

Integrated Master in Bioengineering

Study of an alternative preservative system in liquid soap formulations

Master Dissertation

by

Maria Inês Dias e Sousa Morgado Monteiro

Developed in the scope of the Dissertation course unit

conducted at

Castelbel /LEPABE - Laboratory for Process Engineering Environment, Biotechnology and Energy



FEUP Advisor: **Prof. Vera Homem**

FEUP Co-Advisor: **Prof. Luciana Gomes**

Castelbel Advisor: **Dr. Rita Matias**

Quality Department

February 2022



Acknowledgments

Primeiramente gostaria de agradecer às orientadoras por parte da FEUP, Dr.^a Vera Homem e Dr.^a Luciana Gomes pela compreensão, disponibilidade e partilha constante de conhecimento que me permitiram alcançar os objetivos estabelecidos para este projeto. E obrigada a toda a equipa do laboratório do departamento de engenharia química da FEUP que me auxiliaram com a parte experimental do trabalho. Esta dissertação de mestrado não seria possível sem a equipa de orientação da Castelbel, uma empresa que me acolheu de braços abertos. Gostaria de agradecer à Dr.^a Rita Matias pela oportunidade de desenvolver este tema, que juntamente com Mariana Melo me acompanharam e apoiaram ao longo de todas as etapas deste projeto. Um obrigada especial à Márcia, que durante este semestre fez o papel tanto de orientadora, como de amiga, e viveu esta dissertação como se fosse a sua.

Tenho a sorte de ter vivido cinco anos repletos de alegria e amizades que marcaram a minha vida para sempre. Dessas, tenho de realçar as minhas amigas de Erasmus e muito mais Bia, Carol, Catx e Dri com quem construí tantas memórias e vivi tantas aventuras, foram um apoio constante e incomparável em todos os aspetos da minha vida. Aos meus queridos “fozeiros”, Inês, Rita, Cris, Filipe e Martim, que nunca falham em fazer-me rir, obrigada por serem uma casa no meio de todo o caos que é bioengenharia. E à Fi, Eva, Miguel, Inês e todo o party hits, por quem tenho um carinho tão grande, espero que seja um grupo que mantenha sempre esta união. Não podia deixar de mencionar os amigos que fiz na missão país, que tantos levo para a vida e os clássicos 10, especialmente as minhas companheiras de sempre, Né e Bia. Se estes cinco anos foram “os melhores anos da minha vida” foi graças a todos vocês.

Ao Álvaro, não tenho palavras para agradecer a sorte que é ter-te na minha vida. Tudo o que fiz e superei nestes cinco anos tem uma marca tua e nunca me paras de surpreender com o teu apoio e força que me transmites. Tenho um orgulho imensurável em tudo o que fazes mas ainda mais na pessoa que és e espero que possamos continuar os dois a crescer juntos. E obrigada pelos teus pais e pelo Kiko que me fazem sempre sentir como parte da família.

Tenho também de agradecer à minha própria família, não só aos meus avós, tios e primos mas também à minha família de coração, a minha madrinha que tanto zela por mim, o meu padrinho, a Guiducha e a Cristina que são o meu maior exemplo de amizade.

E aos meus pais, as pessoas mais fortes que conheço, dedico-vos este trabalho. Obrigada por tudo o que fazem e sempre fizeram por mim, todos os sacrifícios, e todas as oportunidades que me deram, toda a paciência, todo o amor e carinho, são as coisas mais importantes que levo destes 23 anos. Tudo o que sou, devo a vocês.

As professoras Vera Homem e Luciana Gomes, orientadoras desta dissertação, são membros integrados do LEPABE – Laboratório de Engenharia de Processos, Ambiente, Biotecnologia e Energia, financiados por: LA/P/0045/2020 (ALiCE) e UIDB/00511/2020 - UIDP /00511/2020 (LEPABE) financiado por fundos nacionais através da FCT/MCTES (PIDDAC).

