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Less preservatives in cosmetics

MiKoKo

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Preface

The project Less preservatives in cosmetics (*"MiKoKo - Mindre Konservering i Kosmetik")* was funded by the Environmental Technology Development and Demonstration Program (MUDP) under the Ministry of Environment and Food of Denmark in 2015.

This report presents the primary results and knowledge gained during the project.

The project was carried out in the period late December 2015 to February 2018 in a collaboration between DermaPharm A/S (DermaPharm) and Danish Technological Institute. The project was supervised by Bettina Ørsnes Larsen and Jette Rud Heltved at the Danish Environmental Protection Agency.

Summary and conclusion

Background
Preservatives are used in cosmetic products to protect the product, and thus the consumer, against contamination by microorganisms during storage and use. Almost everyone uses cosmetic products such as lotions, shampoos and sunscreen regularly, which causes direct exposure to the chemical substances used in cosmetic products. Some preservatives used in cosmetics as well as other consumer products are recognized as a major cause of contact allergy and can as such lead to problems with life-long sensibilisation and potentially severe and even chronically cases of contact allergy. According to The National Allergy Research Centre, at least ten percent of the Danish population suffers from contact allergy to one or more chemical substances. Overall, about six percent of those who have been tested for allergy in Denmark do not tolerate one or more common preservatives. These conditions can have a serious impact on human health, and from a health perspective a reduction of the use of preservatives would therefore be very beneficial.

The project was initiated and carried out in collaboration between DermaPharm and Danish Technological Institute in the period 2015-2018.

Purpose
The purpose of the project was to investigate possibilities that minimize the amount of preservatives in cosmetic products, and thereby minimize the consumer's exposure to preservatives while retaining product safety and quality as required by the European Union (EU) Cosmetics Regulation.

The overall project goals were:

- To develop methods to reduce the amount of preservatives in cosmetic products without compromising consumer safety
- To document consumer safety for one or more packaging solution(s)

The approach - *Hurdle technology*
The approach used throughout the project is based on the concept the *Hurdle Technology*. The *Hurdle Technology* describes intelligent product development that uses different factors (or hurdles) that have an impact on the growth of microorganisms in order to reduce the need for preservatives. Good manufacturing practices (GMP), appropriate packaging, careful choice of the ingredients and formulation type, low water activity and low or high pH values are all known parameters in the control of microbial growth in the absence of, or with a minimum of, preservatives. In the project the approach has been to increase existing specialist knowledge and intelligently combine optimal parameters in the development of cosmetic products with reduced preservation.

Reducing the need for preservation through formulation development
Initially a screening of potential parameters expected to influence the microbial growth in the formulation was performed. The parameters considered most relevant were chosen for further development work in the project. Parameters such as pH and the use of ingredients with more than one function (multifunctionals) in combination with preservatives at reduced levels were studied in great detail, whereas selected ingredients such as oils and their influence on the product protection were studied briefly.

The influence of pH on the effect of preservatives has proven a valuable tool for improving the product protection. It was possible to implement the use of a narrow pH range (5-5.5) in a

number of formulations currently in production at DermaPharm with a significant positive effect. This mere process adjustment is easily implemented, and although changes in pH can affect other formulation properties like viscosity negatively, extensive in-house experience with such formulation adjustments makes pH control very beneficial.

For some formulations, the use of multifunctional ingredients combined with preservatives can further improve product protection. It has been demonstrated that by controlling pH and utilizing multifunctionals, a significant reduction of the preservative level can often be obtained without compromising the product protection.

All in all – the work within formulation development has shown that, by using the *Hurdle Technology* and the parameters studied in this project, a reduction in the use of preservatives can be obtained. A tailored solution for each formulation is, however, often still needed due to the complex nature of cosmetic formulations, and a deeper understanding of the underlying mechanisms could be beneficial – e.g., knowledge of the effect of interactions between ingredients such as preservatives and multifunctionals on the microbial growth in the formulation.

The effect of packaging

Microorganisms are ubiquitous in the environment and on the human body and can be introduced into a product at any given time. Therefore, packaging design plays an important role in the choice of cosmetic preservative systems. Containers and bottles may be designed to make the entry of microorganisms into the products very difficult, for example by using dispensing mechanisms.

Hurdle technology applies concurrent optimisation of several parameters, which ideally can lead to a better protected product. One parameter that is considered important in this respect is the packaging, which to a great extent can constitute a physical barrier for microbial contamination.

Controlled experiments were set up in which one cosmetic product, a body lotion, was dispensed in four different packaging systems and exposed to the same amount of stress in terms of repeated exposures to bacterial contamination. The packaging systems were a jar, a tube, a pump and an airless dispenser. A worst case scenario laboratory test and a consumer test showed that after the body lotion in the jar (used as a positive reference), the body lotion in the tube was the most exposed to contamination. Neither body lotions provided with a pump nor with airless dispensers were affected by contamination despite being subjected to extensive stress of bacteria colonies and containing no preservatives. By using a packaging system such as a pump or an airless dispenser that protects the product from contamination during use, the primary function of any added preservative is to kill contaminating microorganisms brought into the products through raw materials and by the production processes. Therefore, these results showcase the potential decrease of preservatives required by using a pump or airless dispenser.

Albeit complete protection of the body lotion was observed when a pump or airless dispenser was used, using these results in an overall safety assessment of any other cosmetic products provided with a pump or airless dispenser is not readily transferrable. Due to the limited dataset in this study, further evaluation including more products and formulations and more repetitions etc. are recommended, before the impact of the protective capacity of these packaging options can be fully integrated and contribute to the safety assessment of the cosmetic products.

Tools and methods used
During the development work, a number of tools and methods were applied in order to increase the understanding of the ingredients and formulations and to speed up the development process.

Multivariate data analysis was used on a dataset compiled by DermaPharm consisting of formulations developed and challenge tested. The results of the analysis showed clear groupings of ingredients expected to affect microbial growth and of ingredients not expected to affect microbial growth; however, the results conflicted with existing knowledge of some of the ingredients in the groups. The conclusion of the analysis was that the compiled dataset was insufficient for multivariate data analysis and no definite conclusions were drawn. Effective use of multivariate data analysis could show unknown interactions in formulation work, but it would require building a much larger and specific dataset for the purpose. A different approach could be to carry out a design-of-experiments specifically aimed at studying interaction between chosen “hurdles”; however, resources in the current project were allocated other tasks. The possibility of looking deeper into the use of multivariate data analysis for product development of cosmetics is considered a potential worth investigating further in another setting.

A quick analysis method for testing preservative solutions in-house at DermaPharm, ideally comparable to currently used standardised challenge testing, would speed up the development work, and possibilities for developing such a test have been screened. During screening, two different apathogenic organisms were evaluated as possible candidates for challenging the cosmetic formulations, but none of these were evaluated to be acceptable. The overall conclusion of the work is that the development of a quick test was not possible within the means of this project. The specific demands of the challenge tests and the practical performance of these tests as they take place in the test laboratories may very well lead to the development of products with a disproportionally high degree of preservation, and thus a disproportionally high content of preservatives in cosmetic products. However, a more thorough investigation of these matters and the development of a quick test methodology matching commonly used challenge tests will require a substantial amount of time and dedicated resources. Such an effort was beyond the scope of this project.

Implementing *Hurdle Technology* elements in product development at DermaPharm
In this project, it was demonstrated how one can apply the concept of the *Hurdle Technology,* to reduce the concentration of preservatives in cosmetics. DermaPharm used the approach during a recent product development with the purpose of developing a new O/W emulsion (body cream) with very low frequency of skin irritation reactions in the users using a number of the elements of the *Hurdle Technology*.

Using a precautionary principle, a formulation with a reduced risk of eliciting skin irritation reactions was developed utilizing a restricted pH interval of the formulation and ingredients boosting the product protection. The system chosen for this cosmetic formulation cannot, however, replace every preservative in every other product, as evident by the instability it created in an W/O emulsion (ointment), hence illustrating that the chemistry of cosmetic formulations is complex, and often a tailored solution based on several parameters of *Hurdle Technology* is required.

Considerations regarding the use of the project results in the safety evaluation of cosmetic products
Every cosmetic product released to end-user has to undergo careful safety evaluation by a duly qualified safety assessor. As a gold standard, the product has to pass a challenge test in which a bacterial and fungal inoculate is reduced at least by a log factor of 3 and 1 (corresponding to a reduction by a factor of 1000 to 10), respectively, after 14 days and there should be no increase in concentration after day 14. It is obvious from our studies and experience

that the consistency of challenge tests relies more on the capability of the used microorganisms to contaminate a specific cosmetic product than on the taxonomic status of the microorganisms, their initial concentrations, or the conditions of incubation and media of recovery used. We for instance found that it was difficult to contaminate of a range of cosmetic products with a non-pathogenic species of *staphylococcus*. This could indicate that the formulation constitutes a non-physiologically favorable environment for microbial growth. Moreover, we found that the results from specific batches of a cosmetic product may vary between passed and not-passed at two test laboratories. Furthermore, it is known that only a few cases have been described in which a contaminated cosmetic product has been the cause of infections in humans. As no legal or universal challenge test method is currently available, it is up to the safety assessor to evaluate if a product is safe to use based on the details as well as the results of the performed challenge test . We argue that the use of preservatives in cosmetic products should primarily be based on an overall assessment of safety of the product.

Sammenfatning og konklusion

Baggrund
Konserveringsmidler bruges i kosmetiske produkter for at beskytte produktet og dermed forbrugerne imod mikrobiel nedbrydning under opbevaring og brug. Næsten alle bruger regelmæssigt kosmetiske produkter som fx lotion, shampoo og solcreme og udsættes dermed for direkte eksponering for de kemiske stoffer, der bruges i kosmetiske produkter. Nogle af de konserveringsmidler, der anvendes i kosmetik samt andre forbrugerprodukter, er kendt for at forårsage kontaktallergi, og kan i nogle tilfælde føre til problemer med livslang overfølsomhed og potentielt alvorlige og tilmed kroniske tilfælde af kontaktallergi. Ifølge Videncenter for Allergi lider mindst 10 procent af den danske befolkning af kontaktallergi overfor en eller flere kemiske stoffer. Generelt kan ca. 6 procent af de danskere, der er blevet testet for allergi, ikke tåle en eller flere almindelige konserveringsmidler. Dette forhold kan få alvorlige konsekvenser for folkesundheden, og fra et sundhedsmæssigt perspektiv vil en reduktion i brugen af konserveringsmidler være gunstig.

Dette projekt blev udført i et samarbejde mellem DermaPharm og Teknologisk Institut fra 2015 til 2018.

Formål
Formålet med projektet var at undersøge mulighederne for at formindske mængden af konserveringsmidler i kosmetiske produkter og dermed minimere forbrugernes eksponering for konserveringsmidler, samtidig med at produktsikkerheden og kvaliteten bevares i henhold til Europa-Parlamentets og rådets forordning (EF) om kosmetiske produkter.

De overordnede projektmål var:

- At udvikle metoder, der kan reducere mængden af konserveringsmiddel i kosmetiske produkter uden at gå på kompromis med forbrugernes sikkerhed.
- At dokumentere forbrugersikkerheden ved en eller flere typer emballage.

Fremgangsmåde – *Hurdle-teknologi*
Fremgangsmåden i hele projektet er baseret på konceptet *hurdle-teknologi*. *Hurdle-teknologi* beskriver den intelligente produktudvikling, der bruger forskellige faktorer (eller forhindringer), som påvirker mikrobiel vækst, så behovet for konserveringsmidler mindskes. God fremstillingspraksis (GMP), hensigtsmæssig emballage, omhyggeligt valg af ingredienser og formuleringer, reduktion af vandaktiviteten og lave eller høje pH-værdier er alle kendte parametre, som kan kontrollere mikrobiel vækst ved mangel på eller ved et min. af konserveringsmidler. I projektet var tilgangen at øge den eksisterende specialistviden og på intelligent vis kombinere optimale parametre i udviklingen af kosmetiske produkter med færre konserveringsmidler.

Reduceret behov for konserveringsmidler gennem udvikling af formuleringer
De potentielle parametre, som forventes at ville påvirke den mikrobielle vækst i formuleringen, blev kortlagt i starten af projektet, og de parametre, som blev vurderet som mest relevante blev udvalgt til den videre udvikling i projektet. Parametre såsom pH og multifunktionelle ingredienser blev i kombination med reducerede mængder af konserveringsmiddel undersøgt i dybden, mens udvalgte ingredienser såsom olier og deres indflydelse på produktbeskyttelse blev undersøgt overordnet.

Indflydelsen af pH på konserveringsmidler har vist sig at være et værdifuldt værktøj, hvormed produktbeskyttelsen kan forbedres. Indførelsen af et smalt pH-område (5-5,5) i flere formuleringer, som p.t. produceres hos DermaPharm, viste en signifikant positiv effekt. Denne relativt

simple procesændring kan let implementeres, og på trods af, at ændringer i pH kan påvirke formuleringernes øvrige egenskaber, fx viskositet, negativt, kan sådanne typiske formuleringsudfordringer håndteres baseret på den store erfaring på området, og pH-kontrollen er derfor meget fordelagtig.

Brugen af multifunktionelle ingredienser kombineret med konserveringsmidler kan forbedre produktbeskyttelsen yderligere i nogle formuleringer. Det har vist sig, at man ved at kontrollere pH og ved at anvende multifunktionelle indholdsstoffer ofte kan opnå en betydelig reduktion i mængden af konserveringsmiddel uden at gå på kompromis med produktbeskyttelsen.

Alt i alt har arbejdet inden for udvikling af formuleringer vist, at der kan opnås en reduktion i brugen af konserveringsmidler ved anvendelse af *hurdle-teknologien* og de parametre, der er blevet undersøgt i dette projekt. Dog er der ofte stadig brug for skræddersyede løsninger til hver formulering, idet kosmetiske formuleringer har en kompleks natur. En endnu dybere forståelse af de grundliggende mekanismer vil være fordelagtig, fx kendskab til vekselvirkningen mellem de forskellige ingredienser såsom konserveringsmidler og multifunktionelle indholdsstoffer vedrørende den mikrobielle vækst i formuleringen.

Emballagens effekt
Mikroorganismer er allestedsnærværende i miljøet og på menneskekroppen, og de på ethvert tidspunkt introduceres i et produkt. Derfor spiller emballagens design en vigtig rolle, når man vælger emballagesystem til et kosmetisk produkt. Beholdere og flasker kan konstrueres, så det er meget vanskeligt for mikroorganismer at trænge ind i produktet, fx hvis forskellige former for dispensere anvendes.

I hurdle-teknologien optimeres flere parametre samtidig, hvilket ideelt set kan resultere i et produkt, der er bedre beskyttet. I denne sammenhæng spiller emballagen en vigtig rolle, idet den i høj grad kan være en fysisk barriere for mikrobiel kontaminering.

Kontrollerede eksperimenter blev opstillet for at teste forskellige emballagetyper. I et eksperiment blev et kosmetisk product (bodylotion) fyldt i fire forskellige emballagesystemer og udsat for gentagen eksponering over for samme mængde bakteriel kontaminering. Emballagesystemerne var en krukke, en tube, en pumpe og en airless dispenser. En worst-case laboratorietest og en forbrugertest viste, at næst efter bodylotion i en krukke (brugt som positiv reference) var bodylotion i en tube mest udsat for kontaminering. Hverken bodylotion i pumpen eller airless dispenseren var kontamineret på trods af kraftig udsættelse for bakterier og at bodylotionen ikke indeholdt konserveringsmidler. Ved at bruge et emballagesystem som fx en pumpe eller en airless dispenser, som beskytter produktet mod kontaminering under brug, er den primære funktion af konserveringsmidlet at dræbe de mikroorganismer i produkterne, der stammer fra råmaterialer og produktionsprocessen. Resultaterne her demonstrerer dermed potentialet for at kunne reducere den nødvendige mængde konserveringsmiddel ved at anvende emballage med pumpe eller airless dispenser.

Til trods for der blev fundet en fuldstændig beskyttelse af produktet i en pumpe eller airless dispenser i forsøget kan resultaterne dog pt. ikke overføres direkte til en generel sikkerhedsvurdering af andre kosmetiske produkter i emballage med en pumpe eller airless dispenser. På grund af den begrænsede mængde data i denne undersøgelse, anbefales det at udføre flere evalueringer af produkter og formuleringer samt flere gentagelser, før egenskaberne af den beskyttende emballage kan integreres fuldstændig og bidrage til sikkerhedsvurderingen af de kosmetiske produkter.

Anvendte værktøjer og metoder
Under udviklingsarbejdet blev et antal værktøjer og metoder anvendt for at øge forståelsen af ingredienserne og formuleringerne og for at fremskynde udviklingsprocessen.

En multivariat dataanalyse blev udført på data indsamlet af DermaPharm for færdigudviklede formuleringer med resultater af *challenge tests*. Resultaterne af analysen viste tydelige grupperinger af ingredienser, som forventes at påvirke mikrobiel vækst, og ingredienser, som ikke forventes at påvirke mikrobiel vækst, men analyseresultaterne var dog ikke i overensstemmelse med den eksisterende viden om nogle af ingredienserne i grupperne. Konklusionen på analysen var, at det indsamlede datasæt var utilstrækkeligt til multivariat dataanalyse, og derfor blev ingen klare konklusioner draget. En multivariat dataanalyse ville kunne vise ukendte sammenhænge i formuleringsarbejdet, men det vil kræve opbygningen af et langt større og mere specifikt datasæt i forhold til formålet. En anden fremgangsmåde kunne være at udføre et *design-of-experiments* set-up specielt rettet mod undersøgelsen af sammenhænge mellem udvalgte "hurdler"; ressourcerne blev i dette projekt dog allokeret til andre opgaver. Anvendelsen af multivariat dataanalyse til produktudvikling inden for kosmetik anses dog stadig som en mulighed med væsentligt potentiale, og det kunne overvejes at fortsætte arbejdet i andet regi.

En hurtig analysemetode, hvormed konserveringsformuleringer kunne testes in-house hos DermaPharm, og som ideelt kunne sammenlignes med den forhåndenværende og standardiserede *challenge test*, ville fremskynde udviklingsarbejdet, og mulighederne for at udvikle en sådan test er blevet screenet. Under screeningen blev to forskellige apatogene organismer vurderet som værende mulige kandidater, hvormed de kosmetiske formuleringer kunne udfordres, men ingen af disse blev evalueret som egnede. Den overordnede konklusion af screeningen er, at udviklingen af en hurtig analysemetode ikke var mulig inden for nærværende projekts økonomiske rammer. De specifikke krav i *challenge testene* og den praktiske udførsel af tests i laboratorierne kunne meget vel føre til udvikling af produkter med en uforholdsmæssig høj grad af konservering, og dermed en uforholdsmæssig høj grad af konserveringsmidler i kosmetiske produkter. En mere gennemgribende undersøgelse af disse forhold og udviklingen af en hurtig testmetodik, der svarer til de typisk anvendte *challenge tests*, ville kræve mange timer og dedikerede ressourcer. Sådan en indsats vurderes at være udenfor nærværende projekts horisont.

Implementering af *hurdle-teknologien* i produktudviklingen hos DermaPharm
For at demonstrere, hvordan konceptet *hurdle-teknologi* kan bruges til at reducere koncentrationen af konserveringsmidler i kosmetik, brugte DermaPharm konceptet til at udvikle et nyt produkt. Formålet var at udvikle en ny olie-i-vand-emulsion (bodycreme) med meget lav hyppighed af skinirritation hos forbrugerne, og flere dele fra *hurdle-teknologien* blev anvendt.

Ved at anvende et forsigtighedsprincip blev en formulering med en reduceret risiko for at forårsage hudirritation udviklet ved at anvende et begrænset pH-interval i formuleringen og ingredienser, der forstærker produktbeskyttelsen. Systemet valgt til denne kosmetiske formulering kan dog ikke erstatte hvert eneste konserveringsmiddel i ethvert andet produkt, som det blev observeret af den ustabilitet, der blev skabt i en vand-i-olie emulgator (salve), hvilket illustrerede, at kemien i kosmetiske formuleringer er kompleks, og at en skræddersyet løsning baseret på flere parametre i *hurdle-teknologien* ofte er nødvendig.

Overvejelser til sikkerhedsevalueringen af kosmetiske produkter forbundet med brugen af projektresultater
Hvert kosmetisk produkt, der frigives til slutbrugeren, skal gennemgå en grundig sikkerhedsevaluering af en behørigt kvalificeret sikkerhedsassessor. Som en gylden regel skal produktet bestå en såkaldt *challenge test*, hvori bakterie- og svampeinokulat reduceres med mindst en faktor 3 og 1 på en logaritmisk skala (svarende til en reduktion ved en faktor på henholdsvis 1000 til 10) efter 14 dage, og der bør ikke være nogen stigning i koncentrationen efter dag 14. Vore undersøgelser og erfaringer viser tydeligt, at sammenhængen mellem resultater af *challenge testene* mere afhænger af, hvorvidt de anvendte mikroorganismer kan kontaminere et bestemt kosmetisk produkt end af mikroorganismernes taxonomi, deres oprindelige koncentrationer eller betingelserne for inkubering og dyrkningsmediet efter de 14 dage. Vi fandt bl.a., at

det er svært at kontaminere en række kosmetiske produkter med en ikke-patogen art af *Staphylococcus*. Dette kunne indikere, at formuleringen er et ikke-fysiologisk favorabelt miljø for mikrobiel vækst. Endvidere fandt vi, at testresultaterne fra specifikke batches af et kosmetisk produkt i to forskellige testlaboratorier kunne være forskellige. Det er også velkendt, at der kun er beskrevet få tilfælde, hvor et kontamineret kosmetisk produkt har været årsag til infektioner i mennesker. Da der pt. ikke eksisterer én fælles metode til *challenge test*, er det op til sikkerhedsassessoren at vurdere, hvorvidt det er sikkert at anvende et produkt i henhold til detaljerne og resultaterne af den udførte *challenge test*. Vi argumenterer for, at brugen af konserveringsmidler i kosmetiske produkter især bør baseres på en helhedsvurdering af produktets sikkerhed.

1. Introduction

1.1 Purpose

The purpose of the project was to explore new methods, which minimize the amount of preservatives in cosmetic products but retain product safety and quality as required by the European Cosmetics Regulation (EC, 2009). A reduction in preservatives will reduce consumer exposure to preservatives and reduce the risk of contact allergy.

The overall project goals were:

- To develop methods to reduce the amount of preservatives in cosmetic products without compromising consumer safety
- To document consumer safety for one or more packaging solution(s)

As a tool for faster product development at DermaPharm, a quick analysis method for screening preservative solutions was also studied.

1.2 Background

Almost everyone uses cosmetic products such as lotion, shampoo and sunscreen, which causes direct exposure to the chemical substances included. Chemical substances comprise preservatives that are used in cosmetic products to protect the product, and thus the consumer, against contamination by microorganisms during storage and use. A responsible person must be designated for each cosmetic product placed on the EU market and must make sure that the product is safe for human health under normal and reasonably foreseeable conditions (EC, 2009). The responsible person is therefore obliged to let each cosmetic product be assessed for safety by a duly qualified safety assessor before it is made available to the public. However, some cosmetic products might lead to harm from, e.g., allergic reactions due to exposure to allergens including some preservatives. According to The National Allergy Research Centre, at least ten percent of the Danish population suffers from contact allergy to one or more chemical substances. Preservatives in cosmetics are a major cause of contact allergy. In general, about six percent of those who have been allergy tested in Denmark do not tolerate one or more common preservatives, with the frequency varying from substance to substance[1]. These conditions can have a serious impact on human health.

