to standard guidelines (ISO 21149).

As previously mentioned, selecting a fertile medium was a simpler target. In order to keep close to the formulation of the bacteriostatic medium, its composition included MgSO$_4$, glycerol and phenol
red at the same concentrations. In order to favor bacterial growth DPBS was replaced by Trypcase Soja Broth (TSB). However, the use of TSB modified the fluidity of the medium and it was necessary to increase the amount of reticulation agent (HCE) to 25.7 g/L to preserve the same rheological properties.

Comparative tests of overprotective and barrier packaging
A total of 16 series of 50 packs were submitted to in-use tests in duplicate by 5 different cosmetics producers. These tests were performed by 7 different operators. They included 5 types of packaging of different protective levels (supposed to be overprotective or barrier) from 3 different producers. These packs were anonymized and identified by letters. They were transmitted without origin reference to the laboratory in charge of the tests. Results are presented in Table 3. One of the pack (A) provided a limited protection level as since all delivered doses 1 and 2 were contaminated and generally with a high number of microorganisms (less than 30% of the delivered doses were under 1000 UFC of contamination). Pack B provided a better protection although some of the delivered doses were upper 1000 UFC of contamination. Pack D reached higher results with one series of tests were all delivered doses showed less that 1000 UFC of contamination, but these performances were irregular. Pack C and finally E showed the best protection efficiency in these tests. As expected by overprotective or barrier packs, all the devices provided total protection of the reservoir content.

Discussion
Faced to the diversity of technical solutions proposed to preserve low-level and preservative free cosmetic products, both packaging and cosmetic industries should gain from the existence of harmonized standards. In addition to regulatory texts, some rare studies have been realized to validate the microbial safety of cosmetic packaging (Crozier, 2018a). However, it is the first time that an experimental protocol was validated over a wide range of devices and associated to a classification grid allowing to distinguish the different degree of microbiological safety conferred by the pack.

Pseudomonas aeruginosa was selected as a model because of its listing as a challenge tests microorganism (ISO11930 2019) and therefore is present in the microbial library of most industrial
sites. In addition, this bacterium is easily growing, it is of mean size, widespread distributed in the environment and mobile through polar flagella. This bacterial model has the drawback to be a safety level 2 microorganism and therefore to require specific installations. It was preferred to a safety level 1 and also ubiquitous species such as Pseudomonas fluorescens (Bossis et al. 2000) since this bacterium is not usually present and manipulated in industry and our approach was aimed at being applicable by packaging users and producers. The choice of P. aeruginosa, besides and because of its versatility, was also probably at the origin of the difficulty to formulate a medium preserving its bacteriostatic activity over 7 days. This can appear trivial, but whereas bactericidal and fertile medium can be easily found in the literature, to our knowledge it is the first time that an exhaustive study was realized on a P. aeruginosa bacteriostatic medium, and particularly over a long time. The efficiency of boric acid to formulate a bacteriostatic medium in comparison to all other tested substances, is supported by its ancient use as a preservative for the transport of urine samples for clinical observations (Lum and Meers, 1989). Boric acid was more recently proposed to preserve urine samples for veterinary applications (Rowlands et al., 2011). The major limitation to the use of boric acid is its classification as hazardous (CMR2 or 1 depending on the exposure concentration) by European Chemical Agency (ECHA, 2010). In the present case, boric acid was employed at a maximum of 0.485% (mass/vol). Then, only manipulations of the bulk should require CMR protection protocols.

Another important aspect of the protocol concerned the development an in-use contamination procedure aimed at reproducing the gesture of the consumer. This is essential since the contact with skin and its natural microbiota is the major source of contamination for cosmetics products. In addition, in case of incomplete closure, the wiping gesture of the outlet can generate a limited pressure and favor a reflux of the product into the container and a contamination of the reservoir. Another problem, is that if a significant amount of product remains at the level of the outlet between successive actuations, if the product is not self-protected this can be sufficient to permit an active bacterial growth and the delivery of a contaminated dose. The contamination protocole presented in Fig. 1 and Table 1 was established in order to evaluate the two parameters, i.e the microbial quality of
the delivered dose and the potential contamination of the reservoir. Although it was impossible to investigate the microbiological protection of the packaging during its whole life, including production and storage, an in-use a test over 7 days appeared coherent with validation protocols in regulatory texts. In addition, validation tests were realized over series of 50 packs in order to take into account the normal variability of the quality. Indeed, generally packs are not produced one by one, but using molds with multiple forms and in spite of the controls all the forms are not strictly equivalent in performances.