Resumo

Os produtos cosméticos desempenham um papel essencial na vida humana há milhares de anos e atualmente são uma característica indispensável do estilo de vida moderno. A produção de cosméticos é obrigada a cumprir um conjunto de diretrizes que visam regular o processo, bem como a comercialização do produto, a fim de garantir qualidade e segurança aos consumidores. A estabilidade de um produto cosmético relaciona-se com a sua capacidade de manter suas propriedades físico-químicas, microbiológicas, funcionais e estéticas ao longo do tempo dentro de padrões aceitáveis.

Os conservantes são substâncias adicionadas aos cosméticos para evitar o crescimento microbiano. Por serem compostos biologicamente ativos, podem causar um efeito irritante, tóxico ou sensibilizante. Por esse motivo, a segurança desses compostos é continuamente questionada e qualquer evidência científica de possíveis efeitos nocivos à saúde dos consumidores resulta em crescentes restrições impostas por diversas regulamentações internacionais.

Este projeto de dissertação teve como objetivo substituir em sabonetes líquidos os conservantes metilisotiazolinona (MI) e clorometilisotiazolinona (CMI), conhecidas pelo seu potencial alergizante, por uma mistura de conservantes contendo fenoxietanol, ácido benzóico e ácido desidroacético (BDP) que é considerada mais segura. Para avaliar a nova formulação, a estabilidade de ambos os sabonetes líquidos foi testada em condições aceleradas. A atividade antimicrobiana do conservante foi testada através de estudos microbiológicos (método da difusão em agar e teste de eficácia antimicrobiana(CT)). A avaliação de estabilidade acelerada envolveu a avaliação dos atributos físico-químicos e segurança microbiológica dos produtos durante 8 semanas com momentos de avaliação em 0 (t_0), 4 (t_1) e 8 (t_2) semanas. Este período sob estabilidade acelerada corresponde a 1 ano de estabilidade em tempo real.

Em relação à avaliação física e química, o pH, densidade e capacidade e estabilidade de espuma estavam de acordo com as especificações do fabricante desde o início até o final do período de avaliação. A avaliação de centrifugação também confirmou a estabilidade de ambas as formulações. No entanto, a viscosidade de cada formulação apresentou valores inferiores às especificações do fabricante, e as condições aceleradas na nova formulação introduziram uma mudança de cor para amarelo. Na avaliação microbiológica, nenhuma formulação apresentou contaminação. Em geral, pode-se considerar que as propriedades físicas, químicas e microbiológicas permaneceram estáveis durante o período de teste. Em relação à avaliação do conservante, tanto o método de difusão em poços de agar como o teste de eficácia antimicrobiana (CT) confirmaram a capacidade antimicrobiana de cada formulação contra *Staphylococcus aureus*, *Escherichia coli* e *Staphylococcus epidermidis*. De acordo com todos os resultados obtidos, a nova formulação é considerada um substituto adequado.

Palavras-chave: cosméticos, conservantes, condições aceleradas, estudo de estabilidade, sabonete líquido, metilisotiazolinona (MI), clorometilisotiazolinona (CMI), fenoxietanol, ácido benzoico, ácido desidroacético

Abstract

Cosmetic products have played an essential role in human life for thousands of years and presently, are an indispensable feature of modern lifestyle of individuals. The cosmetics production is required to comply with a set of guidelines that aim to regulate the process as well as the product's marketing in order to ensure quality and safety to consumers. Stability is related to the ability of a cosmetic product to maintain its physicochemical, microbiological, functional and aesthetic properties over time within acceptable standards.

Preservatives are substances added to cosmetics to prevent microbial growth. Since they are biologically active compounds, they can cause an irritating, toxic or sensitizing effect. For this reason, the safe use of these compounds is always being questioned and scientific evidence of possible harmful effects on consumers' health has resulted in increasing restrictions imposed by various international regulations.