Cosmetic products may be applied several times a day and may come into contact with various external parts of the human body, e.g., the skin (epidermis), hair, nails, lips or external genital organs. Here they leave molecular traces that can be readily detected. This was recently demonstrated in two human volunteers, whose entire skin surfaces were analyzed and mapped. The results revealed that the dominant molecular features on the human surface stem from cosmetic products (Bouslimani et al. 2015). Noticeably, substances from cosmetic formulations were found on the skin of persons who had refrained from showering and applying hygiene and beauty products for three days prior to sampling of the skin surface. This molecular integration of cosmetic substances into our bodies highlights the importance of ensuring a high product safety with the lowest possible health risk for the consumer.

[1] National Allergy Research Centre: https://www.videncenterforallergi.dk/allergi-og-eksem/konserveringsmidler/allergi-konserveringsmidler-kosmetik/allergi-konserveringsmidler-kosmetik-hyppighed/ accessed on 2018.03.18

It is important to emphasize that allergy to a certain preservative may not necessarily arise from the use of cosmetics only, as the same preservatives may be used in cosmetics as well as in other products such as painting, glue or food. Therefore, the total exposure of a specific preservative can originate from many sources, and reducing specific preservatives in cosmetics will only solve part of the problem.

Preservatives are used to protect against contamination and growth of microorganisms and thereby ensure the safe use of cosmetic products. This is an essential aspect to keep in mind when reducing the use of specific preservatives.

1.2.1 Regulation of preservatives in cosmetic products

Regulations concerning cosmetic products in the EU are contained in the European Cosmetics Regulation – Regulation (EC) no. 1223/2009 (EC, 2009) that includes a list of approved preservatives in Annex V. This positive list was started in 1976 to guarantee consumer safety in relation to cosmetic products. The list covers more than fifty compounds belonging to a variety of different chemical families and every compound must be used according to the conditions set in the Annex. It comprises limitations, requirements, label warnings, and the maximum concentrations allowed in ready to use products. The restrictions given in Annex V may be based on a review commissioned by regulatory bodies (e.g. SCCS opinions), but further restriction may also be self-imposed by the formulators based on a concern for public perception of the safety of individual preservatives. For instance, isothiazolinones became a popular alternative to parabens that have been under scrutiny for their alleged estrogenic and antiandrogenic effects, as well as their putative role in promoting cancerogenesis through endocrine disruption (Sasseville et al. 2015). Despite the fact that not all parabens (e.g., methylparaben and ethylparaben) have been linked to these effects, the cosmetic industry is under an enormous pressure from the media that have adopted the alarmist tone of some scientific publications (Darbre PD, 2003). Therefore, many cosmetic manufacturers responded by replacing all parabens with other preservatives such as isothiazolinones that instead have caused multiple cases of contact allergy and in worst case may have induced chronic eczema in some susceptible individuals (Poulsen and Nielsen, 2016).

Additionally, DermaPharm complies with the requirements of several product certifications, such as the Nordic Swan Ecolabel and Asthma-Allergy Denmark, since these certifications are in high demand by customers (see section 1.2.2). The criteria set by these certifications further limits the number of preservatives that DermaPharm can use in their certified formulations.

An important issue is that narrowing down the range of allowed preservatives to the ones in Annex V might, together with other demands set by customers, lead to the risk that the cosmetic manufacturers limit their use to one or few preservative(s), which in turn may lead to sensibilisation and allergic reactions.

1.2.2 Preservatives

Microorganisms are omnipresent and often it is not a matter of “if” but “when” a cosmetic product will encounter microorganisms. Cosmetics can become contaminated with bacteria or fungi for many reasons, such as:

- Contaminated raw materials, water or other ingredients
- Poor manufacturing conditions, e.g., from poor personal hygiene among production workers
- Ingredients that encourage growth of microorganisms, without an effective preservative system
- Packaging that is contaminated or does not protect a product adequately
- Poor shipping or storage conditions
- Consumer use, such as the need to dip fingers into the product

Preservation efficacy testing (challenge tests) is performed to assure that each cosmetic product that is susceptible to microbial growth is not affected by the introduction of microorganisms during normal or reasonably anticipated use by the consumer. However, no method can guarantee adequate microbiological control under any possible conditions.

There are eight major groups of antimicrobial preservatives included in Annex V: paraben esters, phenol derivatives, alcohols, organic acids and their salts, isothiazolinone compounds, formaldehyde releasers, bromonitro compounds and chelating agents. An increase in the prevalence of contact allergy is one out of several concerns that has caused re-evaluation of some preservatives leading to a decrease in the allowed maximal concentration or, in some cases, a total ban of use. A green company policy with an increased focus on skin- and environmentally-friendly personal care products, further limits the choices of preservatives, and DermaPharm uses only the following functional categories of cosmetic preservatives: 'alcohols' and 'organic acids and their salts'.

In contrast to the mode of action of antibiotics, which is usually known at a very detailed level, far less is known about the action of many cosmetic preservatives. Preservatives damage many components of the microbial cell, and it is often difficult to pinpoint the specific events that are responsible for the microbiocidal or microbiostatic activity of preservatives. Below is a brief description of the mode of action of alcohols, and organic acids and their salts.

Alcohols: Phenoxyethanol is one of the most widely used preservative alcohols in cosmetic products (Steinberg DC, 2010, Dayan N, 2016). Phenoxyethanol appears to exert its antimicrobial action via effects on membrane permeability. However, inhibition of essential enzymes may also be involved.

Organic acids and their salts: Weak acids are neutral molecules that can dissociate from a proton to form an anion (e.g., benzoic acid), and therefore they exist in either charged (ionized) or uncharged (nonionized) forms (Figure 1).

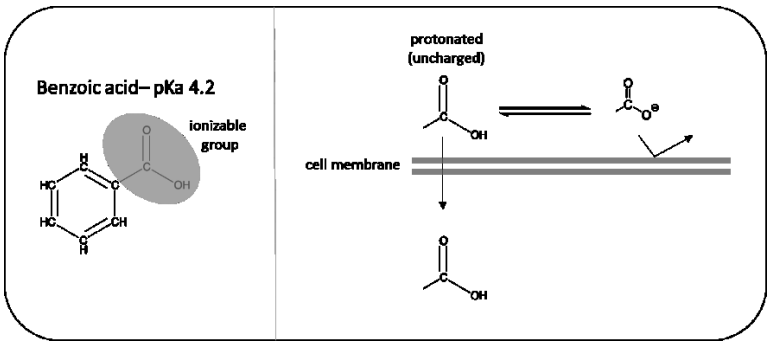


Figure 1 Example of ionization of a weak acid. Weak acids contain groups that can donate protons. Only the fraction of the weak acid that is uncharged can diffuse across membranes (right side of the figure – only the ionizable portion of benzoic acid is shown). The extent of ionization at a given pH is determined by the preservative´s pKa. For example, at a pH of less than 4.2, the uncharged form of benzoic acid predominates, and therefore the majority of the preservative can diffuse across membranes.

Since diffusion across a lipid bilayer requires a molecule to be lipid-soluble, the ionized form of a molecule cannot cross membranes. Therefore, weak acids that are non-protonated cannot diffuse across membranes. At a pH that is equal to a preservative's pKa, equal amounts of the protonated and non-protonated forms are present. The most widely used organic acids and their salts are, according to DermaPharm's knowledge, benzoic acid and its sodium salt, sorbic acid and its potassium salt, and dehydroacetic acid and its sodium salt. Depending on the

specific product and the need for preservation they are used either alone or in combination with other preservatives (Andersen et al. 2015). The salts have a greater aqueous solubility, and can therefore be easier to formulate with, but their addition can affect the pH of weakly buffered acidic products. Sorbates are more effective at higher pH ranges than other organic acids used as preservatives due to their higher pKa value.

Since the cytoplasmic pH is generally higher than that of the growth medium, the weak acid dissociates, releasing a proton and leading to acidification of the cytoplasm, and thus disrupts the proton-motive force, which is essential for cellular metabolism.

The proper use of preservatives to prevent microbial contamination of cosmetics seems to be somewhat arbitrary. As with any other class of raw material, there are a number of factors to consider when choosing the right preservative for a cosmetic formulation – other ingredients in the formula, pH, price, type of packaging, and company policy – among others. Many technical issues are involved with product preservation. The preservative must go well with the other raw materials in the formulation, i.e., it does not colour the product, give it a particular odour, or in other ways affect the appearance and stability of the product. Since DermaPharm strives to develop both environmentally- and skin-friendly products, the preservative must further meet the criteria of several different certifications, including the Nordic Swan Ecolabel, the Asthma and Allergy label, the Allergy Certified label, and the ECOCERT/COSMOS label (see Figure 2**Fejl! Henvisningskilde ikke fundet.**).

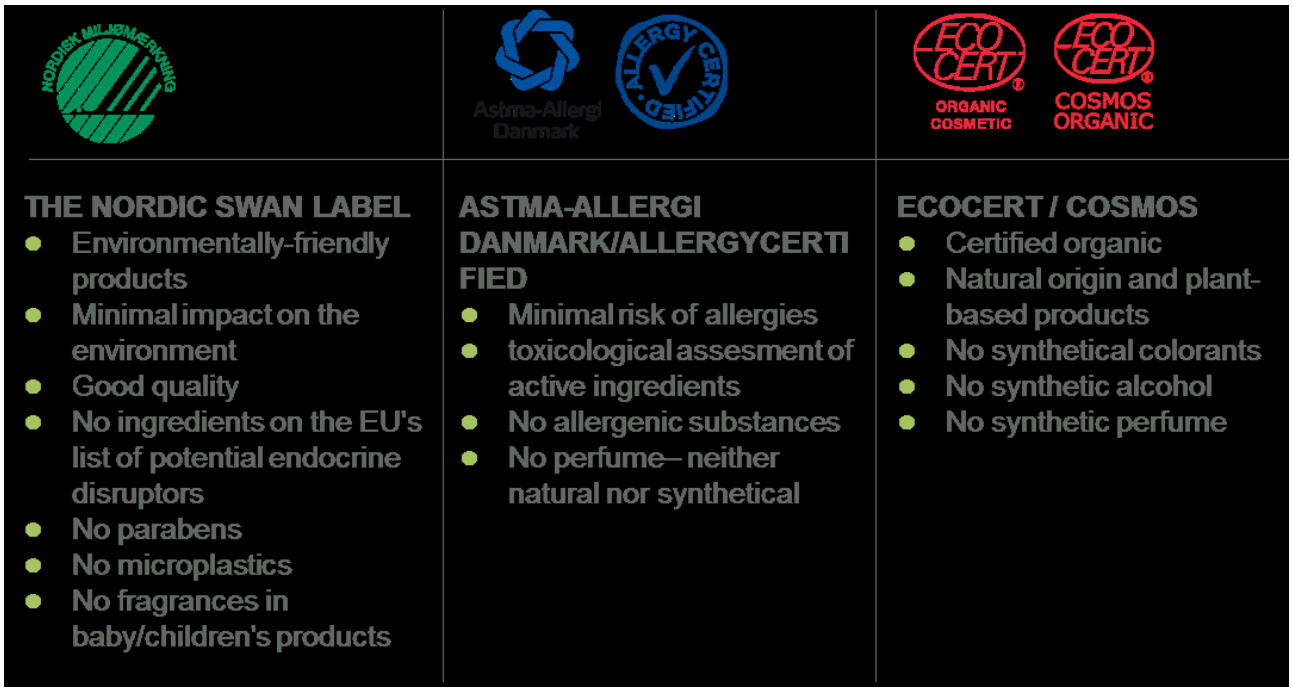


Figure 2 Logos, information and some of the requirements from the different certifications.

Table 1 lists the preservatives that DermaPharm currently use.

Table 1 INCI names of the preservatives used by DermaPharm. All meet the requirements of the Nordic Swan Ecolabel, Asthma-Allergy Denmark, Allergy Certified and the ECOCERT/COSMOS labels, except phenoxyethanol that cannot be used in ECOCERT/COSMOS labelled products.

Single preservatives
Phenoxyethanol
Dehydroacetic Acid and its sodium salt
Benzoic Acid and its sodium salt
Sorbic Acid and its potassium salt

Preservative blends
Phenoxyethanol, Benzoic Acid, Dehydroacetic Acid
Sodium Levulinate, Potassium Sorbate

When all the legal, organizational, and functional requirements have been met, the preservative must act on four different kinds of microorganisms: bacteria (Gram-positive and Gram-negative) and fungi (yeasts and molds). Since these organisms differ widely in their physiology, different preservatives/or blends of preservatives are often needed to obtain a broad spectrum of antimicrobial action.

In the end, finding a preservative that complies with the general consumer perception as well as the different certifications (e.g., Asthma-Allergy Denmark, Allergy Certified, the Nordic Swan Ecolabel, ECOCERT/COSMOS) is somewhat challenging. The focus of Asthma-Allergy Denmark and Allergy Certified is on skin friendly products that minimize the risk of developing skin allergy, the focus of the Nordic Swan Ecolabel is on low environmental impact, biodegradation, and health-issues such as endocrine disruptors, and the focus of ECOCERT/COSMOS is on organic manufacturing and sustainability. In order to comply with all of the above-mentioned certifications, the preservatives used by DermaPharm must meet very different demands (see Figure 2).

1.2.3 Challenge testing

Challenge testing is used for the assessment of growth, survival or reduction of microorganisms when added to cosmetic products under well-controlled conditions. Cosmetic products must be adequately preserved so microorganisms cannot grow and/or survive and thereby pose a health risk to the consumer. According to Annex I of the Cosmetic Regulation, the results of a challenge test should be given in the cosmetic product safety report, and the results of the challenge test should be emphasized in the overall safety evaluation of a cosmetic product. The anti-microbial qualities of a cosmetic product establish and validate the safety of the product over a set shelf-life (EC, 2013). Products with a low microbiological risk (e.g. with a high alcohol level) and single-use products or products that cannot be opened are exempt from the requirement of a challenge test. According to the SCCS notes of guidance, no legal or universal challenge test method is currently available; it is up to the responsible person to decide on the details of the test to be used (SCCS, 2016).

The logic and arguments that form the basis of the challenge test protocols are primarily based on consensus. The test protocols used for cosmetic products among the test labs used by DermaPharm is often derived from the European Pharmacopoeia (Ph. Eur. 5.1.3). The antimicrobial activity of a formulation needs to be broad spectrum, including bacteria (Gram-positive and Gram-negative), yeasts and molds; but not viruses. However, the regulation does not specify the test procedure for the challenge test. According to the guidelines in the European

Pharmacopoeia, the preservative effect of a cosmetic product must be tested against aerobic mesophilic bacteria and fungi recognized as potential skin pathogen species that may be harmful to human health; these are *Pseudomonas aeruginosa* (Gram-negative bacterium), *Staphylococcus aureus* (Gram-positive bacterium), *Candida albicans* (yeast) and *Aspergillus brasiliensis* (mold). An effective preservative system must reduce a microbial population significantly and prevent regrowth. Table 2 shows the requirements for a passed challenge test according to the European Pharmacopoeia.

Table 2 Requirements for a passed challenge test according to the European Pharmacopoeia (Ph. Eur. 5.1.3).

	Criteria	Log reduction			
		2 d	7 d	14 d	28 d
Bacteria	A	2	3	-	No increase
	B	-	-	3	No increase
Fungi	A	-	-	2	No increase
	B	-	-	1	No increase

In a challenge test, a cosmetic formulation is inoculated with the relevant strains at a specific inoculation level, according to the guidelines in, e.g., the European Pharmacopoeia. The inoculated products are stored under well-defined temperature conditions and sampled at the different time intervals specified in Table 2. The survival and/or growth of the inoculated microorganisms is measured over a given time period, and the acceptance criteria must be met. The criteria of acceptance vary between individual challenge test protocols. The European, but not the British nor the Japanese Pharmacopoeias, operates with A and B criteria for acceptance (Ph. Eur. 5.1.3). The A and B differentiation for the European Pharmacopoeia test is related to the chemical risk. The A criteria express the recommended efficacy to be achieved. In justified cases where the A criteria cannot be attained, for example for reasons of an increased risk of adverse reactions, the B criteria must be satisfied.

The ISO 11930 challenge test protocol also operates with A and B criteria. But here, the A and B criteria are related to microbiological risk. If the formulation meets criteria B, the microbiological risk analysis shall demonstrate the existence of control factors not related to the formulation; for example, a protective package such as a pump that provides a higher level of protection than a jar (ISO 11930).

1.3 Proposed strategies for reduction of preservatives

1.3.1 Hurdle technology

One technique to achieve self-preserving products or to reduce the need for preservatives is known as the *Hurdle Technology*. The *Hurdle Technology* describes the intelligent formulation using different preservation factors. Good manufacturing practices (GMP), appropriate packaging, careful choice of formulation and ingredients, low water activity and low or high pH values can all be used to control microbial growth in the absence of, or with a minimum of, preservatives (Varvaresou et al. 2009). Figure 2 illustrates the different parameters (hurdles) that contribute to product protection. Concerning GMP, DermaPharm already upholds a very high level of hygiene in every step of the production and handling of the products. Therefore, it was not necessary to explore these matters as part of the project.

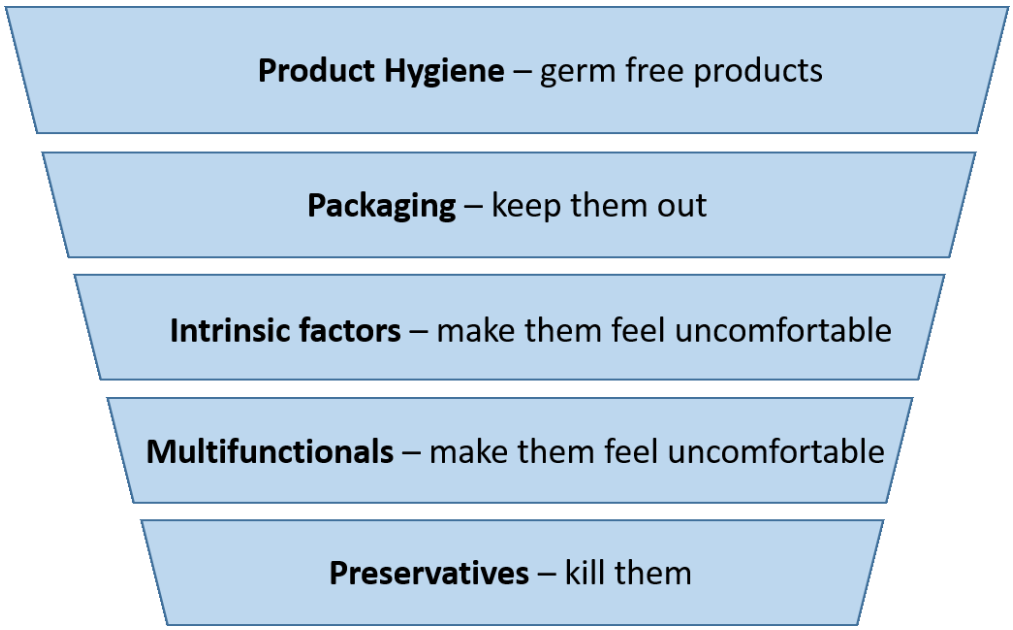


Figure 3 Factors that contribute to product protection – the *Hurdle Technology* approach.

According to the *Hurdle Technology*, the most successful way to increase the barrier towards microbial growth is to consider several parameters at once (stacking the hurdles) and thereby limit the risk (illustrated in **Figure 3**).

There is no universal standard that can be applied to all cosmetic products to assure prolonged shelf-life and consumer safety. Each new cosmetic product needs to be evaluated separately. Formulators are often limited and cannot implement all factors of the *Hurdle Technology*, because development of new cosmetic products is often driven by marketing requirements. Targeting as many appropriate factors affecting the product protection as possible should ensure the best possible protection of the product and hence reduce the overall need for preservatives. Therefore, the *Hurdle Technology* approach will be pursued in this project and selected hurdles will be studied in detail.

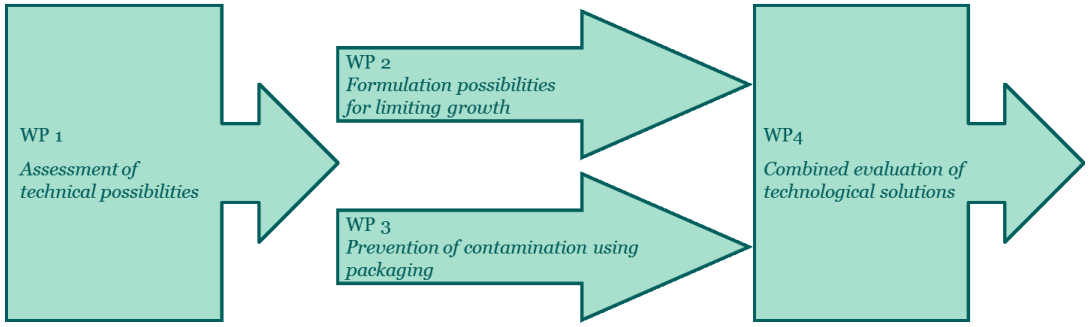
1.4 Project structure

The development work was organised in work packages (WP) and activities under each work package, see Figure 4. In the first phase of the project, WP1, an expert assessment of the technical possibilities within the two areas of focus in the project was made. Development within each focus area was then carried out:

1. WP2. Formulation possibilities to limit growth of microorganisms
2. WP3. Effective prevention of microbiological contamination using suitable packaging

The results of WP2 and WP3 were then combined, and the knowledge used during a specific product developed for DermaPharm's product range (WP4).

Figure 4 Project structure – flow of the four work packages.



2. Screening of technological solutions to reduce preservatives in cosmetics (work package 1)

Work Package 1 (WP1) formed the basis for the two subsequent WPs and included a review of the scientific literature as well as the state-of-the-art within individual cosmetic products, packaging and preservatives. This included knowledge from suppliers, market information and know-how from the relevant industries.

2.1 Evaluation of the technological possibilities for formulations

The survival of microorganisms in cosmetic products depends on many different factors imposed by the formulations (pH, water activity, lack of nutrients, membrane-destabilizing surfactants or chelating agents, etc.), storage conditions, including temperature as well as consumer handling.

During WP1, possible methods to lower the concentration of preservative were discussed and investigated. This process included looking at the correlation between the product formulation and the preserving effect, the impact of specific ingredients on the microbiological growth and the effect of pH on microbiological growth. The investigated approaches are described below and show the results and relevant discussions.

Throughout the project, different product types were chosen, depending on the focus of the specific purpose of activity. Product types have included, e.g., shampoo, lotion and sunscreen. The choice was based on the facts that:

- The product types represent a wide variation within cosmetics
- They are commonly used products, and there is an extensive knowledge of their formulation chemistry as well as many possible alternative ingredients. This knowledge assists in the possible and successful change of the formulation
- They all constitute a significant number/amount of products produced at DermaPharm

Development of microbiological screening methods that are faster than the standardised challenge tests, which today take up to two months from the sample is sent to the results are received, was also considered. That would be a useful tool in product development. Assessment of possibilities included assessing, which microorganisms that are particularly important to detect in relation to ensuring consumer safety (e.g., human-pathogenic organisms).

2.1.1 Study of the amount of preservatives and their distribution in the cosmetic product

The actual concentration of preservatives in the water phase of cosmetic products is expected to be an important factor for limiting the growth of microorganisms. Therefore, methods for determining the actual concentration of the preservatives and their decay in specific products were reviewed. This proved more complicated than expected. Even though the actual preservative concentration could be quantified, it might not necessarily equal the accessible amount of preservatives, rendering a determination of the preservative concentration useless.

Instead, it was considered investigating the octanol/water-distribution coefficient (the Log Kow). That distribution coefficient states the relationship between how soluble a compound is in an apolar solvent compared to water.

If a compound has a high Log Kow-value, then it is very soluble in octanol and in similar substances and the compound can be expected to have a greater solubility in the oil phase compared to the water phase. That means that depending on the preservative system and emulsifier system used in each cosmetic product, the effect of the preservatives can be affected negatively even though the overall concentration of the preservative is high. It was decided that it would be too complex and costly for this project to pursue an analysis of the accessible amount of preservative(s) in a cosmetic formulation without being more certain of a possible beneficial effect on the outcome of the analysis. However, this activity has increased the awareness of this issue as well as of the effect of the solubility properties of the ingredients used.