Considering the results, it was decided to classify packaging in 6 categories ranging from 0 corresponding to a total absence of protection, to 5 providing a total protection. Devices such as overprotective and barrier, which have been tested in the present study, should be classified between 2 and 4, depending on the percentage of contaminated doses measured during the test. In all these devices, the container is absent contamination. Grade 3 and 4 packaging are differentiated by the tests on fertile medium which are the more discriminant and only level 4 can pass to the tests in respect to the criteria presented in Fig. 3. As this procedure was aimed at been public and used by all interested producers of packaging and cosmetics, it was decided to propose a logotype that should be printed on the identity file of the products. This logotype was deposited to the French organism of control of intellectual property INPI under the property of the cluster Cosmetic Valley. It’s use is free but restricted to the cosmetic packaging and requires that the device was responding the criteria summarized in Table 4.

In conclusion, this work that gathered some of the major industrial partners of cosmetic and packaging industry over five years was leading to the first complete and experimentally validated protocol allowing the selection of cosmetic packaging in regard of the microbial protection level expected. This work has been yet presented in congress to members of international organism for standardization (Crozier 2018; Feuilloley and Roullet, 2018) who will now consider its translation in a new international regulatory text.

Declarations

Ethics approval and consent to participate. Not applicable, this study did not implied clinical
Consent for publication. All authors read and approved the manuscript. LMSM accepts to take in charge publications fees.

Availability of data and material. All experimental results are available upon simple demand to the authors. Results on packaging are kept anonymized in order to avoid any commercial application.

Competing interests. The authors have the following interests. Sylvia Martin is employed by Shiseido, Stéphane Desaint is employed by Groupe Rocher, Christine Borges is employed by RPC Promens, Hélène Lesouhaitier is employed by Johnson & Johnson Santé Beauté, Florence Roullet is employed by Aptar Beauty & Home, Nadine Bresciani is employed by Chanel Parfum Beauté, Anne-Marie Jouault is employed by Sisley, Joelle Luc is employed by Laboratoires Pierre Fabre Dermo Cosmétique, Christophe Masson is employed by Cosmetic Valley. Alain Crozier is heading Clean Cosmetic Consulting. There no product or marketing interest to declare. This does not alter the authors’ adherence to all policies on sharing data and materials, as detailed online in the guide for authors. Other authors have no conflict of interest to declare.

Funding. This work was supported by Evreux Porte de Normandie, Region Normandie and European Union (FEDER).

Authors’ contributions. CC, SM, SD, CB, HL, FL, NB, A-MJ, VP, JL and VJ contributed to the conception of the approach and conducted experiments. AJ contributed to the analysis of the data. CM and AC coordinated the work. AC and MF designed and wrote the manuscript.

Acknowledgements. Authors would like to thank GDR CNRS 3711 Cosm’Actifs and the world cluster Cosmetic Valley for their key role in promoting research in cosmetic sciences.

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Tables
Due to technical limitations, Tables 1-4 are provided in the Supplementary Files section.

Figures
Figure 1

Illustration of the contamination procedure developed in order to reproduce in standardized condition the natural contamination due to the skin contact. For flexible packaging (A), the nozzle of the pack is pressed in contact with the contaminated compress (106 CFU/mL) (1) and the dose (as defined by the provider) is released (2). When it is completely restituted (3), the pressure on the packaging is relieved and the movement is prolonged on a distance of 2 cm (4) to mimic the manual wiping of the tip. This procedure can be adapted to rigid packaging (B) by replacing the pressure on the side of the pack by actuation of the delivery pump.
Comparison of the bacteriostatic activity of media produced in the presence of boric acid
(A), chlorhydric acid (B), phenoxyethanol (C), sorbic acid (D), sodium benzoate (E), methyl paraben (F), methylisothiazolinone (G) and citric acid (H).

Figure 3
Flowchart established to classify the microbial protection potential of packaging.

Supplementary Files
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