This dissertation project aimed to substitute in liquid soaps the preservatives methylisothiazolinone (MI) and chloromethylisothiazolinone (CMI), that can cause allergic reactions, for a preservative mixture containing phenoxyethanol, benzoic acid, and dehydroacetic acid (BDP) which is considered safer and already widely used in many other products of the company. To evaluate the new formulation, the stability of both liquid soaps was assessed through accelerated conditions and the preservative's antimicrobial activity was tested through microbiological studies (agar-well diffusion method and Challenge Test). The accelerated stability evaluation involved the assessment of the physical and chemical attributes and microbiological safety of the products during 8 weeks with assessment time-points at 0 (t₀), 4 (t₁) and 8 (t₂) weeks. This period under accelerated stability corresponds to 1 year of real-time stability.

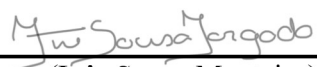
Regarding the physical and chemical evaluation, the pH, density and foam capacity and stability were in accordance with the manufacturer or supplier's specifications from the beginning to the end of the assessment period. The centrifugation assessment also confirmed the stability of both formulations. However, the viscosity of both liquid soaps presented lower values than the manufacturer's specifications, and the accelerated conditions in the new formulation introduced a change of color to yellow. In the microbiological assessment, both samples presented no contamination. Overall, it can be considered that the physical, chemical and microbiological properties remained stable during the testing period. In regards to the preservative's evaluation, both the agar-well diffusion method and the challenge test confirmed each formulation's antimicrobial capacity against *Staphylococcus aureus*, *Escherichia coli* and *Staphylococcus epidermidis*. According to all results obtained, the new liquid soap is an adequate substitute.

Keywords: cosmetics, preservative, accelerated conditions, stability studies, liquid soaps, methylisothiazolinone, chloromethylisothiazolinone, phenoxyethanol, benzoic acid, dehydroacetic acid

Declaration

I hereby declare, on my word of honour, that this work is original and that all non-original contributions were properly referenced with source identification.

Porto, 18th February 2022


(Inês Sousa Monteiro)

Contents

1	Project Framework.....	1
1.1	Project Presentation.....	1
1.2	Presentation of the Company.....	2
2	Introduction.....	3
2.1	Cosmetic Products.....	3
2.1.1	Regulation on Cosmetic Products	4
2.2	Liquid Soaps	5
2.3	Stability Studies.....	6
2.3.1	Physical stability	7
2.3.2	Chemical stability.....	8
2.3.3	Microbiological stability	9
2.4	Preservatives.....	10
2.4.1	Isothiazolinones.....	12
2.4.2	Alternative Preservatives.....	13
2.4.3	Evaluation of Antimicrobial Preservation.....	16
3	State of the Art.....	19
3.1	Contamination on cosmetics	19
3.2	Efficacy of preservatives in cosmetics	20
3.3	Stability Studies in Cosmetics	20
4	Materials and Methods.....	23
4.1	Tested Samples	23
4.2	Accelerated stability assessment.....	23
4.2.1	Organoleptic analysis	24
4.2.2	Weight analysis	24
4.2.3	pH analysis	24
4.2.4	Density Analysis	24
4.2.5	Viscosity Analysis.....	24
4.2.6	Foaming Capacity and Foam Stability Analysis	24

4.2.7	Centrifugation Analysis	25
4.2.8	Contamination Level Analysis	25
4.3	Preservative's Evaluation.....	25
4.3.1	Antimicrobial Activity Analysis	25
4.3.2	Challenge Test.....	26
4.4	Statistical Analysis	27
5	Results and discussion	28
5.1	Accelerated Stability Studies.....	28
5.1.1	Physical, Chemical and Microbiological Properties Evaluation Tests	28
5.2	Preservative's Evaluation.....	38
5.2.1	Antimicrobial activity analysis– Agar-well diffusion method.....	38
5.2.2	Challenge Test.....	39
6	Conclusion	41
7	Future Work.....	42
8	References.....	43
9	Appendices.....	a
	Appendix 1 - Foaming Capacity and Foam Stability Photographic Record	a
	Appendix 2 – Photographic Record of Antimicrobial activity analysis –.....	b
	Appendix 3 – Photographic Record of the Challenge Test	c
	Appendix 4 – Graphic Representation of the Challenge Test	d