2.1.2 Challenges regarding the encapsulation of preservatives

In the project, knowledge from other industries about preservatives has been incorporated, e.g., preservatives in paint. That has made it possible to study the possible effect of stabilizing the preservatives when using encapsulation.

The purpose of encapsulating preservatives is to increase their effect and/or stability. By using encapsulation, it is possible to control the release of preservatives into the cosmetic product and achieve optimal use of the preservative. As the technology is too expensive and time-consuming to implement in the chosen cosmetic systems at DermaPharm, it was decided not to include encapsulation in the future work of this project.

2.1.3 The use of multifunctional ingredients in cosmetics

Multifunctional ingredients are molecules with more than one beneficial effect on the formulation or the skin, and they include ingredients such as glycols, glycerol ethers, fragrance ingredients and essential oils. Some of the multifunctional ingredients display a certain ability to boost the antimicrobial efficacy of preservatives in addition to their primary effect (as emulsifier, wetting agent, humectant, etc.). The synergistic combination of multifunctional ingredients with preservatives can be used to achieve the antimicrobial stabilization of cosmetic formulations and minimize the use of preservatives. Consequently, consumer exposure to each used preservative can be reduced and so can the risk of sensitization due to the widespread use of certain preservatives.

DermaPharm uses the multifunctional ingredient caprylyl glycol in combination with the preservative phenoxyethanol in several of their formulations. Caprylyl glycol's main function is as a humectant. It improves skin hydration, but when used with a preservative it boosts the efficacy of the preservative system. The hydrophilicity and humectant capability of caprylyl glycol can affect the permeability of cell membranes, and the wetting ability of caprylyl glycol may therefore enhance the intracellular penetration of phenoxyethanol.

Even though many multifunctionals are available today to help boosting the effect of the conventional preservative system, the multifunctional ingredient may not be compatible with the emulsion type in a given product. DermaPharm has experienced that for instance the viscosities of several emulsions are affected when caprylyl glycol is used. More knowledge is therefore needed to optimize the use of these multifunctionals.

Multifunctionals were considered a relevant 'hurdle' to study further in the project.

2.1.4 Effect of intrinsic properties in product formulation

Based on experience from other industries, e.g., the paint and food industries, intrinsic parameters such as pH and water activity (a_w) were considered possible 'hurdles' when exploring preservative systems for cosmetic formulations in WP1.

Very high or very low pH tends to inhibit microbial growth, but often it is not suitable in cosmetic products, as it can affect the product stability and/or performance and irritate the skin.

The pH of the outer skin layer, the stratum corneum, ranges from 4.5 to 6. This acidity of the skin is referred to as the "acid mantle" (Lee et al. 2006) (Parra and Paye, 2003). The acidic pH is critical for the barrier properties of the stratum corneum, such as controlling enzymatic activities and skin renewal (Schmid-Wendtner and Korting, 2006). The acidic milieu also functions as a defence mechanism against invading microorganisms that thrive at more neutral pH values (Korting et al. 1990), whereas the growth of the inherent cutaneous microbiota is optimal at acidic pH levels. Investigations in both mice and human models verify the assertion that elevated pH impacts barrier function. *In vivo* studies in hairless mice exposed to acetone insult or adhesive film-stripping demonstrated faster barrier function recovery in the presence of acidic buffer solution compared to neutral buffer solution (Mauro et al. 1998). Increased pH has been observed in several skin diseases, such as in eczematous and atopic skin (Ali and Yosipovitch, 2013). Taking the slightly acidic pH of the skin into consideration, acidic buffer substances or skin cleansing agents applied topically may contribute to the maintenance of the normal barrier function of the skin.

The antimicrobial potency of many cosmetic preservatives is pH-dependent. This is illustrated for the preservative benzoic acid in Figure 5.

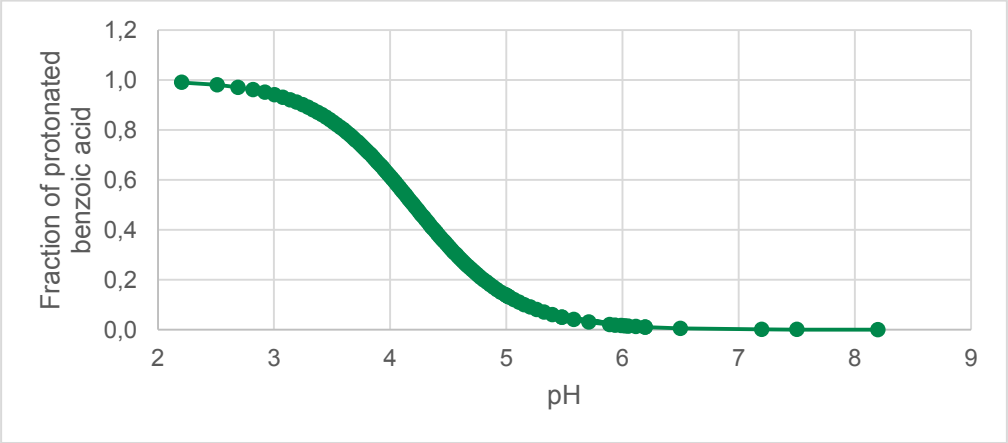


Figure 5 Fraction of protonated (and thus active) benzoic acid at different pH values.

It is the protonated (uncharged) form of benzoic acid that is active, and, as the graph in Figure 5 shows, the fraction of protonated benzoic acid, and therefore the antimicrobial activity of benzoic acid quickly declines with an increase in pH. At a pH above 5.5 the antimicrobial effect of benzoic acid will be very limited.

In the past, many of DermaPharm´s cosmetic products have been formulated with a pH between 5 and 6. In this pH range, a slight decrease of the fraction of protonated benzoic acid is seen (compared to fractions at lover pH values), and therefore a diminished effect on the microbial activity is expected.

Initial results showed that lowering the pH tended to correlate with a decrease in the viscosity of the studied emulsions. That can affect the sensory profile and characteristics of an emulsion

and may not comply with user acceptance. The experiments also showed that decreasing the pH value can affect the stability of some emulsions, as illustrated in Figure 6.

Figure 6 An example of emulsion instability. The three containers have the same oil-in-water (O/W) formulation (a sun spray) at three different pH levels; pH from left to right: 5.9, 5 and 4.5. A phase separation clearly occurs when pH is decreased, and at pH 4.5 the separation of the oil phase (top) from the water phase (bottom) is clearly visible.

This is an example of how a low pH can negatively affect product stability. However, whenever a decrease in pH does not significantly affect the viscosity or product stability, it can be carried out in order to decrease the amount of added preservatives and create a more physiologically undesirable environment for microorganisms. It was considered relevant to further study exploration with reduced pH in this project.

Regulating the water activity (a_w) can be used to prevent the growth of microorganisms, as water is a requirement for growth of all known living organisms. Different microorganisms have different minimum water activity requirements, and Table 3 lists the water activity requirements for the microorganisms used in conventional challenge tests.

Table 3 Water activity requirements for different microorganisms (Barbosa-Cánovaset al 2007).

Test organism	Water activity (a_w)
Staphylococcus aureus	0.9 (anaerobic) 0.86 (aerobic)
Pseudomonas aeruginosa	0.97 (for *P. fluorescens*)
Escherichia coli	0.95
Candida albicans	0.9
Aspergillus brasiliensis	0.85 (for *A. clavatus*)
No microbial proliferation	<0.60

A reduction in the water activity can reduce the growth potential of microorganisms. Therefore, it was investigated if the water activity could be used as a parameter when selecting the preservative level of a formulation.

The measured water activity values of selected cosmetic formulations are reported in Table 4. No apparent correlations were found between water activity values and the outcome of the challenge test.

Table 4 Water activity values for eight selected cosmetic products measured (in triplicates) on a LabMaster Water Activity instrument. Two products were not challenge tested due to their physicochemical characteristics (a body oil and body scrub).

Formulations that passed a challenge test	a_w

Baby cream	0.98
Shampoo	0.97
Sun care	0.97
Acai Serum	0.95
Formulations that failed a challenge test	**a_w**
Sun care	0.98
Sun care	0.97
Formulations that were not challenge tested	**a_w**
Body oil	0.75-0.83
Body scrub	0.43-0.47

In formulations where the water activity was low, such as in the body oil and the body scrub, the measurement accuracy was very low. For each of these formulations, three different measurement values were obtained (hence the range of a_w values). These uncertainties of measurements could be an effect of the high oil content of the formulations, which can hinder the free water in permeating the surface and entering the measuring chamber of the instrument. The instrument may then register a time interval where no water molecules diffuse from the cosmetic product to the air in the measuring chamber, which can lead to a false positive read. This demonstrates that although water activity is a relevant parameter in theory, it can in practice be difficult to rely on the measured values as the oil content increases.

Hygroscopic substances, such as sugars and salts have the ability to attract and hold water molecules from the surrounding environment and have been used to bind free water and preserve food ingredients for centuries. To assess whether the water activity of a formulation can be reduced by the addition of hygroscopic substances, 2% glycerol was added to the sun care formulation with a water activity of 0.97 that did not pass a challenge test (**Table 4**).

The addition of 2% glycerol seemed to increase the water activity slightly (measurements ranging from 0.97 to 0.99), contradicting the theory that glycerol lowers the water activity: The addition of 2% glycerol may not be sufficient to reduce the water activity. However, very high concentrations of hygroscopic substances such as sugars or salts can be challenging in cosmetic products as they do not comply with the sensory profile, aesthetic requirements or the stability of the product. Therefore, water activity may be a more relevant parameter in the food industry where the concentration of added hygroscopic substances can be much higher.

Due to 1) lack of correlation between water activity values and the outcome of challenge tests, 2) the possible high uncertainty of the measurement and 3) the limited possibilities to affect water activity in cosmetic formulations, we conclude that it may not be feasible to use water activity as a formulation parameter (a hurdle) with the purpose of lowering the preservative concentration, at least not based on the approach used here with simple addition of 2 % glycerol.

2.2 Evaluation of technological possibilities within packaging

A number of parameters are considered today when packaging for new formulations are selected. Knowledge of how existing packaging solutions limit the actual microbial contamination packaging seems limited. In the project, a study was carried out regarding the possible packaging solutions that can minimise the risk of contamination followed by chemical or biological breakdown of the products. Focus was given to parameters such as packaging design, material type, oxygen permeability, light permeability and the expected risk of microbial contamination during use. Environmental considerations in the form of resources for packaging were also considered relevant to include in the assessment of packaging (limits to gram packaging per gram formulation).

Microorganisms are ubiquitous in the environment and on the human body and can be introduced into a product at any given time. Therefore, packaging design plays an important role in the design of cosmetic preservative systems. Containers and bottles may be designed to make the entry of the microorganisms into the products very difficult, for example by using dispensing mechanisms.

As described in section 1.3.1 (*Hurdle Technology*), concurrent optimisation of several parameters can ideally lead to a better protected product. One parameter that is considered important in this respect is the packaging, which to a great extent can constitute a physical barrier to microbial contamination.

Controlled experiments with different packaging systems containing the same formulation and exposed to the same amount of stress in terms of repeated exposures to bacterial suspensions, would increase the knowledge of the protection against microbial contamination by packaging and that will be studied further (see section 4).

2.3 Focus of the following work packages

Based on the review of technologies carried out in WP 1, the following focus was chosen for work in WP2 and WP3:

- Effect of increased pH control on growth of microorganisms
- Reducing preservatives and studying the efficacy towards growth of microorganisms and effects on challenge test outcomes
- Use of multifunctional ingredients
- Investigate in-house method for faster screening of microbial stability
- Experiments with different packaging designs to evaluate contamination risk under controlled conditions as well as consumer use

3. Approaches to reduce preservative levels in cosmetics (work package 2)

The work package (WP) objective was to develop formulations with reduced preservatives based on techniques identified in WP1. WP2 includes the development of formulations at laboratory level in a collaboration between Danish Technological Institute and DermaPharm. Relevant tests to document the impact and optimize the level of preservation as well as the quality of the developed formulations were performed. Tests included microbiological tests developed at Danish Technological Institute aimed at screening of the microbial stability before conducting a full challenge test.

3.1 Study of challenge test discrepancies

3.1.1 Background

Cosmetic products are under strict regulation and must be evaluated by a microbial challenge test before they can enter the market. Today, the challenge test criteria set high standards sometimes resulting in the manufacturer to increase the level of preservatives to meet the criteria. This is problematic as many of the consumers that benefit from the functions of cosmetics are individuals suffering from more or less problematic skin conditions such as dry skin or eczema. These individuals often need to use the products continuously for many years and they risk repeated exposure to certain preservatives. The consequence of meeting the high-level acceptance criteria of a challenge test could be that the level of preservative added to meet the acceptance criteria has detrimental effects on the product, as this might induce a higher risk of skin irritation or allergies in the consumers due to high levels and wide spread use of certain preservatives.

3.1.2 Purpose

A current problem that DermaPharm has experienced is that the outcome of the presumably standardized challenge test varies among different test laboratories. These discrepancies have been studied and results are described in the following paragraphs.

3.1.3 Results of challenge tests at different laboratories

The discrepancies of the result of challenge tests performed at different laboratories was evident when the same cosmetic samples were sent to two or three different test laboratories (out of five test laboratories used by DermaPharm) and conflicting results were received, see

Table 5.

Table 5 Discrepancies between challenge test outcome for eight different formulations. Five different laboratories were used for challenge testing. Lab. ID (from 1 to 5) indicate the specific laboratory used for testing the specific product.

Product	Preservative system	Lab ID	Test result
Eye Cream	0,8% blend of phenoxyethanol, benzoic acid and dehydroacetic acid	1	Pass
		2	Fail (mold)
		3	Pass
Face Cream	0,8% blend of phenoxyethanol, benzoic acid and dehydroacetic acid	1	Pass
		2	Fail (mold)
		3	Pass
Baby Ointment	0,4% sodium benzoate	1	Pass
		3	Fail (all*)
Face Sun Care	0,8% blend of phenoxyethanol, benzoic acid and dehydroacetic acid + 0.6% caprylyl glycol	2	Fail (mold)
		4	Pass
Day Cream	0,8% blend of phenoxyethanol, benzoic acid and dehydroacetic acid	2	Pass
		5	Fail (bacteria)
Night Cream	0,8% blend of phenoxyethanol, benzoic acid and dehydroacetic acid	2	Fail (mold)
		5	Pass
Sun Care	0,8% blend of phenoxyethanol, benzoic acid and dehydroacetic acid	2	Fail (mold)
		3	Pass
Sun Care	0,8% blend of phenoxyethanol, benzoic acid and dehydroacetic acid + 0.6% caprylyl glycol	5	Fail (mold)
		4	Pass

Eight products were tested at two to three different test laboratories, and no product gave the same challenge test result in all laboratories.

3.1.4 Discussion of challenge test discrepancies

The discrepancies are most likely the result of small variations in the test method and/or the use of different methods that, among others, differ in the type of microorganisms used, the inoculation of samples, and the acceptance criteria. Discrepancies have been more pronounced when a formulation contains borderline concentrations of a preservative system. The discrepancy is highly problematic, as the result of a challenge test is used in the overall safety assessment of the cosmetic product.

One of the reasons for the variations in the challenge test results could be the different start inoculums that the laboratories use, see

Table 6.

Table 6: Start inoculation of the mold *Aspergillus brasiliensis* for challenge test at the different laboratories used during the project, and which challenge test method they use.

Lab ID	Inoculation of *Aspergillus brasiliensis* (cfu/g or cfu/ml)	Challenge test method
1	1 x 10^4 - 1 x 10^5	ISO 11930:2012
2	approx. 10^5	In House test method
3	approx. 10^4	ISO 11930:2012
4	approx. 10^5	European Pharmacopoeia 2011:5.1.3
5	10^5 of a mixed suspension of yeast and fungi	In House test method in accordance to the European Pharmacopoeia

For instance, for yeast and fungi (mold), lab #1 always uses between 1 x 10^4 – 1 x 10^5 cfu/g (cfu: colony forming unit). Lab # 3 varies from test to test, but mostly inoculates around 10^4 cfu/g of mold. Lab # 2 inoculates around 10^5 cfu/g of mold and lab #5 inoculates a mixed suspension of yeast and fungi (mold). This variation may affect the challenge test outcomes, and perhaps it is easier for a formulation to pass a challenge test on mold at laboratory # 3, since it uses the overall lowest inoculation concentration.

Five of six samples that were tested at laboratory #2, failed the challenge test on mold. The five samples that failed passed the test criteria in the second laboratory they were tested in (lab #1, 3, 4 or 5). A factor that could explain this is the inoculum size of the mold or an unknown factor related to the inoculum or the procedure at lab #2.

It is not specified in the protocol described in the European Pharmacopoeia if pure or mixed challenges should be used (Ph. Eur. 5.1.3). Some of the differences seen in the results can be explained by the test laboratories using pure or mixed challenges . Lab # 5 uses several pure cultures that are mixed together after they have been grown and harvested. The use of a mixed inoculum may be more representative of actual conditions of contamination since microorganisms do not exist as pure cultures in nature, but as interacting populations within microbial communities. However, it introduces the variable of microbial population dynamics into the challenge test. If one assumes that co-metabolism or synergism occurs within a microbial community, mixed cultures may provide greater stress to the preservative system than pure challenges (Brannan, 1995).

In opposition to the above, it has previously been proposed that mixed cultures may be less stringent than pure challenges because one organism may produce metabolic factors that are antagonistic against other microorganisms in the challenge (Frederickson and Stephanopoulos, 1981), or the microorganisms will compete with each other for substrates and growth factors (Hibbing et al. 2010). Also, pure cultures may lead to very resistant and viable cultures as it is well known that many microorganisms secrete autocrine growth factors that stimulate the growth of the specific microorganism under optimal growth conditions.

The growth phase of the challenge inoculum could also influence the result, since the growth phase affects the physiological state of the organisms used, which may again affect the organism´s resistance to preservatives. In the same manner, the number of passages of the stock cultures is an important parameter to control for this test, as continuously propagating cells could lead to changes in phenotypic expression, especially antimicrobial susceptibility.

Another issue is the cultivation of the challenge organisms. Both liquid cultures and confluent

growths on solid media are allowed. A problem with cultivating the bacteria on plates is that biofilms may form, which can make the microorganisms growing in it resistant to antimicrobial agents. This might lead to results were a product fail a challenge test, not because of a failed preservative efficacy but because of the resistance of the microbes growing in the biofilm. This would possibly not reflect a real-life scenario. Liquid growth may be more reproducible as the growth rate can easier be controlled.

3.2 Development of a quick challenge test for screening preservative solutions

3.2.1 Background

The challenge test is a costly and time-consuming method of evaluating the preservative effect of cosmetic products. A survey by GÖCH Arbeitskreis (2011) as well as experience from DermaPharm have shown that the method can yield different results, depending on which laboratory conducts the test, even when the described protocol is used (Ph. Eur. 5.1.3). Furthermore, it takes up to two months from a sample is sent until the test results are received.

The cosmetic market is moving fast and new products are being released frequently, resulting in a need for short timelines in the development process. The time-consuming microbial challenge tests of today do not accommodate the need for fast product development in the industry.

When it comes to identifying the lowest level of preservative that is required in a specific formulation, the challenge test is also not a useful tool. As a tool for effective product development at DermaPharm, the possibility of a new microbiological screening test has been studied. The method should be useful in initial screening of preservation efficiency, the development and optimization of cosmetic products, and it should potentially be possible to correlate it to the results from a challenge test.

A quick challenge test for screening preservative solutions was tested at DermaPharm's laboratories prior to the initiation of the project. The work carried out in this project (MiKoKo) is based on these early results.

The method for a faster in-house analysis was initially based on the apathogenic *Staphylococcus arlettae*, related to the potential pathogenic *Staphylococcus aureus,* which is used as a test organism in the acknowledged challenge tests for cosmetic products, e.g., European Pharmacopoeia (Ph. Eur. 5.1.3).

The challenge test described in the European Pharmacopoeia is based on the addition of 10^5-10^6 microorganisms per gram of product. Sufficient preservation of a given product is evaluated according to the reduction in the concentration of live microorganisms at set time points over a total incubation period of 28 days. To pass the challenge test with A-criteria for bacteria, a reduction in cfus by 2 log units (99%) is required after 2 days, and a reduction in CFUs by 3 log units (99.9%) is required after 7 days. After 14 or 28 days, there must be no increase in cell-numbers. Criteria are also described in the Pharmacopoeia for yeast and fungi.

3.2.2 Purpose

To validate whether an in-house challenge test can be based on the apathogenic *S. arlettae*, tests have been designed to show if equal sensitivity of *S. aureus* and *S. arlettae* to the preservative systems in the tested products can be proven.

3.2.3 Quick in-house challenge test for screening preservative solutions

The quick method was optimized, and comparative tests using *S. aureus* and *S. arlettae* were carried out on products without preservatives to validate whether the method can lead to results comparable to the challenge test according to the European Pharmacopoeia (Ph. Eur. 5.1.3).

Results from incubations in four different products are shown in Figure **7**.

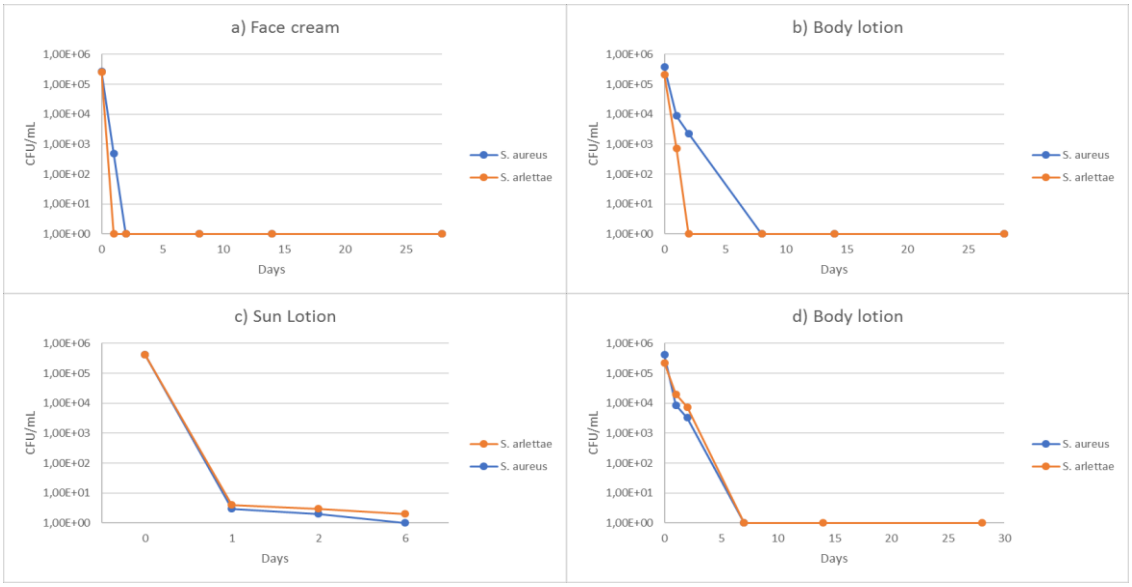


Figure 7 Incubation in four different products: a: face cream, b: body lotion, c: sun lotion and d: body lotion.

In standardized challenge tests, samples are analysed on days 0, 2, 7, 14 and 28. Here, an extra time point at day 1 was included as previous results showed a rapid decrease in cell-numbers within the first two days.

Test results showed a similar decreasing trend in bacterial numbers for both *S. aureus* and *S. arlettae*, confirming a good correlation in biocidal susceptibility for the two bacterial strains (see Figure 7). A further evaluation of the method showed that all added cultures could be retrieved at a satisfactory level (50-130%) on day 0, compared to the initial quantification.

The in-house testing method was also evaluated at the laboratory at DermaPharm. The method was tested on four different formulations based on a body lotion recipe, and with a concentration of preservation lowered from 0.6% to 0.2%. The test formulations were formulated with four different oils: Caprylic/Capric Triglycerides, Dibutyl Adipate, Brassica seed oil or Canola oil. The Brassica seed oil and Canola oil are both rape seed oils, but the two types originate from different sources and vary in their fatty acid composition.

The formulations were inoculated with *S. arlettae* at 10^8 cfu/g product (i.e., heavy contamination) and incubated for 7 days at 37 °C. The product was sampled on day 0, 2 and 7; neutralized to counter the effect of the added preservatives and hereafter analyzed by the pour-plate method using tryptic soy agar.

Results showed that 10^8-10^9 cfu/g product could be retrieved from the four products at day 0. At day 2 and day 7, no viable bacteria could be detected.

The results from the tests at DermaPharm show that although viable bacteria could be retrieved from the products at day 0, *S. arlettae* is not able to survive in the tested products. Survival is not even seen in a product with low concentration of preservative and when *S. arlettae* is added in concentrations 100-1000 times higher than the concentration of *S. aureus* stated in the Ph. Eur. The high sensitivity of *S. arlettae* towards the preservative makes it unsuitable as test organism for in-house challenge testing.

A surprising, but consistent result was the continued decrease of both *S. aureus* and *S. arlettae* to below detection limit in all tested products within a few days - even in the lotion without added preservative. The lotions represent an unfavorable environment to the microorganisms, which could favor their entering into a state with low metabolic activity where cell division is stalled (Oliver 2005, 2010). Therefore, it is not necessarily the effect of the preservative alone that impairs microbial growth and survival, but also other parts of the formulation. This data could indicate that the preservative-free lotion contained other substances with adverse effects on microorganismal growth. Other aspects of the conducted challenge test may also play a role in the outcome, see discussion in section 3.1.

To find an alternative apathogenic test microorganism, further tests were performed on four different body lotions, using *Pseudomonas fluorescens* (DSM50090) as the apathogenic representative of *Pseudomonas aeruginosa* (DSM 1128), see results in Figure 8.

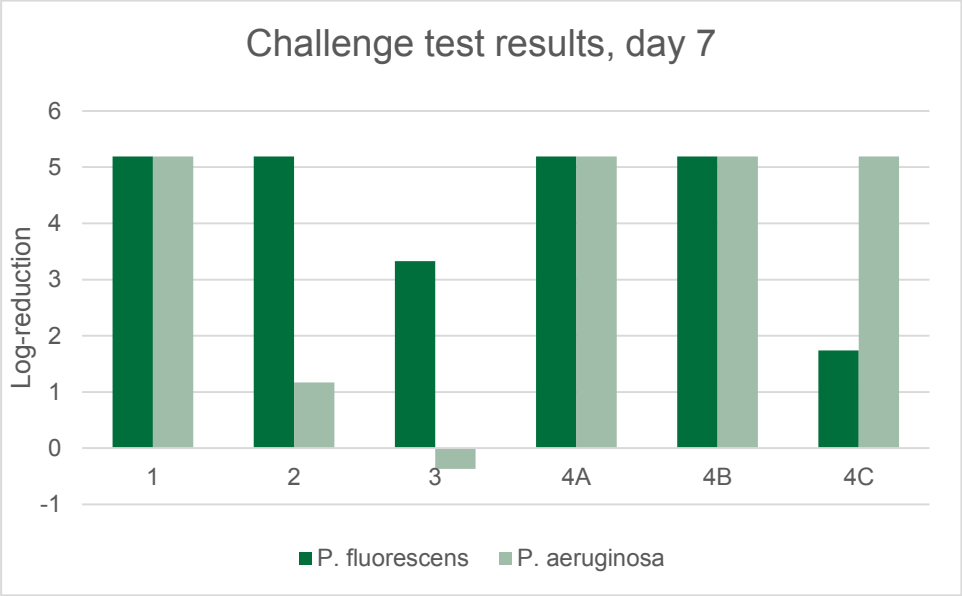


Figure 8 Comparison of *Pseudomonas fluorescens* as an apathogenic representative of *Pseudomonas aeruginosa* for an in-house challenge test. Results show comparative log-reductions after 7 days of incubation in four different formulations. Numbers 1-4 on x-axis represent each formulation, and 4A-C are three replicates.

Although replicates of formulation 4 show some variation, the tests showed that a higher concentration of *P. aeruginosa* could be retrieved at seven days of incubation in two of the four formulations (data not shown).

The tests show that selection and use of apathogenic representatives of the pathogenic microorganisms used in standard challenge tests is not straightforward and might not be possi-

ble. Finding a suitable organism and developing a test protocol will require a more intensive test program.

Also, it is difficult to compare results from this method with external challenge test results due to the described discrepancies between laboratories (see section 3.1).

Advantages of using an in-house test method include the possibilities for faster results possibly comparable to the challenge tests as well as an independence of suppliers, who often perform the challenge test free of charge for their customers. Implementation of the method at DermaPharm does not require any changes in the current facilities, since the test organisms are apathogenic. The time needed to perform the tests is quite high, particularly when the need for suitability testing is considered. Suitability testing is performed in order to get valid results of the test. To prove suitability, each different product needs to be tested for survival and retrieval of the test organisms and this is very time-consuming. Overall, the disadvantages out-weigh the advantages for the time being (see Table 7), and the implementation of the method at DermaPharm is currently postponed.

Table 7 Advantages and disadvantages of implementing the new in-house microbial testing method at DermaPharm.

Advantages	Disadvantages
Fast compared to challenge testing (app. 3-7 days)	Challenge testing still has to be performed to comply with legislation
Possibility for faster product development	High time consumption for in-house testing
Results comparable with *S. aureus* in comparative tests (used in challenge testing)	Low survival rate in additional experiments indicates the need for a new test organism
Possible to implement without significant changes at DermaPharm´s current laboratories	Need for suitability testing for survival and retrieval of test organisms for each different product
Independence of suppliers regarding challenge testing	Less contact with suppliers

3.3 Multivariate data analysis

In cosmetic products, there are often a number of different ingredients and conditions for the formulation. This complicates the investigation of the influence of a single ingredient on the entire formulation; however, multivariate data analysis (using principal component analysis (PCA) and partial least squares (PLS)) was used to find trends of specific factors (ingredients or conditions) that affect the microbial stability of a product and hence the outcome of challenge tests. The purpose of this analysis was to include several of the important factors in the development work following a *Hurdle Technology* approach.

To be able to evaluate the complex nature of cosmetic formulations and their properties, a comprehensive dataset on cosmetic formulations developed at DermaPharm was compiled. The data for each cosmetic formulation included:

- Product type, e.g., shampoo or sunscreen
- Ingredients and amounts
- Approximate pH range of formulation
- Chemical stability
- Approved/disapproved challenge test
- The type of organisms causing disapproved challenge test

80 products have been compared using multivariate data analysis in two separate rounds of analysis work. The first dataset consisted of 28 products, which in the second round was expanded and split into two datasets. Initially the product types were scrubs, sunscreens, shampoos, creams and lotion. Scrubs were not included in the second analysis, since they were considered significantly different from the rest of the formulations and gave a distorted picture of the data.

3.3.1 The results of the multivariate data analysis

The results of the first analysis are based on data from 28 different formulations. The analysis resulted in a grouping of ingredients with possible growth inhibition or possible ingredients with no preserving effect. Some ingredients that often are used as solvents in mixtures, such as water and citric acid belonged to both groups. Their presence in both groups (indicating conflicting effects) may be a result of them being present in almost all formulations or in a subset of formulations with a high challenge test fail ratio.

According to the results from the multivariate data analysis, ceteareths may act as potential inhibitors of microbial growth. They are non-ionic surfactants that function as emulsifiers in the product formulation. Emulsifiers are known to be potential multifunctional ingredients and could possess other functions in the products such as potential inhibitors of microbial growth as suggested by the multivariate data analysis.

It is known that extracts of marine microorganisms can have antibacterial effects, and the ingredient ”Plankton extract” do belong to the group of ingredients with possible antibacterial activity of a formulation according to the multivariate analysis.

Sodium citrate is also a potential growth inhibitor according to the data analysis. Sodium citrate is a pH adjuster, and in literature it has been described as having antimicrobial activities.

Interestingly, the two preservatives sodium benzoate and potassium sorbate were found on the list of ingredients that cannot be associated with antimicrobial activities, according to the multivariate data analysis. Since there is no doubt about the antimicrobial effect of these two preservatives, this result might instead be an indication that multivariate data analysis on the current dataset cannot be fully justified.

The dataset is considered too limited for several reasons - one being the obvious coupling between content of certain ingredient and outcome of the challenge test. The results could also be interpreted as an indication of the complexity of the correlation between the chemical composition of a formulation and the outcome of the challenge test, confirming that it is the entire composition and not only the used preservative that affects the outcome. This illustrates how complex a cosmetic formulation can be, and highlights the potential of working with a *Hurdle Technology* approach.

The second analysis was performed in order to improve the strength of the multivariate analysis by carefully selecting a more relevant dataset. The analysis was based on selected data split into two datasets. The first dataset comprised sunscreen products (51 products in total), and the second dataset contained other products (49 products in total). From this analysis, groups of ingredients that were potential growth inhibitors and ingredients with no effect on growth were again identified. As with the previous analysis, this also gave conflicting results.

Indicative results from the multivariate data analysis showed a tendency for some oils to have an impact on microbial growth. It has been studied briefly in this project with no conclusive outcome of the performed experiments, but an inhibitory effect of certain oils on microbial growth might be a factor worth studying as another “hurdle” for microbial growth in future product development.

Based on the work that was carried out, the multivariate data analysis could be considered a possible tool that will allow us to better understand how individual ingredients interact with each other. Therefore, the analysis can potentially contribute with valuable knowledge in the product development process. However, to obtain useful information it is necessary to compile datasets that cover more formulations and wider ingredient concentration ranges. Due to the lack of data currently available, no further data analyses have been performed as part of this project. However, with an appropriate dataset or even data from a designed set of experiments, the tool can potentially give an overview of trends and important parameters in the product formulations and production and make it possible to gain new information when comparing multiple complex products.

3.4 Effect of pH reduction and reduction of preservatives on preservation

Based on the work carried out in WP1, pH control and reduction of preservatives were focus areas in the development of new formulations. Therefore, the overall aim was to identify optimum pH levels of cosmetic products, as pH has a proven impact on product stability as well as on the microbial resilience of the products.

The objective of the performed work was therefore to lower the pH in fifteen personal care products (O/W emulsions), to assess the effect on overall appearance and stability of the products, and to obtain a better understanding of the correlation between formulation chemistry and physical stability.

There are many reasons why pH is of major relevance to cosmetic products intended for use on the external parts of the human body (epidermis, hair system, nails, lips and external genital organs):

1. It will be easier for products with a slightly acidic pH to pass a microbial challenge test than more pH-neutral products because the acidic environment(s) encountered in many cosmetic products creates a physiological unfavorable environment for microbes.
2. Many of the preservatives used by DermaPharm are organic acids that are significantly more potent at more acidic pH values.
3. Cosmetic products with a slightly acidic pH comply better with the pH of human skin, where a slightly acidic pH regulates key biological activities involved in the maintenance of the stratum corneum (Hachem JP et al. 2003, Ali and Yosipovitch, 2013). Also, slightly acidic products do not notably affect the innate skin microbiota that has evolved to live there (Lambers et al. 2006)

In that way, pH plays a multifunctional role in the preservation of personal care products.
For some medical conditions, skin diseases or skin disorders, the application of a cosmetic product with a low pH might even reduce the impact of the condition. For instance, studies have shown beneficial effects of topical acidic electrolyte water (pH 2-2.7) on the severity of dermatitis and *S. aureus* colonization of the skin in children (Sasai-Takedatsu et al. 1997) and adults (Kubota et al. 1997).

However, it should be noted that low pH could cause irritation in some sensitive individuals, although one study shows that adjusting pH to as low as three does not result in an increased sensitivity towards external stress (sodium lauryl sulphate solution) (Kim et al. 2009).

3.4.1 Influence of pH on emulsion stability

Emulsions are heterogeneous systems consisting of two immiscible or partially immiscible liquids, one of which is dispersed (internal phase) in the other (external phase) in the form of droplets, and stabilized by a third component, an emulsifier. Emulsions are inherently unstable

and they can be subject to instability processes such as flocculation, creaming, coalescence, and in the long term, phase separation. The emulsion system should remain stable throughout the anticipated shelf-life of the final product. Emulsions become unstable depending on the storage conditions such as pH, ionic strength, thermal processing, freeze–thaw cycles, drying and mechanical agitation. Therefore, evaluation criteria for pH-optimization should include both microbial safety as well as product stability during storage.

3.4.2 Influence of pH on microbial growth

pH and acidity are important characteristics that affect the survival and growth of microorganisms in cosmetic products[2]. The pH range for microbial growth and survival is defined by a minimum and maximum value with an optimum pH for growth and survival. Most bacteria are neutrophiles, which means they grow optimally at a pH within one or two pH units of the neutral pH of 7. In comparison, most fungi are more acid-tolerant than bacteria and thrive at slightly acidic pH values and grow optimally at pH 5 or below (Madigan and Martinko, 2006). Neutrophiles, which include the bacteria that are used in the challenge test, have their growth optimum between pH 5.5 and 8.0. Microorganisms grow best at their optimum growth pH. Growth occurs slowly or not at all below the minimum growth pH and above the maximum growth pH. Although microorganisms often grow over wide ranges of pH and far from their optima, there are limits to their tolerance. Drastic variations in cytoplasmic pH can harm microorganisms by disrupting the plasma membrane or inhibiting the activity of enzymes and membrane transport proteins. Furthermore, changes in the external pH might also alter the ionization of nutrient molecules and reduce their availability to the microorganisms. In principle, pH can be used to control microbial growth (Brannan, 1995). Not many cosmetic products are formulated with a pH value that is extreme enough to limit microbial growth of all organisms, as many of the problematic microorganisms can tolerate pH values down to 5. Therefore, pH is not sufficient in itself to adequately control growth in most cosmetic products, and as a consequence other hurdles, such as preservatives, are needed to control growth. However, as a parameter in a *Hurdle Technology* approach, pH is an interesting variable.

3.4.3 Samples and methods

Fifteen O/W emulsions were selected based on product turnover and obtained from the production facility at DermaPharm, Faarup, Denmark. Different emollients and humectants have been used in the 15 emulsions, and the emulsions were of varying viscosities (26,067 cP-211,000 cP), see **Table 8**.

Table 8 Formulations used for testing the effect of reduced pH on emulsion stability. Initial pH values (before pH adjustment) and viscosities are shown for each sample.

ID #	Product type	Inital pH	Initial viscosity (cP)
1	Day cream	5,53	133.000
2	Sun care face	5,4	26.067
3	Bodylotion	5,43	56.333
4	Sun lotion	5,39	49.400
5	Sun lotion	5,26	63.067
6	Night cream	5,75	26.467
7	Sun care face	5,62	46.267
8	Cleansing milk	5,72	34.533
9	Sun lotion	5,37	58.200
10	After sun lotion	5,54	32.000

[2] Acidity is the function of the concentration of hydrogen ions [H^+] in an aqueous solution and is measured as pH.

11	Day cream	5,32	211.000
12	Day cream	5,53	45.667
13	Sun care face	5,3	40.600
14	Sun lotion	5,4	86.000
15	Hand cream	5,36	58.200

pH adjustments and viscosity measurements

For each of the 15 emulsions, samples with two different pH values were prepared (pH 5 and 4.5), and followed over a three-month period. The pH values of the emulsions were adjusted from their initial pH to pH 5.0 and 4.5. The pH was measured with a pH meter (Mettler Toledo Digital pH-meter 1120). The glass electrode was immersed directly into the emulsion, kept for a few seconds to equilibrate, and the pH value noted on the instrument. The viscosities of the emulsions were measured at room temperature with a viscometer (Brookfield Viscometer DV-II+) and subsequently stored under controlled conditions for three months prior to visual inspection. Viscosity of the emulsions was measured at day 1, 7, 30, and 90.

Emulsion stability tests

To determine emulsion stability, the pH-regulated emulsions were stored in the dark in tightly closed, transparent plastic containers under the following conditions: storage at 40°C, storage at room temperature, and storage at 4°C. The physical stabilities of the emulsions were studied by visual inspection for any signs of physical instability such as changes in colour, odour, phase separation, etc., during the storage period.

3.4.4 Results of emulsion stability testing

No considerable changes in colour or odour were observed in the emulsions after 3 months of storage under the specified storage conditions. Ten out of 15 emulsions were stable at both pH levels and under all storage conditions, see **Table 9**.

Table 9 Stability of the 15 tested formulations. Ten out of 15 emulsions were stable under all storage conditions and at both pH levels.

ID #	Product type	Stability at pH 5	Stability at pH 4.5
1	Day cream	Stable	Stable
2	Sun care face	Stable	Unstable at 40 °C
3	Bodylotion	Stable	Stable
4	Sun lotion	Stable	Unstable at all temperatures
5	Sun lotion	Stable	Unstable at 40 °C
6	Night cream	Unstable at 40 °C	Unstable at 40 °C
7	Sun care face	Stable	Stable
8	Cleansing milk	Stable	Stable
9	Sun lotion	Stable	Unstable at 40 °C
10	After sun lotion	Stable	Stable
11	Day cream	Stable	Stable
12	Day cream	Stable	Stable
13	Sun care face	Stable	Stable
14	Sun lotion	Stable	Stable
15	Hand cream	Stable	Stable

The five remaining emulsions showed different degrees of instability under one or more of the specified conditions.

Of the 15 tested formulations, 14 were stable at pH 5. Four out of the five unstable emulsions contained UV filters, indicating a possible connection between emulsion instability and UV filters at low pH values (pH 4.5). One must keep in mind that this was at an elevated temperature of 40°C and that the emulsions in general were stable at both pH 5 and 4.5.

Changes in viscosity

In general, the stabilities of the emulsions were unaffected when the pH was lowered, but the viscosities were affected. Viscosity measurements are important for characterization of emulsions because viscosity could affect the shelf-life of products as well as the user acceptance. The viscosities of the emulsions at the different pH levels are depicted in **Figure 9**.

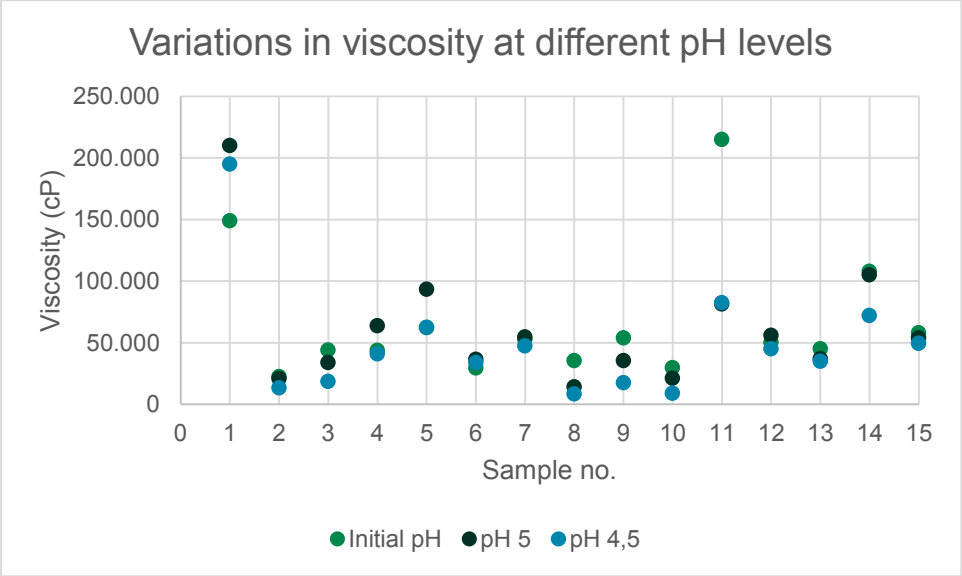


Figure 9 The measured viscosities differ in the individual emulsion samples, depending on pH level. The x-axis represents the individual emulsions (ID # 1-15) and the y-axis represents the viscosity measurement values in centipoise (cP).

The viscosities of some emulsions were significantly affected by the pH reductions, especially at pH 4.5. This is visualized in **Figure 10**, which shows the percentage change in viscosity for each of the fifteen emulsions compared to the emulsions at their initial pH.

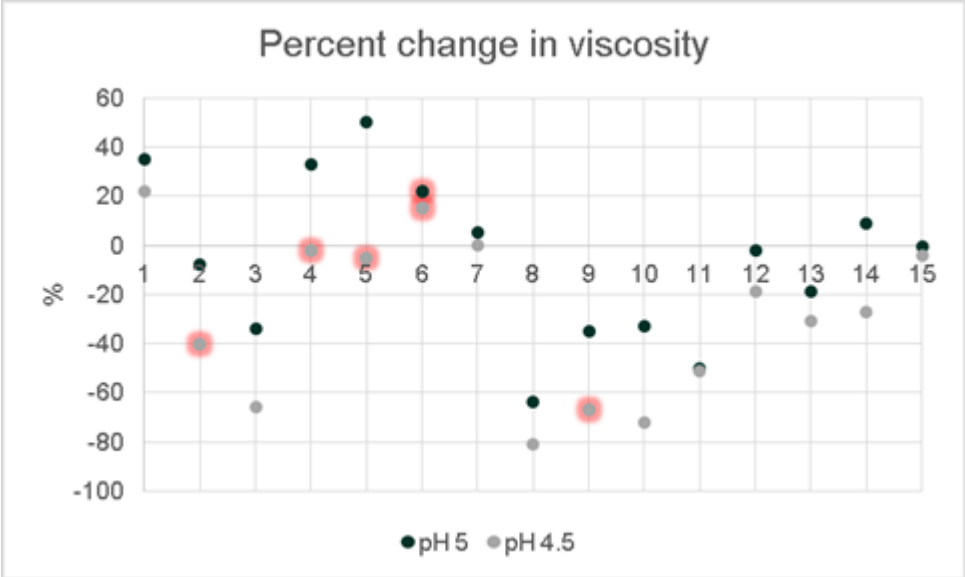


Figure 10 Percentage changes in viscosity with reduced pH in the 15 emulsions compared to their initial pH. The x-axis represents the individual emulsion samples (ID # 1-15) and the y-axis represents the percentage change in viscosity compared to the (individual) emulsions at their initial pH values. Red halos around the dots represent the emulsions that were unstable.

From **Figure 10** It cannot generally be concluded that the viscosity increases or decreases with lower pH values. Nevertheless, the viscosities seemed to decrease in the emulsion samples at pH 4.5, where 12 out of 15 emulsions had reduced viscosities compared to the original emulsions at their initial pH values. Especially the emulsions # 3, 8, 9, 10, and 11 were affected when the pH was reduced to 4.5, where the viscosities dropped more than 50%.

At pH 5, the change in viscosity was not significant enough to change the sensory profile of the emulsions.

The results of the stability tests showed that product stability was, in general, preserved at reduced pH levels (unstable emulsions at pH 4.5 are marked with red halos in **Figure 10**), and that large viscosity changes due to the reductions in pH did not necessarily affect product stability.

Conversely, there were examples of emulsions that maintained the same viscosities at the different pH levels, but nevertheless became unstable at pH 4.5 (emulsions # 4 and 5).

The variation in viscosity could not be accounted for by any specific ingredient.

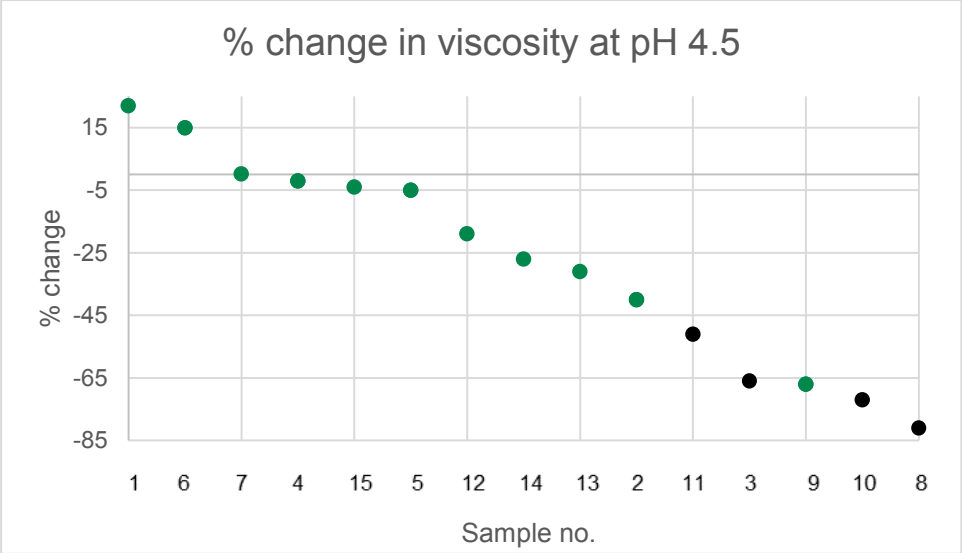


Figure 11 Percentage changes in viscosities at pH 4.5 for the fifteen emulsions compared to their initial pH. The x-axis represents the individual emulsion samples (ID # 1-15). The emulsions are listed from the emulsion with the highest % increase in viscosity to the emulsion with the highest % decrease in viscosity, at pH 4.5. Red halos around the dots represent unstable emulsions. Black dots represent emulsions containing the stabilizer carbomer.

No correlation between viscosity changes and instability or between the amount or type of emulsifier and changes in viscosity was seen (results not shown).Four out of the 15 products contained the emulsion stabilizer and viscosity adjuster carbomer (represented by the black dots in **Figure 11**) and these all had >50% reductions in their viscosities at lower pH values, indicating that the viscosity building effect of the carbomer required higher pH levels. However,

the four carbomer-containing products remained stable. Other two emulsions with >40% reduced viscosity at pH 4.5 that did not contain carbomer became unstable (emulsions # 2 and # 9), indicating that the carbomer could be important for the stability of the emulsions (**Figure 11**).

Other viscosity adjusters that are not pH dependent exist, such as the polymer polyacrylate crosspolymer-6. This was present in emulsions #6 (15% increase in viscosity at pH 4.5) and #7 (0.2% increase in viscosity at pH 4.5), which may explain why their viscosities did not decrease at lower pH levels.

There was no correlation between alterations in viscosities at lower pH levels and the type of product tested (e.g. face cream or body lotion). Neither was there any correlation with the alterations in viscosities at lower pH levels and the emulsifying systems used.

DermaPharm's experience is that, for unknown reasons, emulsions with a high amount of UV filters often fail microbial challenge tests. However, significant lowering of the pH to meet the challenge test criteria does not seem to be a feasible solution for all emulsions containing UV filters, as four out of seven sun care formulations became unstable at a lowered pH. Formulation chemistry is complex, and there is no explanation to why the three other sun care products were stable at a lowered pH.

Nevertheless, DermaPharm has chosen to introduce a narrower pH interval to the production of sun care products along with other cosmetic products (pH 5-5.5) due to the higher efficiency of the used preservatives at lower pH, and the physiologically more unpleasant milieu it creates for both the introduced challenge test microorganisms and other microorganisms that may come into contact with the product during its use. The seemingly small change in the accepted pH range (from 5-6 to 5-5.5) has resulted in a significantly higher percentage of new cosmetic products passing a microbial challenge test.

3.4.5 pH values, preservative reduction and challenge test outcomes

pH values

To confirm that lowering of the pH affects the ability of a formulation to pass a challenge test, we selected four formulations that had all previously passed a challenge test, and increased their pH (Table 10):

The samples were sent to the same test laboratories that performed the original tests and the results are summarized in **Table 10**. Detailed data is given in **Annex 1**.

Table 10 Summary of challenge test results (Ph. Eur) on four formulations at three different pH values. Detailed results are described in **Annex 1.**

Formulation	Test laboratory	Preservation	pH of the tested formulations	Results (Ph. Eur)
Vitamin mask	Lab #5	0.8% blend of phenoxyethanol, benzoic acid and dehydroacetic acid	4.9 - 5.5 - 5.9	Passed with A criteria on all parameters.
Cleansing foam	Lab #5	0.8% blend of phenoxyethanol, benzoic acid and dehydroacetic acid	5.2 - 5.5 - 5.9	Increase in the growth of bacteria with increase in pH. Results (bacteria) pH 5.2: passed with A-criteria

				pH 5.5: passed with B-criteria pH 5.9: failed
Face cream	Lab #4	2% blend of sodium levulinate and potassium sorbate	5.4 - 5.6 - 5.8	Increase in the growth of yeast and fungi with increase in pH. Results (yeast and fungi) pH 5.4: passed with A-criteria pH 5.6: passed with B-criteria pH 5.8: passed with B-criteria
Shower gel	Lab #4	2% blend of sodium levulinate and potassium sorbate	5.2 - 5.5 - 5.8	Increase in the growth of bacteria with increase in pH. Results (bacteria) pH 5.2: passed with B-criteria pH 5.5: passed with B-criteria pH 5.8: failed

Challenge test results on the vitamin mask showed that the formulation can pass the challenge test with A-criteria on all parameters regardless of pH (see Annex I). It is evident that the protection of the vitamin mask is not significantly affected by an increase in pH. This may be due to the relatively high concentration of vitamins in the formulation; some vitamins have previously been reported to have antimicrobial activities or boost the activities of antimicrobial compounds (Youssef DA et al. 2011, Shahzad S et al 2018).

A correlation between an increased pH and a higher bacterial count in the challenge test results (cfu/ml) was found for the cleansing foam. The cleansing foam was not able to sufficiently reduce the bacterial load and therefore failed the challenge test at an increased pH of 5.9.

For the face cream, there was a correlation between an increase in pH and an increase in the growth (cfu/g) of yeast (*Candida albicans*) and fungi (*Aspergillus brasiliensis*), although the face cream still passed the challenge test for these microorganisms (with B-criteria).

The shower gel on the other hand was primarily affected in its ability to kill a Gram-positive bacterium (*Staphylococcus aureus*). Here, the bacterial count (cfu/g) increased with increasing pH to a degree where the bacterial count was too high to pass the challenge test on this microorganism at pH 5.8. The speed of reduction of *S. aureus* is illustrated for the shower gel at the three different pH values in **Figure 12**.

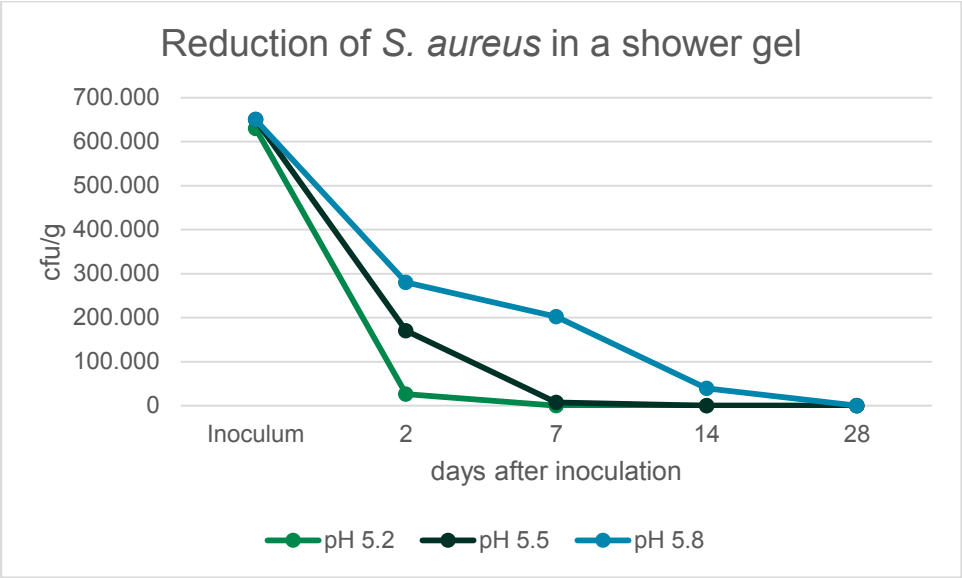


Figure 12 Example of the influence of pH (of a shower gel) on the growth of the Gram-positive bacterium *S. aureus*.

Overall, the results showed that there is a positive correlation between the bacterial count (cfu/g) and pH, and the results confirmed that a low pH increase the ability of a formulation to pass a microbial challenge test.

Preservative reduction

Next, DermaPharm halved the amount of preservative in the same four products at their lowest tested pH values and had them tested at the same challenge test laboratories that performed the original tests. Results are summarized in Table 11 and detailed data is given in **Annex 2.**

Table 11 Summary of challenge test results (Ph. Eur) on four formulations with two different preservative levels at low pH. (Detailed results are described in **Annex 2).**

Formulation	Test laboratory	Preservation	Preservative level of the tested formulations	Pass criteria/Results (Ph. Eur)
Vitamin mask, pH 4.9	Lab #5	Blend of phenoxyethanol, benzoic acid and dehydroacetic acid.	0.8% - 0.4%	Passed with A criteria on all parameters
Cleansing foam, pH 5.2	Lab #5	Blend of phenoxyethanol, benzoic acid and dehydroacetic acid.	0.8% - 0.4%	Increase in the growth of bacteria with a decrease in preservative level Results (bacteria) 0,8% preservative: passed with A-criteria 0,4% preservative: passed with B-criteria
Face cream, pH 5.4	Lab #4	Blend of sodium levulinate and potassium sorbate.	2% - 1%	Increase in the growth of yeast and fungi with a decrease in preservative level Results (yeast and fungi) 2% preservative: passed with A-criteria

				1% preservative: passed with B-criteria
Shower gel, pH 5.2	Lab #4	Blend of sodium levulinate and potassium sorbate.	2% - 1%	Increase in the growth of yeast and fungi with a decrease in preservative level, but not enough to change the pass-criteria (A-criteria)

The results indicated that the microbial count was a little higher in the products with halved preservative, but the microorganisms were still reduced sufficiently to pass the challenge test in all four formulations.

For the vitamin mask, A-criteria were obtained in the original formulation as well as in the formulation with halved preservative amount, although the bacterial count was higher in the formulation with 0,4% preservative. (Table 11).

For the cleansing foam, there was an increase in growth (cfu/g) of bacteria at halved preservative level. Here, the pass-criteria changed from A-criteria in the formulation with 0,8% preservative to B-criteria in the formulation with 0,4% preservative.

For the face cream, there was an increase in growth (cfu/g) of yeast and fungi at halved preservative level. Here, the pass-criteria changed from A-criteria in the formulation with 2 % preservative to B-criteria in the formulation with 1 % preservative.

For the shower gel, an increase in growth of yeast and fungi was also observed, but this increase did not alter the pass criteria (A-criteria).

It should be noted that different inoculum sizes were used for challenge testing of these samples, not only in the different test laboratories, but also among different samples in the same laboratory. For instance, the face cream and shower gel samples were inoculated with 10^6 cfu/g of *C. albicans* in some samples and with $5{,}7 \times 10^5$ cfu/g of *C. albicans* in other samples. This reflects the lack of a specific requirement for inoculum concentration and can affect the number of colony forming units per gram (cfu/g) at the different incubation times, and ultimately the challenge test outcome.

However, in spite of discrepancies and uncertainties regarding the challenge test method (see also section 3.1), the challenge test is for many formulation types mandatory as input in the required safety assessment of cosmetic products.

3.5 Multifunctional ingredients

Multifunctional ingredients (multifunctionals) are substances with more than one beneficial effect on the formulation or on the skin; examples are glycols, glycerol ethers, fragrance ingredients, and essential oils. Some of these widely-used multifunctional ingredients also display a certain antimicrobial efficacy - often only in combination with true preservatives. Multifunctionals may therefore contribute favorable to the restriction of preservatives (Varvaresou et al. 2009).

The below results demonstrate how the multifunctionals caprylyl glycol and glyceryl caprylate can be used to achieve formulations containing less preservatives or no preservatives at all, Multifunctionals such as the glycol caprylyl glycol (wetting agent, humectant) and the glyceryl monoester glyceryl caprylate (emollient, surfactant) are used in a number of DermaPharm´s products.

In section 3.4.5, it was shown that formulating with a slightly acidic pH allowed one to reduce the amount of preservatives by 50% and still have the formulations pass a challenge test. However, when the amount of preservative was decreased, an acceptance of the challenge test with B-criteria was more prevalent than with A-criteria. To investigate if the addition of a multifunctional could affect the challenge test results, a small percentage of either glyceryl caprylate or caprylyl glycol was added to the formulation.

A small percentage of caprylyl glycol was added to three of the same formulations used in section 3.4.5 (a cleansing foam, a vitamin mask and a shower gel). The below results showed that a small percentage of caprylyl glycol improved the microbial protection of the used preservative for all three formulations so they would pass the challenge test with A-criteria (for detailed data, see **Annex 3**).

Table 12 Summary of challenge test results (Ph. Eur) on four formulations with different levels of preservation and multifunctionals as well as low pH. Detailed results are described in **Annex 3.**

Formulation	Lab	Preservation	Preservative level of tested formulations	Result of challenge test (Ph. Eur)
Vitamin mask, pH 4.9	Lab #5	Blend of phenoxyethanol, benzoic acid and dehydroacetic acid	0.8% preservative 0.4% preservative 0.2 % preservative + 0.6% caprylyl glycol	Increase in the growth of yeast and fungi with reduced preservative. Reduced preservative + multifunctional showed equally good results as with maximum amount of preservative. No change in the criteria reached (A-criteria)
Cleansing foam, pH 5.2	Lab #5	Blend of phenoxyethanol, benzoic acid and dehydroacetic acid	0.8% preservative 0.4% preservative 0.4 % preservative + 0.6% caprylyl glycol 0.2 % preservative + 0.6% caprylyl glycol	Addition of multifunctionals lead to A-criteria on all parameters
Shower gel, pH 5.2	Lab #4	Blend of sodium levulinate and potassium sorbate.	2% preservative 1% preservative 1% preservative + 0.6% caprylyl glycol 0.5 % preservative + 0.6% caprylyl glycol 1% preservative + 0.6% glyceryl caprylate 0.5 % preservative + 0.6% glyceryl caprylate	Caprylyl glycol: Growth of yeast and fungi is decreased with 1% preservation + 0.6% caprylyl glycol, leading to an improvement (A-criteria reached). Further reduction of preservative to 0.5% lead to fail on fungi. Glyceryl caprylate: Similar criteria reached, but less effective than caprylyl glycol regardless
Sun care SPF30, pH 5,5	Lab #3		1.2% preservative 0.8% preservative + 0.6% caprylyl glycol	Failed with maximum amount of preservative.

	Reduced preservative + multifunctional lead to pass criteria (B)

The challenge tests summarized in **Table 12** showed that the vitamin mask met A-criteria in a formulation with 0.8% as well as 0.4% preservative. The amount of preservative in the vitamin mask was further reduced to 0.2%, and 0.6% caprylyl glycol was added to the formulation. Challenge test results showed that the bacterial count was comparable with the vitamin mask containing 0.8% preservative.

When the preservative system was reduced from 0.8% to 0.4% in the cleansing foam there was an increase in growth (cfu/g) of bacteria at halved preservative level. Here, the pass-criteria changed from A-criteria in the formulation with 0.8% preservative to B-criteria in the formulation with 0.4% preservative.

When caprylyl glycol was added (0.4% preservative + 0.6% caprylyl glycol) the cleansing foam a very strong protection against all inoculated microorganisms was obtained, and the formulation passed with A-criteria. Adding 0.6% caprylyl glycol allowed us to further reduce the amount of preservative to 0.2% and still meet A-criteria. The addition of 0.6% caprylyl glycol thus allowed for a 75% decrease in the amount of preservative used. Caprylyl glycol is only expected to boost the effect of the preservative and it is not expected that the substance in itself can protect the product adequately, i.e., it is not considered a stand-alone preservative.

The challenge test results of the different variants of the two formulations, the cleansing foam and the vitamin mask, are good examples of how both a physiochemical factor such as pH and the addition of a multifunctional can contribute to the microbiological stability of a formulation, compared to the use of preservatives alone.

To compare the boosting ability of caprylyl glycol to another multifunctional, the glyceryl monoester glyceryl caprylate, a shower gel was challenge tested with different concentrations of preservative and caprylyl glycol or glyceryl caprylate.

When caprylyl glycol was added together with 1% preservative, A-criteria could be obtained, which was better than the challenge test outcome with 2% preservative alone.

When glyceryl caprylate (0.6%) was added together with 1% preservative, there was an increase in growth of bacteria (cfu/g of both *S. aureus* and *A. brasiliensis*) leading to a challenge test result passing only the B-criteria for this formulation. When the preservative was lowered to 0.5% none of the multifunctionals were sufficient for the shower gels to meet the requirements of the Ph. Eur. Criteria, and both samples failed on *E. coli* and *A. brasiliensis*.

Another example of how caprylyl glycol can assist in the preservative efficacy was demonstrated with an SPF30 sun care product that had failed a challenge test with 1.2% of a preservative. With the addition of 0.6% caprylyl glycol to the formulation one could reduce the amount of preservative from 1.2% to 0.8% and pass the challenge test (with A-criteria) (**Table 12** and **Figure 13**).

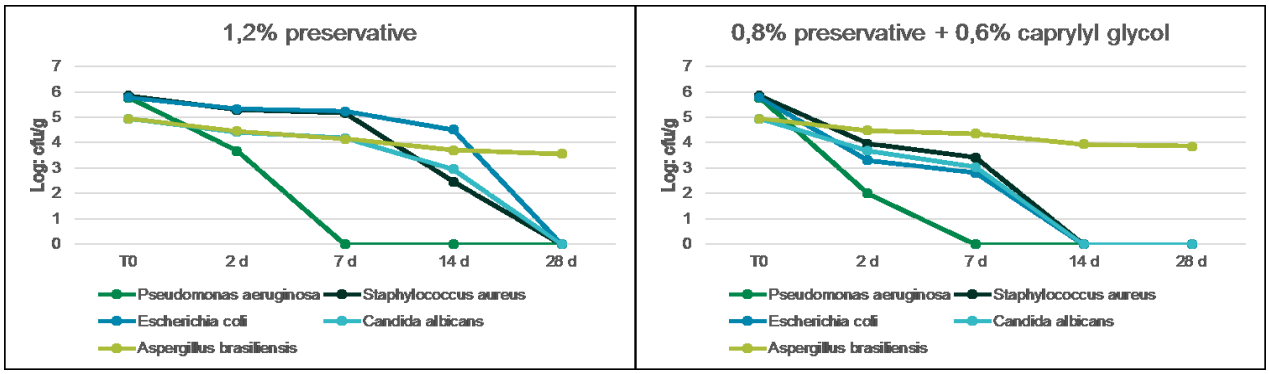


Figure 13. Log reduction of microbial growth in sun care product with (right) and without (left) the presence of multifunctional ingredient.

Overall, the challenge test results indicate that caprylyl glycol (with its suitable chain length) helps to destabilize and disrupt the microbial cell membrane, hereby boosting the activity of the preservative system.

However, it is not always evident that adding a multifunctional will influence the challenge test result in a positive way. Glyceryl caprylate or caprylyl glycol was added to several different formulations that had failed a challenge test, where they did not aid in the microbial stability (results not shown). The reason for caprylyl glycol doing a little better than glyceryl caprylate might be that the preservative used in this formulation contains organic acids, and caprylyl glycol is known to work synergistically with organic acids (Thiemann A and Jänichen J, 2014).

The work with multifunctionals has shown an effect in some formulations and with some preservative systems, but further knowledge is needed to benefit fully from these effects when formulating new products.

An interesting approach to identify new potential multifunctionals is given by the Hansen Solubility Parameters, which is a tool for identifying ingredients with similar solubility properties. The tool might be capable of predicting multifunctional properties of common cosmetic ingredients by comparing solubility parameters of known multifunctional ingredients with solubility parameters of other substances (Hyldgaard & Hyldgaard, not dated). It has not been possible to pursue this approach in this project, but the approach is considered an interesting tool to study in relation to further work with *Hurdle Technology* and the reduction of preservatives.

3.6 Conclusion on formulation possibilities to limit growth of microorganisms

pH

The influence of pH on the effect of preservatives studied in this project has proven a useful tool for improving the product protection. The results showed that it was possible to formulate with lower concentrations of preservative when the pH was lowered. This is likely because pH levels around 5.5 is often a turning point for were many microorganisms thrive. In addition, a reduced pH allows for the lowering of added preservative concentrations in some formulations due to a higher efficacy of the preservative at low pH, which complies with the overall goal of this project. It has been possible to implement a strict pH regulation (pH 5-5.5 compared to previously pH 5-6) in a number of formulations currently in production at DermaPharm with a positive effect, although changes in pH can affect other formulation properties like viscosity negatively. Regarding the safe use of the product it is noted that a pH between 5 and 5.5 generally complies well with the skin pH (4.5-6), but some sensitive individuals might experience irritation.

Multifunctionals
The work on multifunctionals incorporates the knowledge gained in the study of other formulation parameters earlier in the development work. In line with the *Hurdle Technology* concept, it has been shown that utilizing multifunctional ingredients in combination with known preservatives and low pH can improve the product protection further, but tailored solutions to individual products are still needed to obtain the optimal protection because of the complex nature of the chemistry of cosmetic products. Overall – the work within formulation development has shown that using hurdle technology and the parameters studied in this project has a very positive effect on the reduction of preservative use, but a tailored solution for each formulation is often still needed and a deeper understanding of the underlying mechanisms could be beneficial.

Multivariate analysis
During the development work, a number of tools and methods have been studied and applied in order to increase the understanding of the ingredients and formulation as well as speed up the development process.

Multivariate data analysis was used on dataset compiled at DermaPharm consisting of formulations that had already been challenge tested. The analysis showed clear groupings of ingredients expected to affect the microbial growth and ingredients not expected to affect microbial growth, but the results conflicted with existing knowledge of some of the ingredients in the groups and results of the analysis are considered to be inconclusive. Effective use of multivariate data analysis could show unknown interactions in formulation work, but it would require building a specific dataset for the purpose, which was not possible within the scope of this project.

Microbial screening test
The high demands of the challenge tests and the practical performance of these tests as it takes place in the test laboratories may very well lead to development of products with a disproportional high degree of preservation and thus preservatives in cosmetic products. A quick challenge test method for screening preservative solutions comparable to full challenge testing would speed up the development work and possibilities for developing such a test was screened. However, a more thorough investigation of these matters and the development of a quick challenge test for screening that matches these requirements will be very time-consuming and will require a separate project focused on the criteria and standard procedures of the challenge tests. The overall conclusion of the work is that the development of a quick test was not possible within the means of this project.

4. How packaging effects the need for preservation (work package 3)

Optimum packaging protects the formulation from microbiological contamination and thereby reduces the need for preservatives. Products in disposable packaging without risk of contamination and subsequent microbial growth do not have to be preserved. Therefore, we see a great potential in developing products (packaging + formulation) that likewise hinder contamination and can be considered comparable with the disposable containers.

The packaging may itself be a source of microbial contamination. Tests performed by DermaPharm suggest that packaging today can be contaminated before adding the formulation. This may increase the need for preservation of the cosmetic products. Microbiological tests at DermaPharm have shown that up to 36% of the tested packaging types were contaminated (results not shown). Sources of contamination are expected to come from handling the packaging, e.g., during production when packaging is unwrapped and placed on the filling line by production workers.

The impact of contamination of the packaging before filling it with cosmetic products has not been studied in detail. Focus has been on the evaluation of the level of contamination by the end-user.

The amount of preservative can be greatly reduced if it can be demonstrated that the type of packaging used protects against microbial contamination. An essential part of the development of a preservative-free product is the development of packaging solutions that protect against contamination. Therefore, various types of packaging solutions for cosmetic products were studied.

4.1 Evaluation of packaging solutions

Four types of packaging were selected for testing from DermaPharm's current range of packaging solutions (see Figure 14):

- Airless dispenser
- Pump
- Tube
- Jar (as a worst case reference)

Figure 14 Different closures and types of packaging were studied. From left to right: pump, tube, airless dispenser and jar.

DermaPharm is very aware of their choice of packaging material, and many of the solutions live up to the criteria set by, e.g., the Nordic Swan Ecolabel or ECOCERT/COSMOS. The Nordic Swan Ecolabel is very focused on the environment, which is reflected by their criteria for packaging materials for cosmetic products (Nordic Ecolabelling, 2016). Below are some of the requirements set for packaging:

- Requirement 026 Amount of packaging: Packaging quantity used must be justified based on the amount of cream or shampoo it contains to minimize amount of packaging. This calculation takes the content, the weight of the package, the material type and the use of recycled materials in the production of the package (amount of product contained versus the environmental impact of packaging) into account.
- Requirement 027 Type of packaging: It must be possible to sort all types of materials (paper, cardboard, plastic, metal and glass) without tools, with the exception of pump parts.
- Requirement 028 Packaging material metal: metallic packaging or components must only be used in pump parts, sealing foil and spray bottles for hairstyling and shaving cream.
- Requirement 029 Dosability / Dosing systems and emptying level: The device for application must be designed so the optimal dosage is easy. Likewise, liquid soap dispensers must not give more than 2 grams of soap per application to avoid overdosing. Bottles with a pump and bottles for cream and conditioner must have an emptying level of 90 % or have a lid that can be removed without tools.

DermaPharm is aware of all criteria, and the packaging selected for testing lives up to the new requirements.

All four types of packaging (see Figure 14) comply with the above requirements, the volumes and weight of each packaging solution are given in Table 13.

Table 13 Data for selected packaging used for the study of contamination risk, product protection and preservation need.

Packaging type	Volume (ml)	Weight (g)	Ratio (g/ml)
Airless dispenser	50	31	0.62
Pump	785	70	0.09
Tube	50	11	0.22
Jar	50	34	0.68

For plastic jars, it can in general be difficult to fulfill criteria 26 regarding weight because of the need to use a certain amount of plastic to get a sense of a solid container, and the frequent need for an inner and outer wall. Tubes almost always meet the weight criteria, while this is an important parameter when choosing an airless device. Criteria 27 is also important to consider when choosing an airless device, since these can be made of glass combined with other materials. Some manufacturers of packaging have taken that into account in the design. Ordinary bottles with a pump normally comply with the requirements.

All of the chosen types of packaging are today being used at DermaPharm, and they are expected to cover a wide range of protection (low to high) as well as environmental impact (Table 13) and price (data not shown). All four types have been used as packaging for cosmetic formulations and comply with the current needs for oxygen permeability, light permeability and the expected risk of microbial contamination during use.

4.2 Test of packaging regarding the need for preservation

4.2.1 Background for lab scale testing of packaging

A database search using PubMed, Google and Google Scholar on cosmetic packaging revealed a number of different patents, but only a single peer-reviewed study on bacterial contamination and the correlation to the type of packaging and closure type (Brannan & Dille 1990).

Testing two unpreserved cosmetic formulations, a shampoo and a body lotion (Brannan & Dille 1990) showed differences in contamination level as well as in the number of contaminated products for containers with different closures. The unpreserved formulations in the different containers were analysed after 3 weeks (shampoo) and 2 weeks (body lotion) of use by randomized groups of users (18-28 persons).

These tests were conducted using randomized test groups under "normal" use and therefore showed contamination under realistic conditions with naturally occurring microorganisms. For the shampoo, a flip-cap design significantly reduced bacterial contamination, whereas the pump system was most efficient for reducing contamination in the skin lotion (**Table 14**).

Table 14 The relative number of contaminated products in a shampoo and body lotion as well as the average bacterial concentration in the contaminated products (Brannan & Dille 1990). [a] indicates that the results are not significantly different.

Product and closure	% In-use contamination	Log-normalized average contamination (cfu/g product)
Shampoo		
Screw-cap	29 % [a]	$2.37 \cdot 10^5$
Slit-cap	21 % [a]	$1.97 \cdot 10^3$
Flip-cap	0 %	0
Body Lotion		
Screw-cap	71 %	$2.61 \cdot 10^4$
Slit-cap	39 %	$5.62 \cdot 10^3$
Flip-cap	10 %	$9.49 \cdot 10^5$

As part of the study, the mass of used products along with the number of uses were recorded. User data from Brannan & Dille (1990) and Brannan et al. (1987) is shown in Table 15.

Table 15 User data showing average mass of product used, as well as the average number of uses.

Product type	Average used (g)	Average no. of uses	Test period (days)	Study
Body Lotion	32	18	14	Brannan & Dille 1990
Body Lotion	36	23	14	Brannan & Dille 1990
Body Lotion	35	22	14	Brannan & Dille 1990
Body Lotion	25	17	14	Brannan et al. 1987

Body Lotion	25	16	14	Brannan et al. 1987
Body Lotion	42	22	14	Brannan et al. 1987
Average	32.5	19.7	14	
Lotion/day (g)	2.32			

Based on the average amount of product used (in grams) and the test period (in days), an average of 2.32 g sample per day can be calculated. Based on data from EU consumers, the Scientific Committee on Consumer Safety (SCCS, 2016) has estimated the daily use of creams and lotions for an average person a little higher: Body lotion 7.82 g, face cream 1.54, hand cream 2.16 g. The values correspond to the 75th percentile of a European average use (Hall et al. 2011) – i.e., above the average amount used (50 percentile). For the laboratory testing we wish to perform the experiments using a realistic worst case scenario with respect to contamination during normal use of the products. The risk of contamination from the user is considered highest at a low dosage each time the product is used, so for this purpose the average of 2.32 grams/day from **Table 15** is considered the most relevant to use.

4.2.2 Designing a laboratory-based test

A laboratory-based test with artificially contaminated cosmetic products should be based on a realistic use. Although use depends on product type, 2-3 grams/day could be considered as "normal use" for creams and lotions and therefor form the basis of a laboratory-based contamination test. For pump-systems a realistic use could be 1-2 portions.

The chosen test conditions should simulate worst case within the range of normal use. To estimate the degree of bacterial contamination, the formulation should initially be sterile and without preservatives (or preserved with very low concentrations).
The bacterial concentrations on the skin of hands and fingers will depend on the hand hygiene of the user, but will in average contain 10^7 bacteria/cm^2. The Family *Staphylococaceae* has been shown to constitute 1.7-28.2% of this (Edmonds-Wilson et al. 2015). As a starting point for the laboratory tests, it is assumed that 10% of the total skin microbiota will be deposited on the contact zones of the packaging.

A primary problem of concern with respect to performing such experiments is to have a sterile start-up product. To this end a formulation was identified, which was stable during the process of sterilization by autoclave (results not shown).

Pretests were performed to evaluate bacterial survival in the test product after 6 days of incubation. A body lotion, which is produced and sold in large quantities, was used as the test product. No preservatives were added to the formulation as this test was designed to test the packaging (as a barrier for contamination) and not preservative efficacy. The bacterial challenge solution contained a 1:1 mix of non-motile *Staphylococcus aureus* and motile *Pseudomonas aeruginosa*.

The pretests showed that *S. aureus* could not be retrieved from the product after several days of incubation. The products were therefore incubated solely with the motile *P. aeruginosa*.

4.2.3 Consumer type testing

To validate the results achieved under the controlled laboratory tests of contamination of a body lotion in different packaging systems, DermaPharm performed user tests on selected packaging types. The results from the laboratory and consumer tests were compared, and both tests were used in the assessment of the most promising packaging solution to be used as a hurdle for microbial growth in cosmetic products.

4.2.4 Results from packaging tests in the lab and through consumer testing

Consumer testing was divided into two parts:

- Worst case scenario in the laboratory
- Consumer test at DermaPharm and Danish Technological Institute

For both tests, the packaging material was sterilised with ethanol and the cosmetic formulations were sterilized to ensure a completely clean product before the contamination test. A standard body lotion was used for the tests.

4.2.4.1 Packaging test – Worst case scenario laboratory test

The packaging and cosmetic formulations were artificially contaminated in the laboratory by exposing the opening of three different packaging types (tube, pump and airless dispenser) to high concentrations of *Pseudomonas aeruginosa*. Each packaging was tested in four replicas containing the same formulation.

The exposure was based on a worst case exposure compared to the normal use patterns, where the packaging was contaminated 12 times over 8 days. Each contamination was carried out twice a day on six of the eight days the test period lasted, and subsequently stored four weeks with no use to let the bacteria grow. A jar was used as a positive control, as it was determined to be the most vulnerable packaging type. It has a high degree of contact with microbial contaminated skin and air.

After the four weeks without additional contamination, the degree of contamination was determined. Body lotion from each of the containers was analyzed for the presence of viable *P. aeruginosa.* Body lotion was analyzed from the (1) nozzle of the packaging and (2) the bulk of the product.

A picture of the plating results for each packaging type appears in Figure 15 and an overview of the results is shown in Table 16.

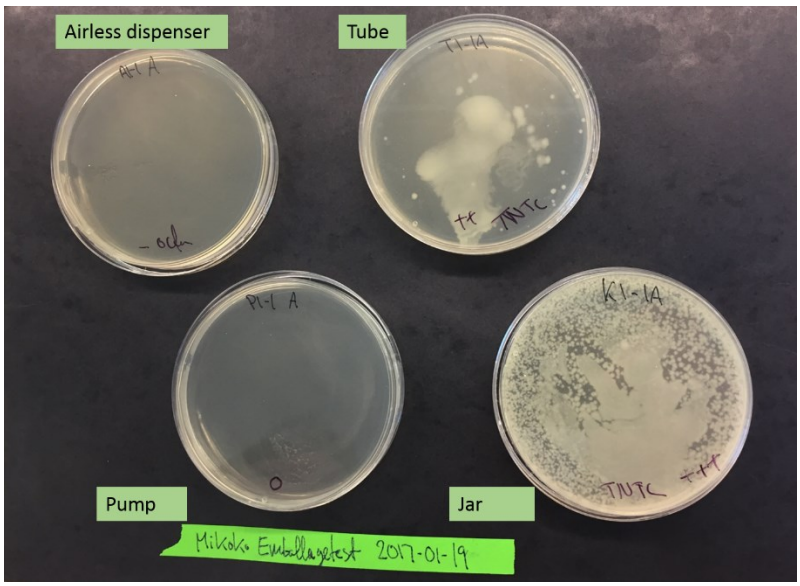


Figure 15 Agar plates with sample platings from the four different packaging types.

Table 16 Results from the worst case scenario packaging test. TNTC: Too numerous to count. 0 denotes no visual colonies. + denotes less than 20 colonies. ++ denotes 20-100 colonies. +++ denotes above 100 colonies. The detection limit of the method is <10 cfu/g.

	Bacteria colonies			
	Jar	**Tube**	**Pump**	**Airless dispenser**
Material from the opening of the packaging (nozzle)	TNTC	TNTC/++	0	0
Material from the middle of the packaging (bulk)	TNTC	+	0	0

The pump and airless containers could protect the bulk of the body lotion as well as the body lotion in the nozzle.

4.2.4.2 Packaging test – realistic consumer test

Contamination risk of consumer products like body lotions is dependent on the pattern of use, the contamination level of the users and the storage environment. Validation of the laboratory tests was therefore deemed necessary through a consumer use test.

The consumer use test was set-up at DermaPharm and Danish Technological Institute where the body lotion samples in three different packaging types were placed in rest rooms. It was prioritized to use locations, which were known to be used multiple times on a daily basis. The tests were conducted from the 25th April to the 16th May 2017 at DermaPharm and from the 15th May to the 6th June 2017 at Danish Technological Institute.

From each location, two packaging systems of each type were selected based on were the largest amount of body lotion was used. The degree of contamination was determined by using the same method as in the laboratory contamination test; analyzing the number of viable bacteria from the opening at the packaging and at the bulk of the product. The number of viable bacteria was enumerated after 24 and 48 hours of incubation at 37 °C to allow for the growth and detection of slower growing bacteria.

A picture of the plating results for each packaging type appears in Figure 16 and an overview of the results is shown in Table 17.

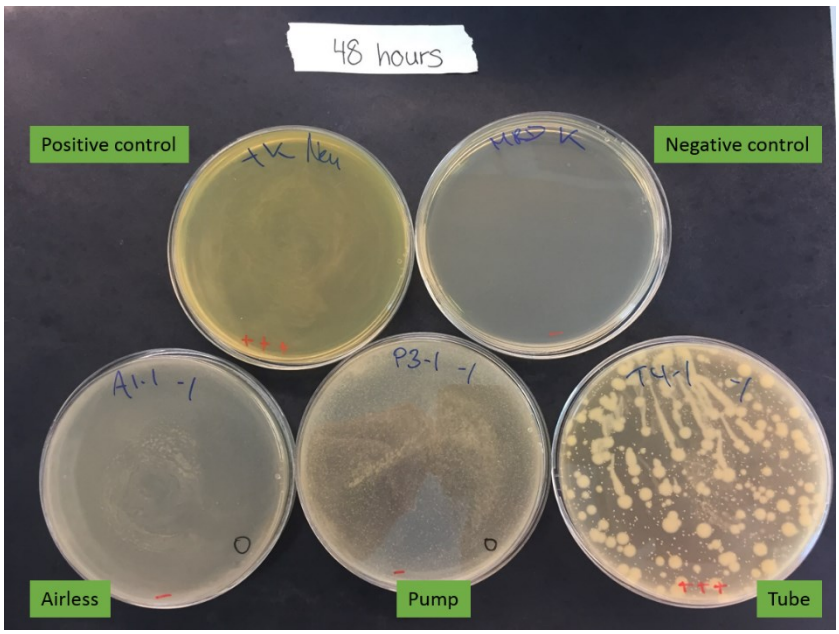


Figure 16 - An agar plate from the three different packaging types together with the positive and negative control.

Table 17 - Results from consumer packaging test after 24 hours. 0 denotes no visual colonies. + denotes less than 20 colonies. ++ denotes 20-100 colonies. +++ denotes above 100 colonies. The detection limit of the method is <10 cfu/g.

	Bacteria colonies after 24 hours		
	Tube	**Pump**	**Airless dispenser**
Material from the opening of the packaging (nozzle)	+++/0	0/0	0/0
Material from the middle of the packaging (bulk)	+++/0	0/0	0/0

As seen in the laboratory tests, no contamination was seen in the pump and airless dispenser. For the tube, a high degree of contamination was seen in two out of four replicates of test in a tube in the lotion from both nozzle and bulk.

4.2.5 Conclusion on packaging tests

The worst case scenario test and the consumer test showed that the tube packaging was most exposed to contamination. The pump and airless dispenser were unaffected by the contamination even though no preservatives were added to the body lotion.

By using a packaging system like the pump and airless dispenser that protects the product from contamination during use, the primary function of the added preservative is therefore to kill contaminating microorganisms brought into the products through raw materials and the production process. Therefore, using a pump or airless dispenser allows for a potential decrease in the needed level of preservative. Albeit complete protection of the products was observed when using a pump or airless dispensers for this particular formulation in this limited study, this might not always be expected. In this study, the packaging was sterilized with ethanol and the formulation was autoclaved before filling. Further evaluation on more products and formulations is required before these results potentially can be used as a general parameter in assessing the need for preservation, e.g., in a safety assessment of a given cosmetic product.

5. Using the knowledge gained for product develeopment at DermaPharm (Work Package 4)

The overall purpose of work package 4 (WP4) was to combine and use the knowledge gained in the previous work packages in development of a cosmetic product at DermaPharm – there by documenting the effect of using some of the different aspects of *Hurdle Technology* studied during the project.

5.1 Confirming the effect of reduced preservation, multifunctionals and pH on chemical and microbiological stability

In WP2 optimal preservation was obtained by using multiple factors (hurdles). Experimental design has the benefit of making efficient and focused experiments to collect useful information while varying factors simultaneously. The aim of this experimental design was therefore to evaluate the correlation and the possible interactions between three variables in the formulation: preservative concentration, multifunctional concentration and pH value. All three variables have been shown to have a positive effect on product protection during formulation development work. The purpose of the design was to confirm this knowledge and ideally result in further knowledge on the interaction between these variables.

5.1.1 The experimental design

A basic body lotion was formulated for the experimental design without preservative, multifunctionals and with no final pH adjustment. Each variable; preservative concentration, multifunctional concentration and pH, was then adjusted to three different levels, which were chosen based on knowledge gained earlier in the project (see section 3). A total of eleven experiments were set up with different formulations as well as three repetitions to evaluate the uncertainty of each response (see Table 18).

Table 18 Overview of the formulations in the experimental design

Formulation no.	Multifunctional weight%	Preservative weight%	pH
1	0	0	5
2	0.6	0	5
3	0	0.8	5
4	0.6	0.8	5
5	0	0	6
6	0.6	0	6
7	0	0.8	6
8	0.6	0.8	6

9	0.3	0.4	5.5
10	0.3	0.4	5.5
11	0.3	0.4	5.5

Each formulation was tested to evaluate: the level of microbial resilience (selected formulations only), viscosity, conductivity and pH. The physical stability of the formulation was also evaluated visually for formulations stored at room temperature and 40°C. The test proceeded for a period of 2½ months. An overview of the tests performed during this time appears in **Table 19**.

Table 19 Overview of the tests conducted in the experimental design setup. *Microbial tests only on samples 1, 4 and 5. RT = Room temperature.

	Start	2 days	7 days	1 month	End – 2½ months
Microbial contamination (RT)*	*	*	*	*	*
Stability (RT)	*	-	*	*	*
pH (RT) Conductivity (RT) Viscosity (RT)	*	-	*	*	*
Stability (40 °C)	*	-	*	*	*

Test of the microbial stability of all samples was planned, but unfortunately was not executed within the timeframe of the project because the initial inoculation of each sample that was needed to perform the test failed and there was no time to repeat the study. Three samples were inoculated correctly and were analysed for microbial activity (samples 1, 4 and 5).

5.1.2 Results of the experimental design

As shown in Table 19, different properties were measured in the eleven formulations and the results are summarised below.

5.1.2.1 Microbial growth

Three samples were studied regarding microbial growth, formulation 1,4 and 5. *Pseudomonas aeruginosa* (DSM1128) was added as bacterial culture to the body lotion at the start of the experiment with a concentration equivalent to 10^6 cells per ml. The surviving cells of the added bacterial culture were measured at day 0, 2, 7, 24 and the end of the experiment.

From the results shown in Figure 17 it is obvious that the formulations with low pH (pH 5; formulation 1 and 4) clearly perform better than the formulation with high pH (pH 6, formulation 5).

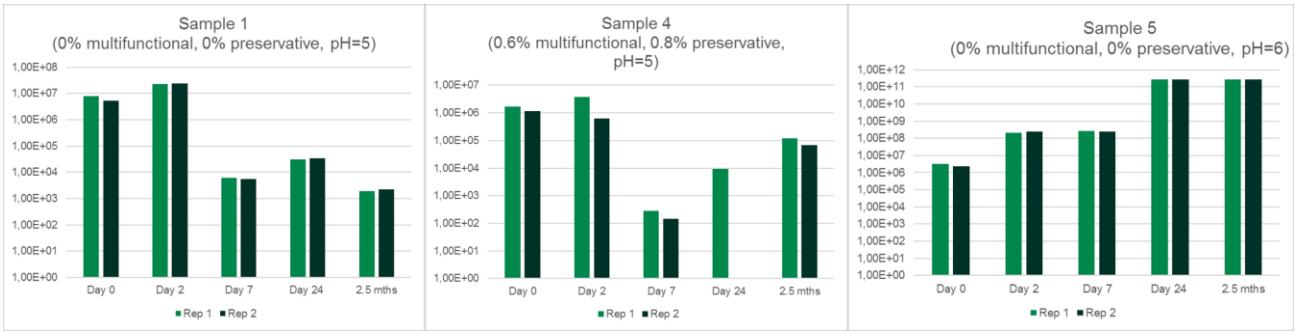


Figure 17 Microbial growth in samples 1, 4 and 5 (two repetitions). The graphs illustrate the growth of *Pseudomonas aeruginosa* (y-axis) at the time points day 0, day 2, day 7, day 24 and after 2,5 months (x-axis). *Pseudomonas aeruginosa* was inoculated with a start concentration of 10^6 cells per ml.

Based on these results the effect of preservative and multifunctional added to a formulation of low pH (comparing formulation 1 to formulation 4), does not seem obvious. But since no full challenge test has been carried out and there are only two samples, no firm conclusions can be drawn.

The overall purpose of the experimental design was to study the effect of the factors pH, preservative and multifunctional on the microbial protection of the product as well as any interactions between these factors. Due to lack of data on all formulations, this was however not possible.

5.1.2.2 pH, conductivity and viscosity

The pH, conductivity and viscosity were measured at the start of the experiment, after 1 week, after a month and at the end of the experiment. The results are shown in Figure 18, Figure 19 and Figure 21.

The pH is quite constant for most of the formulations. The largest change is seen in formulation 5, where the pH increases from 6 to 6.5 after a week. Formulation 5 contains no preservative or multifunctional ingredients and the elevated pH is expected to result in an increased bacterial growth. However an increase in pH can also be an indication of bacterial growth, which might explain the change in pH for formulation 5 (the microbial tests show bacterial growth, see Figure 17). Formulation 2 and 8 both show a significant increase in pH at the end of the experiment. Whether this has any connection to the microbial growth was not confirmed.

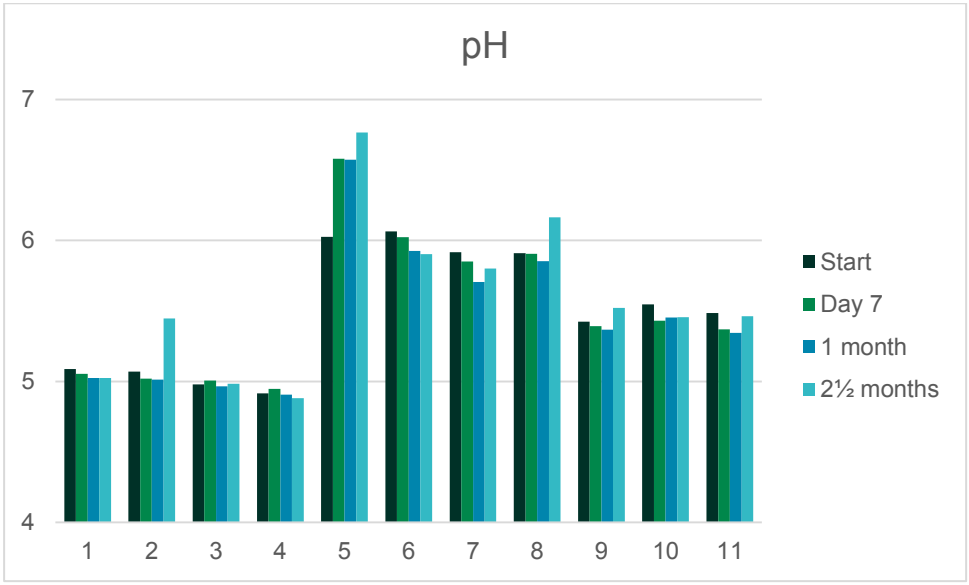


Figure 18 Changes in pH during the experimental design period. The X-axis shows sample number.

The conductivity varies significantly between the different samples initially (start), see Figure 19. For each sample this is, however, quite constant throughout the time of the experiments. There seems to be a correlation between the conductivity and the preservative concentration as well as the pH initially.

This correlation is confirmed when trying to make a mathematical modelling of the design data and the conductivity results, see Figure 20. The modelling shows a high predictivity and model validity for the initial conductivity of the formulations (see bottom graph in Figure 20), which indicates that the conductivity can be predicted using pH and the concentration of multifunctional and preservative used. The pH has the greatest affect on the conductivity, which is to be expected due to the chemical nature of the pH regulator used (top graph in Figure 20).

Regarding the physical and microbial stability, changes in the conductivity of an emulsion can be a sign of phase separation as well as microbial growth. And while the model validity is high for the prediction of conductivity at the beginning of the experiments, a decrease is seen over time (results not shown) indicating that other factors begin to affect the conductivity. This could be because of microbial growth. Formulations 1 and 2 have 0 % preservative, whereas formulation 7 and 8 have 0.8 % preservative. The difference in conductivity could be due to the fact that the preservative contains sodium benzoate. When there is a high amount of preservative, there are more cations from sodium benzoate in the formulation, which explains the higher conductivity. A slight drop in conductivity for formulation 5 from start to 2½ months is seen. Whether this has any connection to the microbial growth was not confirmed.

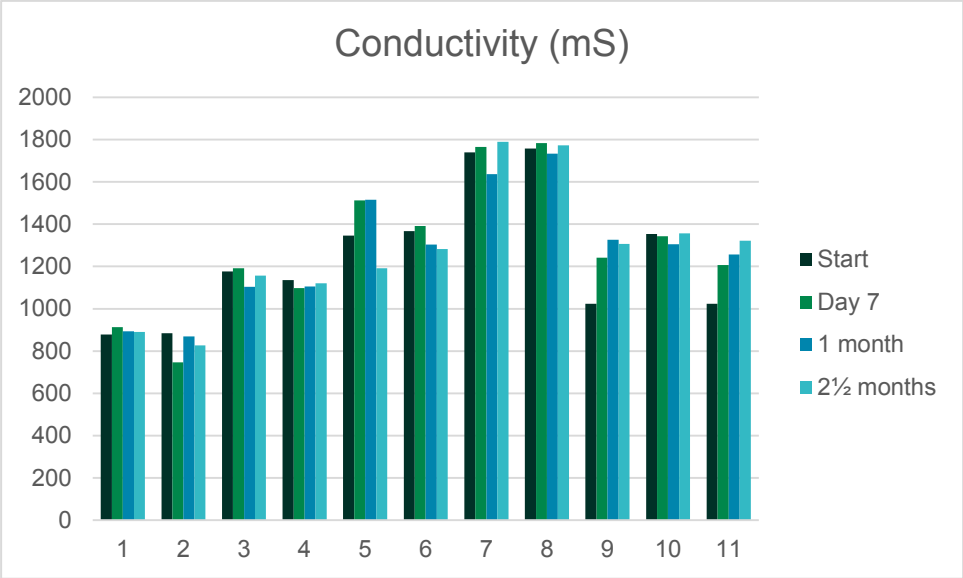


Figure 19 Changes in conductivity during the experimental design period. The X-axis shows sample number.

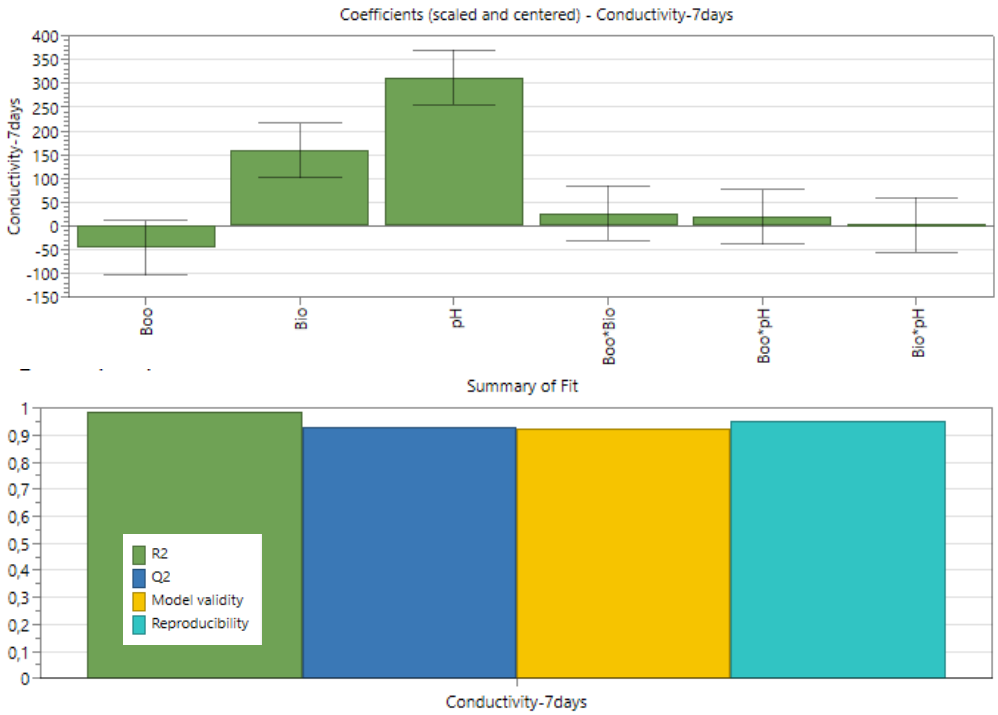


Figure 20 Modelling conductivity data (day 7). The model is based on the input variables: concentration of multivariate ingredient (Boo), concentration of preservative (Bio) and pH. The plot on top shows coefficients for the model – e.g., which parameters that significantly affect the conductivity and how much. The plot below shows the 'summary of fit' values – e.g., how well the model predicts the conductivity using the input variables.

Viscosity was measured as a function of shear rate for all the formulations at the four different time periods. All formulations show a decreasing viscosity at increasing shear rate as expected (results not shown). In the below figure, the viscosity at a shear rate of 10 s^{-1} is shown for all the formulations. This shear rate was chosen in order to compare between formulations and changes over time. The viscosity is considered constant for most of the formulations, although a slight decrease is seen over time for all formulations.

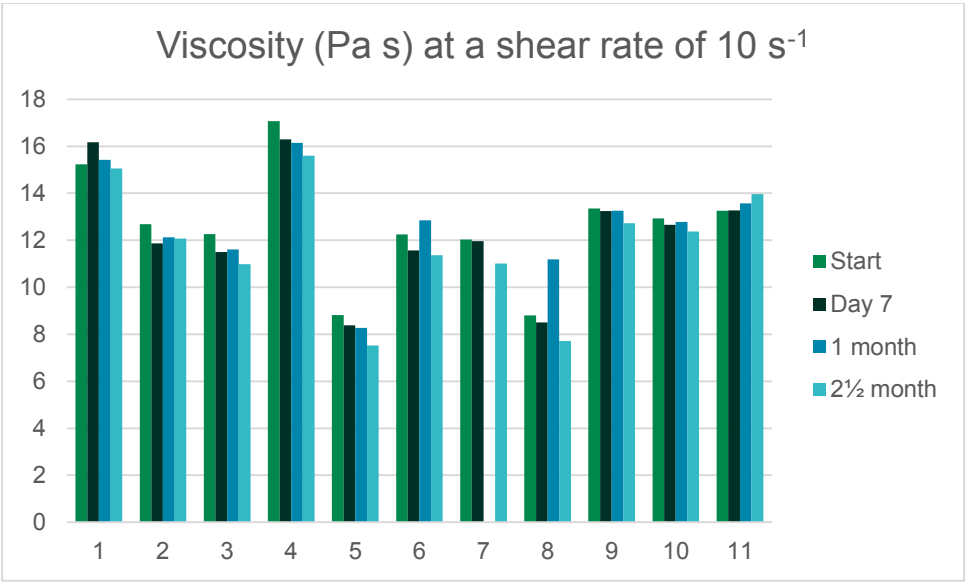


Figure 21 Changes in viscosity during the experimental design period. The X-axis shows sample number.

5.1.2.3 Stability

The stability was evaluated by studying the visual changes in the formulation over time. The formulations were placed under two different sets of storage conditions; room temperature and 40 °C. During the time of the experiment the stability was evaluated four times as described above. An example of the visual changes is shown in Figure 22 and the results are shown in Table 20.

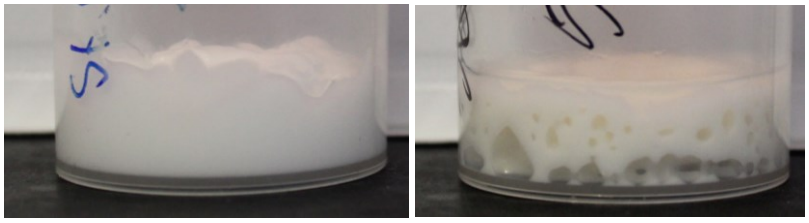


Figure 22 Difference in visual changes during the stability experiment of formulation 5 (left) and 7 (right) at 40 °C.

Table 20 Results of stability experiments at room temperature and 40°C. (-) denotes no visible change registered during the experimental period.

	Room temperature	40 ^{0} C
1	-	Visual change after 1 month
2	-	-
3	-	Visual change after 1 month
4	Visual change after 2½ months	-
5	-	-
6	-	-
7	-	Visual change after 1 month
8	-	-
9	-	-
10	-	-
11	-	-

Only one formulation (formulation 4) showed changes in physical appearance at room temperature during the test period, but no changes were seen when this formulation was stored at 40°C. At 40 °C formulation 1, 3 and 7 all showed signs of changes in the visible appearance (see Figure 22). The common factor in these formulations was that none of them contained multifunctionals, indicating that the multifunctionals might have a positive effect on stability for this formulation. A change in visual appearance was however not seen in formulation 5 which also does not contain multifunctionals, so it is unlikely that the presence of the multifunctional alone was the triggering effect.

5.1.3 Conclusion on design of experiment

The overall purpose of the experimental design was to study the effect of the pH, preservative and multifunctional on the microbial protection of the product as well as any interactions between these factors. Due to lacking microbial test data on all formulations in this design, a full data analysis was not possible.

The experimental design shows that the conductivity of the formulations to a large extent can be explained by the pH and preservative, which is also expected due to the content of ionic compounds in both ingredients.

The microbial testing showed that an otherwise identical formulation at pH 5 with and without a combination of preservative and multifunctional seemed comparable in microbial resistance to growth (*Pseudomonas aeruginosa),* indicating that the effect of reducing pH plays a major role in product protection.

5.2 Using *Hurdle Technology* in specific product development at DermaPharm

5.2.1 Introduction

To demonstrate how the concepts of the *Hurdle Technology* can be applied to reduce the concentration of preservatives in cosmetics DermaPharm used the technology during a recent product development. The background for the development was an O/W emulsion (body cream) suspected for eliciting skin irritation reactions. The purpose of the development was to develop an O/W emulsion with similar properties and with a minimum of preservative by using one or more of the elements of *Hurdle Technology* studied during this project.

5.2.2 Selection of preservative and multifunctional

Due to DermaPharm´s vision that strives to develop both environmentally- and skin-friendly products, the formulation of choice must meet the criteria of no less than four different certifications; the Nordic Swan Ecolabel, the Asthma and Allergy label, the Allergy Certified label, and the ECOCERT/COSMOS label, see figure 2.

Hence, all individual ingredients, including the preservatives or multifunctionals used, must meet the requirements of all the above certifications. This limits the possibilities for preservation and hence the use of hurdles such as pH and multifunctionals are considered extremely relevant when working in the framework of all certifications concurrently.

A preliminary search for preservatives and multifunctionals identified eleven different preservative agents and/or multifunctional ingredients that met the requirements of all four labels; these were explored further in order to reach the appropriate microbial protection of the O/W emulsion.

5.2.3 Methods

A basic O/W formulation (body cream) was used for screening of the microbial efficacy and skin irritation potential of formulations prepared during the product development. The INCI of the O/W formulation is listed below.
Aqua, Aloe Barbadensis Leaf Extract, Cocos Nucifera Oil, Butyrospermum Parkii Butter, Canola Oil, Glycerin, Cetearyl Glucoside, Cetearyl Alcohol, Stearyl Alcohol, Sodium Stearoyl Glutamate, Tocopherol, Xanthan Gum, Citric Acid

Eleven different formulations were prepared at slightly acidic pH (pH 5-5,5), as pH was considered an important *Hurdle Technology* factor that could contribute positively to the microbiological stability of the formulations. Preservatives and multifunctionals were chosen among those approved under the four certifications and levels and combinations adjusted during the development process.

The formulations were followed for 3 months, where their physical stability was monitored at 5 °C, room temperature (20 °C) and 40 °C, respectively.

Unfortunately, as it is often the case when dealing with customer driven product development, it was not possible to use packaging as a *Hurdle Technology* factor in this particular development, since the customer of the product requested that it was packed in a tube.

5.2.4 Skin application test

Although a product has met the safety requirements to cosmetic products in general, and the requirements to four different certifications, namely the Nordic Swan Ecolabel, the Asthma and Allergy label, the Allergy Certified label, and the ECOCERT/COSMOS label, there is still no guarantee that you will not have one or more individuals reacting to it. Therefore, the formulations were applied on the cheeks of 10 volunteers (whereof at least 2 were self-assessed as sensitive individuals). If any form of irritation occurred after 15 minutes or less, it was considered as a skin irritation reaction. To minimize the risk of a skin irritation reaction (redness or a prickling sensation) in the final product, every formulation that gave rise to 1 or more skin irritation reactions was excluded from the product development. The formulations did not elicit any form of irritative response when applied on the skin (neither redness nor a prickling sensation). In the evaluation we distinguish between visible/objective skin irritation (redness) and non-visible/subjective skin irritation reactions (prickling sensation). Even though you cannot observe a non-visible skin reaction, you may still "feel" an unpleasant sensation when the product is applied, which is not a desirable effect.

Four out of eleven formulations passed the skin application tests. After having passed the initial criterion not to cause any skin irritation in the applied setting, other exclusion criteria were applied as well, such as alterations in smell, or discoloration of the formulations. One formulation contained sodium dehydroacetate, the sodium salt of dehydroacetic acid, which tends to discolor some formulations over time. A discoloration was seen for this formulation and thus, it was excluded from the development. A preservative system can sometimes greatly affect the viscosity or stability of an emulsion. However, all three formulations were within an acceptable viscosity range for a body cream, and all three formulations were stable after 3 months at 5 °C, room temperature (app. 20 °C) and 40 °C, respectively.

5.2.5 Microbial stability of the remaining formulations

Three formulations met all the requirements of the four different certifications**Fejl! Henvisningskilde ikke fundet.**, and did not elicit skin irritation, or had in any other way affected the physical appearance of the formulation.

Since none of the three remaining formulations contain preservative agents that are on the preservative list in Annex V of the European Cosmetics Regulation (EC, 2009), it was considered uncertain that these formulations were adequately protected against microbiological activity. Moreover, addition of a skin absorption enhancer was considered to further increase the moisturising effect of the product. Therefore, the wetting agent and multifunctional ingredient glyceryl caprylate was introduced in the next formulations tested as another hurdle approach. A total of four formulations were prepared and the composition of these four formulations combined with the slightly acidic pH of the formulas was expected to contribute to the microbiological stability of the products, complying with the *Hurdle Technology* concept.

Each of the four adjusted formulations was then challenge tested to confirm an adequate product protection. To ensure reproducibility, each formulation was challenge tested at two different test laboratories with testing done according to the European Pharmacopeia (Ph. Eur. 5.1.3). All four formulations passed the challenge test with A-criteria (except one formulation that passed with B-criteria on *E. coli* and *A. brasiliensis*) according to the European Pharmacopoeia (results not shown).

The challenge test results show that all the tested formulations exerted excellent activity against microorganisms in the slightly acidic (pH=5-5.5) environment of the formulations.

All four formulations were equally resistant against bacteria (*S. aureus, P. aeruginosa* and *E. coli*). It was the same pattern against the yeast (*C. albicans*), and fungi (*A. brasiliensis*) where they all passed, although one formulation has a bit weaker resistance against fungi compared to the other formulations.

Two formulations had a characteristic sweet odor caused by the content of one multifunctional ingredient. This might be unacceptable for the consumer. Another formulation had a slightly lower viscosity than the other three formulations. It had a more watery consistency, whereas the last formulation had a much more rich consistency, which was considered more acceptable for consumers.

All parameters considered, an O/W emulsion (body cream) was developed using *Hurdle Technology* with the right sensory profile, color, smell, stability, ingredients (accepted by all four certifications) and which passed a microbial challenge test with A-criteria (Ph. Eur.)

5.2.6 Conclusion on product development

The described product development is an example of how the knowledge of *Hurdle Technology* and its underlying principles lead DermaPharm to develop a formulation with an overall adequate protection against microbial growth with a minimum of preservative and thus complying with the MiKoKo project goal.

The solution developed for this particular O/W emulsion (body cream) cannot be directly transferred to other formulations. Indeed, DermaPharm tried to implement the used hurdles from this formulation development in an W/O emulsion (ointment) as well. However, the stability of the W/O emulsion was affected and it did not meet the stability requirements after two months, where a phase separation was apparent. Using *Hurdle Technology* in formulation product development will thus always require a case by case evaluation.

Using a precautionary principle, where formulations that gave rise to skin irritation reactions were excluded, a formulation was developed with a reduced risk of eliciting skin irritation reactions. Furthermore, the formulation was easily up scaled from the laboratory to the large-scale production facilities at DermaPharm.

5.3 Considerations regarding use of the results in the safety evaluation of cosmetic products

The preservation of cosmetic products is important. Preservation ensures stability of the product as well as safety for the consumers. The question raised in this study is how the use of preservatives in cosmetics can be contained at relevant levels and types and not exceed what is reasonable in order to obtain its purpose. In this perspective, it is important to stress that only a very few cases have been described in which cosmetic products have been shown or suspected of being the source of human infection (SCCS, 2016). Moreover, almost all such reported cases have been observed in neonates or severely immunocompromised patients (Becks and Lorenzoni 1995; Itin et al. 1998; Álvarez-Lerma 2008). The interaction between the skin and lubricants may be more complex than just the intrinsic preservation capacity of the cream. It has for instance been demonstrated that treatment with petrolatum in infants with extremely low birth weight increases the incidence of systemic candidiasis. That in spite of the presumption that *candida* is not able to grow under conditions with no water activity (as in petrolatum). There has been some speculation that the petrolatum increased the adherence of the mold to the skin, and thus facilitated a trans-cutaneous infection to the fragile newborns (Campbell et al. 2000). However, in every day clinical practice, contamination of cosmetic products as source of human infection is never seen. Therefore, the medical society in Denmark does not encounter a general problem with low or insufficient preservation of cosmetic products as such. The reason for this is probably the present and experience-based degree of preservation in cosmetic products and the high standard of hygiene in Denmark.

In this report, we have pointed out the issue of inconsistent results from some of the different laboratories that offer microbial challenge testing of cosmetics. The variation in results of the challenge tests even within the same laboratory may incline the producers to choose the easiest way to ensure a passed challenge test at first passage, namely to add high and probably more than sufficient doses of preservatives of maybe the strongest preservatives. If such a strategy is followed it may result in unnecessary use of preservatives, which will put a load on both the users (risking allergy) and the environment (risking disturbance of the ecological systems). Therefore, DermaPharm promotes a strategy were sufficient preservation is obtained by use of as little preservation as possible. As covered by this report, the so-called *Hurdle Technology* shows a basic route to go, by constantly thinking along the lines of successively adding inhibitory means to the products in terms of making it difficult for microorganisms to survive. Lowering the pH and using multifunctional ingredients whenever suitable are two examples of “active” *Hurdle Technology* while the use of packaging with no or very little risk of contamination is an example of “passive” *Hurdle Technology*. With respect to the recommended challenge test it is important to stress, that legislation does not define a specific test method and often challenge tests developed in-house based on but not identical to method described in the acknowledged standards are used in practice.

According to the cosmetics regulation, the safety assessor must be duly qualified to perform a risk assessment of cosmetic products and must have all the needed information available - including the results of a challenge test for products where this is a requirement. The safety assessor is required to take into account all the hazards identified for the product and the exposure to it, as well as explain the reasoning behind the conclusion of the assessment. This is important. Rather than a blind focus on certain log reductions of the growth of certain microorganisms under rather uncontrolled and only semi-standardized conditions, the safety assessor must take all parameters into account including the nature of the product, the results of the challenge test, the intended use of the product, the packaging of the product and the experience with the product or very similar products. Hence, a *passed* microbial challenge test is not mentioned as a basic requirement in the cosmetic regulation (or the SCCS notes of guidance) as long as the overall safety assessment concludes the safe use of the specific product taking all known hazards and information into consideration. If parameters such as the packaging or years of experience with a similar product containing the same preservatives (and amounts of

preservatives), point to an almost zero risk for contamination of the product, this knowledge should be used in the reasoning written by the safety assessor. On the other hand, if a product is easily contaminated with, e.g., scales of dry human skin favoring the growth of certain molds, a passed challenge test may not be the only consideration for the safety assessor in order to approve the product. A global evaluation of the product must always take place, and the use of preservatives in cosmetic products should primarily be based on an overall assessment of safety of the product.

6. Conclusion

Reducing the need for preservation through formulation development
The influence of pH on the effect of preservatives has proven a useful tool for improving the product protection. It was possible to implement the use of a narrow pH range (5-5.5) in a number of formulations currently in production at DermaPharm with a very positive effect, that increased the number of formulations passing the challenge test. The changes in pH can affect other formulation properties like viscosity negatively; however, such challenges can be overcome by a number of well-known approaches within formulation of products.

For some formulations, the use of multifunctional ingredients combined with preservatives can further improve product protection. It has been demonstrated that by controlling pH and utilizing multifunctionals, a significant reduction of the preservative level can often be obtained without compromising the product protection.

All in all – the work within formulation development has shown that, by using the *Hurdle Technology* and the parameters studied in this project, a reduction in the use of preservatives can be obtained without compromising the consumers' safety. The approach can be used in product development at DermaPharm, but a tailored solution for each formulation is, however, often still needed due to the complex nature of cosmetic formulations. The experience obtained with the approach is valuable to the continued work with product development using *Hurdle Technology* as well as other parameters for cosmetics formulation.

The effect of packaging
Microorganisms are ubiquitous in the environment and on the human body and can be introduced into a product at any given time. Therefore, packaging design plays an important role in the choice of cosmetic preservative systems, since the packaging to a great extent can constitute a physical barrier for microbial contamination. Controlled experiments were set up in which product protection in a jar, a tube, a pump and an airless dispenser was studied. A worst-case scenario laboratory test and a consumer test showed that next after the body lotion in the jar (positive reference) the body lotion in the tube was the most exposed to contamination. Neither body lotions provided with a pump nor with airless dispensers were affected by contamination despite being subjected to extensive stress of bacteria colonies. The pump and airless dispenser protect the product from contamination during use, and this type of packaging, therefore, showcases a potential decrease in the needed level of preservation. Due to the limited dataset in this study, further evaluation, including more products and formulations, and more repetitions etc. are recommended to verify the potential to achieve protection from contamination through packaging and evaluate the contribution to the safety assessment of the cosmetic products.

Multivariate data analysis was used on a dataset compiled by DermaPharm. The results of the analysis showed clear groupings of ingredients expected to affect microbial growth and ingredients not expected to affect microbial growth. However, the results conflicted with existing knowledge and, hence, the compiled dataset was considered unsuited and no definite conclusions could be taken. Effective use of multivariate data analysis could show unknown interactions in formulation work, but it would require a dataset fitting for the purpose.

A **quick analysis method for testing preservation**, ideally comparable to currently used standardised challenge testing, would speed up the development work. Two different apathogenic organisms were evaluated as possible candidates for a quick challenge test of the cos-

metic formulations, but none of these were considered acceptable and no method was developed in this project.

Hurdle Technology and the safety evaluation of cosmetic products

It is obvious from our studies and experience that the result and consistency of challenge tests relies more on the capability of the used microorganisms to contaminate a specific cosmetic product than on the taxonomic status of the microorganisms, their initial concentrations, or the conditions of incubation and media of recovery used. It is, however, a requirement in the safety assessment that a challenge test is performed, and the results are given and discussed in the safety assessment. Adapting the formulation using several hurdles such as pH and multifunctionals has generally resulted in a higher ratio of passed challenge tests regardless of uncertainties and previous inconsistencies in results. Thus, the knowledge gained in the project contribute to an easier product approval, which is of great value to the product development process by increasing success rate and decreasing product development time for each product.

During the work, it has been proven that different approaches made it possible to lower the amount of preservatives in cosmetic products without putting the consumers' health at risk. This can be accomplished by defining hurdle technologies and thereby finding – in this case - pH, multifunctionals and smart packaging as important parameters that individually and by combination reduce the need for preservation. In this project DermaPharm's formulation expertise and know-how combined with Danish Technological Institute's multidisciplinary skills made it possible to achieve this during the project and give a better prospect for lowering the amount of preservatives in future cosmetic products.

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8. Annex 1 – challenge test results on formulations with adjusted pH

This annex includes the challenge test results described in section 3.4.5, where the pH of four formulations was adjusted to study the effect on microbial protection of the product.

Table 21 Challenge test results from test laboratory # 5. A cleansing foam and a vitamin mask were challenge tested at three different pH values. Both were preserved with a 0.8% blend of phenoxyethanol, benzoic acid and dehydroacetic acid. The numbers show cfu/ml at different time after inoculation.

Challenge test results, cleansing foam	Colony forming units per ml after incubation time:					Result
	Inoculum	**2 days**	**7 days**	**14 days**	**28 days**	**Ph. Eur. Criteria**
pH 5,2						
Bacteria	1.000.000*	ca. 1.000	< 10	< 10	< 10	A
Yeast	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
Fungi	100.000 (yeast + fungi)**	ca. 100	< 10	< 10	< 10	A
pH 5,5						
Bacteria	1.000.000*	ca. 100.000	ca. 10.000	< 10	< 10	B
Yeast	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
Fungi	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
pH 5,9						
Bacteria	1.000.000*	ca. 10.000	ca. 10.000	ca. 10.000	< 10	Failed
Yeast	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
Fungi	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
Challenge test results, vitamin mask	Colony forming units per ml after incubation time:					Result
	Inoculum	**2 days**	**7 days**	**14 days**	**28 days**	**Ph. Eur. Criteria**
pH 4,9						
Bacteria	1.000.000*	< 10	< 10	< 10	< 10	A
Yeast	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
Fungi	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
pH 5,5						
Bacteria	1.000.000*	< 10	< 10	< 10	< 10	B
Yeast	100.000 (yeast + fungi)**	ca. 100	< 10	< 10	< 10	A
Fungi	100.000 (yeast + fungi)**	ca. 100	ca. 100	< 10	< 10	A
pH 5,9						
Bacteria	1.000.000*	< 10	< 10	ca. 10.000	< 10	Failed
Yeast	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
Fungi	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A

* Each sample was inoculated with a mixed suspension of the bacteria *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Eschericia coli*, *Proteus mirabilis*, and *Pluribacter gergoviae*. The initial germ-count thus was adjusted to 1.000.000 cfu/ml.
** Each sample was inoculated with a mixed suspension of the yeast *Candida albicans* and the fungi *Aspergillus brasiliensis*, *Penicillium expansum*, and *Trichoderma viride*. The initial germ-count was adjusted to 100.000 cfu/ml.

Table 22 Challenge test results from test laboratory # 4. A face cream and a shower gel were challenge tested at three different pH values. Both were preserved with a 2% blend of sodium levulinate and potassium sorbate. The numbers show cfu/g at different time points after inoculation.

Challenge test results, face cream	Colony forming units per g after incubation time:					Result
	Inoculum	**2 days**	**7 days**	**14 days**	**28 days**	**Ph. Eur. Criteria**
pH 5.4						
Staphylococcus aureus	630.000	44.000	< 10	< 10	< 10	B
Pseudomonas aeruginosa	440.000	< 10	< 10	< 10	< 10	A
Escherichia coli	470.000	100.000	2.000	< 10	< 10	B
Candida albicans	1.000.000	120.000	136.000	1.000	< 10	A
Aspergillus brasiliensis	170.000	58.000	2.000	1.600	20	A
pH 5.6						
Staphylococcus aureus	650.000	240.000	< 10	< 10	< 10	B
Pseudomonas aeruginosa	570.000	4.400	< 10	< 10	< 10	A
Escherichia coli	490.000	200.000	48.000	< 10	< 10	B
Candida albicans	570.000	180.000	150.000	25.000	< 10	B
Aspergillus brasiliensis	450.000	100.000	33.000	20.000	3.000	B
pH 5.8						
Staphylococcus aureus	650.000	200.000	120	< 10	< 10	B
Pseudomonas aeruginosa	570.000	5.700	10	< 10	< 10	A
Escherichia coli	490.000	205.000	54.000	< 10	< 10	B
Candida albicans	570.000	250.000	142.000	19.000	< 10	B
Aspergillus brasiliensis	450.000	100.000	65.000	45.000	45.000	B
Challenge test results, shower gel	Colony forming units per g after incubation time:					Result
	Inoculum	**2 days**	**7 days**	**14 days**	**28 days**	**Ph. Eur. Criteria**
pH 5.2						
Staphylococcus aureus	630.000	26.000	50	< 10	< 10	B
Pseudomonas aeruginosa	440.000	< 10	< 10	< 10	< 10	A
Escherichia coli	470.000	14.000	< 10	< 10	< 10	B
Candida albicans	1.000.000	90.000	640	50	< 10	A
Aspergillus brasiliensis	170.000	4.000	< 10	< 10	< 10	A
pH 5.5						
Staphylococcus aureus	650.000	170.000	7.000	< 10	< 10	B
Pseudomonas aeruginosa	570.000	20	< 10	< 10	< 10	A
Escherichia coli	490.000	46.000	< 10	< 10	< 10	B
Candida albicans	570.000	140.000	1.200	110	< 10	A
Aspergillus brasiliensis	450.000	6.000	< 10	< 10	< 10	A
pH 5.8						
Staphylococcus aureus	650.000	280.000	202.000	39.000	30	Failed
Pseudomonas aeruginosa	570.000	40	< 10	< 10	< 10	A
Escherichia coli	490.000	260.000	15.000	< 10	< 10	B
Candida albicans	570.000	90.000	3.800	120	< 10	A
Aspergillus brasiliensis	450.000	6.000	< 10	< 10	< 10	A

Challenge test results, face cream	Colony forming units per g after incubation time:					Result
	Inoculum	**2 days**	**7 days**	**14 days**	**28 days**	**Ph. Eur. Criteria**

pH 5.4						
Staphylococcus aureus	630.000	44.000	< 10	< 10	< 10	B
Pseudomonas aeruginosa	440.000	< 10	< 10	< 10	< 10	A
Escherichia coli	470.000	100.000	2.000	< 10	< 10	B
Candida albicans	1.000.000	120.000	136.000	1.000	< 10	A
Aspergillus brasiliensis	170.000	58.000	2.000	1.600	20	A
pH 5.6						
Staphylococcus aureus	650.000	240.000	< 10	< 10	< 10	B
Pseudomonas aeruginosa	570.000	4.400	< 10	< 10	< 10	A
Escherichia coli	490.000	200.000	48.000	< 10	< 10	B
Candida albicans	570.000	180.000	150.000	25.000	< 10	B
Aspergillus brasiliensis	450.000	100.000	33.000	20.000	3.000	B
pH 5.8						
Staphylococcus aureus	650.000	200.000	120	< 10	< 10	B
Pseudomonas aeruginosa	570.000	5.700	10	< 10	< 10	A
Escherichia coli	490.000	205.000	54.000	< 10	< 10	B
Candida albicans	570.000	250.000	142.000	19.000	< 10	B
Aspergillus brasiliensis	450.000	100.000	65.000	45.000	45.000	B
Challenge test results, shower gel	Colony forming units per g after incubation time:					Result
	Inoculum	**2 days**	**7 days**	**14 days**	**28 days**	**Ph. Eur. Criteria**
pH 5.2						
Staphylococcus aureus	630.000	26.000	50	< 10	< 10	B
Pseudomonas aeruginosa	440.000	< 10	< 10	< 10	< 10	A
Escherichia coli	470.000	14.000	< 10	< 10	< 10	B
Candida albicans	1.000.000	90.000	640	50	< 10	A
Aspergillus brasiliensis	170.000	4.000	< 10	< 10	< 10	A
pH 5.5						
Staphylococcus aureus	650.000	170.000	7.000	< 10	< 10	B
Pseudomonas aeruginosa	570.000	20	< 10	< 10	< 10	A
Escherichia coli	490.000	46.000	< 10	< 10	< 10	B
Candida albicans	570.000	140.000	1.200	110	< 10	A
Aspergillus brasiliensis	450.000	6.000	< 10	< 10	< 10	A
pH 5.8						
Staphylococcus aureus	650.000	280.000	202.000	39.000	30	Failed
Pseudomonas aeruginosa	570.000	40	< 10	< 10	< 10	A
Escherichia coli	490.000	260.000	15.000	< 10	< 10	B
Candida albicans	570.000	90.000	3.800	120	< 10	A
Aspergillus brasiliensis	450.000	6.000	< 10	< 10	< 10	A

9. Annex 2 - challenge test results on formulations with reduced pH and reduced preservation

This annex includes the challenge test results described in section 3.4.5, where the preservative level of four formulations was reduced concurrently with a reduction in pH to study the effect on microbial protection of the product.

Table 23 Challenge test results from test laboratory # 5. A cleansing foam and a vitamin mask with pH 5.2 and 4.9 respectively were challenge tested with two different concentrations of preservative (either 0.8% or 0.4% of a mixture of phenoxyethanol, benzoic acid and dehydroacetic acid). The numbers show cfu/ml at different time points after inoculation.

Challenge test results, cleansing foam, pH 5,2	Colony forming units per ml after incubation time:					Result
	Inoculum	**2 days**	**7 days**	**14 days**	**28 days**	**Ph. Eur. Criteria**
0,8% preservative						
Bacteria	1.000.000*	ca. 1.000	< 10	< 10	< 10	A
Yeast	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
Fungi	100.000 (yeast + fungi)**	ca. 100	< 10	< 10	< 10	A
0,4% preservative						
Bacteria	1.000.000*	ca. 100.000	ca. 100	< 10	< 10	B
Yeast	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
Fungi	100.000 (yeast + fungi)**	ca. 100	< 10	< 10	< 10	A
Challenge test results, vitamin mask, pH 4,9	Colony forming units per ml after incubation time:					Result
	Inoculum	**2 days**	**7 days**	**14 days**	**28 days**	**Ph. Eur. Criteria**
0,8% preservative						
Bacteria	1.000.000*	< 10	< 10	< 10	< 10	A
Yeast	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
Fungi	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
0,4% preservative						
Bacteria	1.000.000*	< 10	< 10	< 10	< 10	A
Yeast	100.000 (yeast + fungi)**	ca. 10.000	< 10	< 10	< 10	A
Fungi	100.000 (yeast + fungi)**	ca. 100	ca. 100	< 10	< 10	A

* Each sample was inoculated with a mixed suspension of the bacteria *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Eschericia coli*, *Proteus mirabilis*, and *Pluribacter gergoviae*. The initial germ-count thus was adjusted to 1.000.000 cfu/ml.

** Each sample was inoculated with a mixed suspension of the yeast *Candida albicans* and the fungi *Aspergillus brasiliensis*, *Penicillium expansum*, and *Trichoderma viride*. The initial germ-count was adjusted to 100.000 cfu/ml.

Table 24 Challenge test results from test laboratory # 4. A face cream and a shower gel with pH 5.4 and 5.2 respectively were challenge tested with two different concentrations of preservative (either 2% or 1% of a mixture of sodium levulinate and potassium sorbate). The numbers show cfu/g at different time points after inoculation.

Challenge test results, face cream, pH 5,4	Colony forming units per g after incubation time:					Result
	Inoculum	**2 days**	**7 days**	**14 days**	**28 days**	**Ph. Eur. Criteria**
2% preservative						
Staphylococcus aureus	630.000	44.000	< 10	< 10	< 10	B
Pseudomonas aeruginosa	440.000	< 10	< 10	< 10	< 10	A
Escherichia coli	470.000	100.000	2.000	< 10	< 10	B
Candida albicans	1.000.000	120.000	136.000	1.000	< 10	A
Aspergillus brasiliensis	170.000	58.000	2.000	1.600	20	A
1% preservative						
Staphylococcus aureus	650.000	140.000	1.000	< 10	< 10	B
Pseudomonas aeruginosa	570.000	< 10	< 10	< 10	< 10	A
Escherichia coli	490.000	120.000	18.000	< 10	< 10	B
Candida albicans	570.000	235.000	13.000	16.100	< 10	B
Aspergillus brasiliensis	450.000	104.000	78.000	45.000	45.000	B
Challenge test results, shower gel, pH 5,2	Colony forming units per g after incubation time:					Result
	Inoculum	**2 days**	**7 days**	**14 days**	**28 days**	**Ph. Eur. Criteria**
2% preservative						
Staphylococcus aureus	630.000	26.000	50	< 10	< 10	B
Pseudomonas aeruginosa	440.000	< 10	< 10	< 10	< 10	A
Escherichia coli	470.000	14.000	< 10	< 10	< 10	B
Candida albicans	1.000.000	90.000	640	50	< 10	A
Aspergillus brasiliensis	170.000	4.000	< 10	< 10	< 10	A
1% preservative						
Staphylococcus aureus	650.000	60.000	4.000	20	< 10	B
Pseudomonas aeruginosa	570.000	60	20	< 10	< 10	A
Escherichia coli	490.000	120.000	20	< 10	< 10	B
Candida albicans	570.000	240.000	< 10	< 10	< 10	A
Aspergillus brasiliensis	450.000	32.000	< 10	< 10	< 10	A

10. Annex 3 – challenge test results on formulations with multifunctionals

This annex includes the challenge test results described in section 3.5, where the preservative level of four formulations was reduced concurrently with a reduction in pH and a multifunctional was added to study the overall effect on microbial protection of the product.

Table 25 Challenge test results for a cleansing foam, pH 5.2, with different levels of preservative (either 0.8%, 0.4% or 0.2% of a mixture of phenoxyethanol, benzoic acid and dehydroacetic acid) and/or multifunctional (either no caprylyl glycol or 0.6% caprylyl glycol). The formulations with caprylyl glycol were challenge tested at the same test laboratory as they had been tested in previously (section 3.4.5). The numbers show cfu/ml after inoculation.

Challenge test results, cleansing foam, pH 5,2	Colony forming units per ml after incubation time:					Result
	Inoculum	**2 days**	**7 days**	**14 days**	**28 days**	**Ph. Eur. Criteria**
0.8% preservative (from section 3.5.5)						
Bacteria	1.000.000*	ca. 1.000	< 10	< 10	< 10	A
Yeast	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
Fungi	100.000 (yeast + fungi)**	ca. 100	< 10	< 10	< 10	A
0.4% preservative (from section 3.5.5)						
Bacteria	1.000.000*	ca. 100.000	ca. 100	< 10	< 10	B
Yeast	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
Fungi	100.000 (yeast + fungi)**	ca. 100	< 10	< 10	< 10	A
0.4% preservative + 0.6% caprylyl glycol						
Bacteria	1.000.000*	< 10	< 10	< 10	< 10	A
Yeast	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
Fungi	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
0.2% preservative + 0.6% caprylyl glycol						
Bacteria	1.000.000*	ca. 10.000	< 10	< 10	< 10	A
Yeast	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
Fungi	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A

* Each sample was inoculated with a mixed suspension of the bacteria *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Eschericia coli*, *Proteus mirabilis*, and *Pluribacter gergoviae*. The initial germ-count thus was adjusted to 1.000.000 cfu/ml.

** Each sample was inoculated with a mixed suspension of the yeast *Candida albicans* and the fungi *Aspergillus brasiliensis*, *Penicillium expansum*, and *Trichoderma viride*. The initial germ-count was adjusted to 100.000 cfu/ml.

Table 26. Challenge test results for a vitamin mask, pH 4.9, with different levels of preservative (either 0.8%, 0.4% or 0.2% of a mixture of phenoxyethanol, benzoic acid and dehydroacetic acid) and/or multi-functional (either no caprylyl glycol or 0.6% caprylyl glycol). The formulations with caprylyl glycol were challenge tested at the same test laboratory as they had been tested n previously (**section 3.4.5**). The numbers show cfu/ml after inoculation.

Challenge test results, vitamin mask, pH 4.9	Colony forming units per ml after incubation time:					Result
	Inoculum	**2 days**	**7 days**	**14 days**	**28 days**	**Ph. Eur. Criteria**
0.8% preservative (section 3.4.5).						
Bacteria	1.000.000*	< 10	< 10	< 10	< 10	A
Yeast	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
Fungi	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
0.4% preservative (section 3.4.5).						
Bacteria	1.000.000*	< 10	< 10	< 10	< 10	A
Yeast	100.000 (yeast + fungi)**	ca. 10.000	< 10	< 10	< 10	A
Fungi	100.000 (yeast + fungi)**	ca. 100	ca. 100	< 10	< 10	A
0.2% preservative + 0.6% caprylyl glycol						
Bacteria	1.000.000*	< 10	< 10	< 10	< 10	A
Yeast	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A
Fungi	100.000 (yeast + fungi)**	< 10	< 10	< 10	< 10	A

* Each sample was inoculated with a mixed suspension of the bacteria *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Eschericia coli*, *Proteus mirabilis*, and *Pluribacter gergoviae*. The initial germ-count thus was adjusted to 1.000.000 cfu/ml.
** Each sample was inoculated with a mixed suspension of the yeast *Candida albicans* and the fungi *Aspergillus brasiliensis*, *Penicillium expansum*, and *Trichoderma viride*. The initial germ-count was adjusted to 100.000 cfu/ml.

Table 27. Challenge test results for a shower gel, pH 5.2, with different levels of preservative (either 2% or 1% of a mixture of sodium levulinate and potassium sorbate) and/or multifunctional (either no caprylyl glycol/glyceryl caprylate or 0.6% caprylyl glycol/glyceryl caprylate). The numbers show cfu/g at different time points after inoculation. The formulations with caprylyl glycol and glyceryl caprylate were challenge tested at the same test laboratories as they had been tested in previously (**section 3.4.5**).

Challenge test results, shower gel, pH 5.2	Colony forming units per g after incubation time:					Result
	Inoculum	**2 days**	**7 days**	**14 days**	**28 days**	**Ph. Eur. Criteria**
2% preservative						
Staphylococcus aureus	630.000	26.000	50	< 10	< 10	B
Pseudomonas aeruginosa	440.000	< 10	< 10	< 10	< 10	A
Escherichia coli	470.000	14.000	< 10	< 10	< 10	B
Candida albicans	1.000.000	90.000	640	50	< 10	A
Aspergillus brasiliensis	170.000	4.000	< 10	< 10	< 10	A
1% preservative						
Staphylococcus aureus	650.000	60.000	4.000	20	< 10	B
Pseudomonas aeruginosa	570.000	60	20	< 10	< 10	A
Escherichia coli	490.000	120.000	20	< 10	< 10	B
Candida albicans	570.000	240.000	< 10	< 10	< 10	A
Aspergillus brasiliensis	450.000	32.000	< 10	< 10	< 10	A

1% preservative + 0.6% caprylyl glycol						
Staphylococcus aureus	490.000	540	490	36	< 10	A
Pseudomonas aeruginosa	440.000	< 100	< 10	< 10	< 10	A
Escherichia coli	690.000	2900	< 10	< 10	< 10	A
Candida albicans	460.000	< 100	< 10	< 10	< 10	A
Aspergillus brasiliensis	380.000	59.000	32.000	2.400	740	A
0.5% preservative + 0.6% caprylyl glycol						
Staphylococcus aureus	490.000	480	320	270	< 10	A
Pseudomonas aeruginosa	440.000	< 100	< 10	< 10	< 10	A
Escherichia coli	690.000	120.000	2.900	1.400	< 10	Fail
Candida albicans	460.000	< 100	< 10	< 10	< 10	A
Aspergillus brasiliensis	380.000	70.000	65.000	8.600	3.400	Fail
1% preservative + 0.6% glyceryl caprylate						
Staphylococcus aureus	490.000	19.000	1.700	290	< 10	B
Pseudomonas aeruginosa	440.000	< 100	< 10	< 10	< 10	A
Escherichia coli	690.000	3.000	< 10	< 10	< 10	A
Candida albicans	460.000	7.000	200	< 10	< 10	A
Aspergillus brasiliensis	380.000	80.000	8.500	3.600	2.200	A
0.5% preservative + 0.6% glyceryl caprylate						
Staphylococcus aureus	490.000	730	510	430	< 10	B
Pseudomonas aeruginosa	440.000	< 100	< 10	< 10	< 10	A
Escherichia coli	690.000	89.000	40.000	1.300	340	Fail
Candida albicans	460.000	900	< 10	< 10	< 10	A
Aspergillus brasiliensis	380.000	51.000	22.000	17.000	7.700	Fail

Table 28. Challenge test results from test laboratory # 3 for a sun care product SPF 30, pH 5.5, with different levels of preservative (either 1.2% or 0.8% of a mixture of phenoxyethanol, benzoic acid and dehydroacetic acid) and/or multifunctional (either no caprylyl glycol or 0.6% caprylyl glycol).

Challenge test results, sun care SPF 30, pH 5.5	Colony forming units per g after incubation time:					Result
	Inoculum	**2 days**	**7 days**	**14 days**	**28 days**	**Ph. Eur. Crite**
1.2% preservative						
Pseudomonas aeruginosa	590.000	4.700	< 10	< 10	< 10	A
Staphylococcus aureus	720.000	200.000	150.000	290	< 10	B
Escherichia coli	620.000	210.000	170.000	33.000	< 10	Fail
Candida albicans	89.000	26.000	15.000	910	< 10	B
Aspergillus brasiliensis	89.000	28.000	14.000	5.000	3.600	B
0.8% preservative + 0.6% caprylyl glycol						
Pseudomonas aeruginosa	590.000	< 100	< 10	< 10	< 10	A
Staphylococcus aureus	720.000	9.000	2.600	< 10	< 10	B
Escherichia coli	620.000	2.000	660	< 10	< 10	B
Candida albicans	89.000	4.900	1.100	< 10	< 10	A

Aspergillus brasiliensis	89.000	30.000	23.000	8.700	7.300	A

Less preservatives in cosmetics. Mikoko

DermaPharm A/S and Danish Technological Institute have in the project “less preservatives in cosmetics” developed new methods to minimize the amount of preservatives in cosmetic products and thereby minimize the exposure of preservatives to consumers while retaining product safety and quality required by the Cosmetics Regulation. A *Hurdle Technology* approach has been key and several parameters and methods have been applied to reduce the need for preservatives. A combination of formulation aspects like pH and multifunctional ingredients with the right choice of packaging attribute to a greater protection of the products from microbiological growth and contamination. Many aspects are directly applicable for implementation and the hurdle technology approach has been demonstrated in the development of new products at production scale at DermaPharm.

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