List of Figures

Figure 1. European market for cosmetic products in 2020, adapted from ⁸ .	4
Figure 2. Chemical structure of benzoic acid (1) and dehydroacetic acid (2).	11
Figure 3. Chemical structure of phenoxyethanol.	11
Figure 4. Chemical structure of methylchloroisothiazolinone (1) and methylisothiazolinone (2).	11
Figure 5. Formulation B at 50 °C.	29
Figure 6. Weight loss (%) of formulations A and B during the accelerated stability evaluation. Since the organoleptic analysis was performed only on one sample the standard deviations are not presented.	30
Figure 7. pH measurements of formulations A and B during the accelerated stability evaluation. The means $\pm$ SD are presented and the symbol * indicate statistically different values for $p < 0.05$.	31
Figure 8. Density measurements of formulations A and B during the accelerated stability evaluation. The means $\pm$ SD are presented and the symbol * indicate statistically different values for $p < 0.05$.	32
Figure 9. Viscosity measurements of formulations A and B during the accelerated stability evaluation. The means $\pm$ SD are presented and the symbol * indicate statistically different values for $p < 0.05$.	33
Figure 10. Foaming Capacity measurements of formulations A and B during the accelerated stability evaluation. The means $\pm$ SD are presented and the symbol * indicate statistically different values for $p < 0.05$.	35
Figure 11. Foaming Stability measurements of formulations A and B during the accelerated stability evaluation. The means $\pm$ SD are presented and the symbol * indicate statistically different values for $p < 0.05$.	36
Figure 12. Centrifugation results of formulations A and B at RT in t0, t1 e t2 during the accelerated stability evaluation.	37
Figure 13. Contamination levels results of formulations A and B in the accelerated stability evaluation.	37
Figure A1. Photographic record of formulation A in the foaming capacity and foam stability analysis immediately after homogenization (1) and after 30 minutes (2).	a
Figure A2. Photographic record of formulation B in the foaming capacity and foam stability analysis immediately after homogenization (1) and after 30 minutes (2).	a
Figure A3. Photographic record of the inhibition halos of formulation A for <i>E. coli</i> (1), <i>S. aureus</i> (2) and <i>S. epidermidis</i> (3).	b
Figure A4. Photographic record of the inhibition halos of formulation B for <i>E. coli</i> (1), <i>S. aureus</i> (2) and <i>S. epidermidis</i> (3).	b
Figure A5. Photographic record of the CT in the first attempt.	c
Figure A6. Photographic record of the CT in the second attempt.	c
Figure A7. Graphic representation of the Challenge Test.	d

List of Tables

Table 1. Components of Castelbel's liquid soaps ¹⁴	6
Table 2. Effect of various factors on preservative efficacy ⁵⁷	16
Table 3. Choice of test organisms for CT on water-miscible cosmetics ⁵⁷	17
Table 4. Acceptance criteria for evaluation of antimicrobial activity of cutaneous formulations for bacteria and fungi ⁵⁸ . NI: no increase in number of viable microorganisms compared to the previous reading.....	18
Table 5. Organoleptic analysis of Formulation A and B.....	28
Table 6. Inhibition halo (diameter values in millimeter) obtained for each bacterial strain with formulations A and B.	38
Table 7. CT results (CFU/mL).	39
Table 8. CT with neutralizer results (CFU/mL). un: uncountable.	40

Notation and Glossary

<i>% foaming capacity</i>	Percentage of foaming capacity	%
<i>% foam stability</i>	Percentage of foam stability	%
$V_{foam\ after\ homogenization}$	Foam volume immediately after homogenization	mL
$V_{foam\ after\ 30min}$	Foam volume after resting for 30 minutes	mL
$V_{initial\ solution}$	Volume of initial solution before homogenization	mL
<i>% water recovered</i>	Percentage of water recovered	%
<i>mass water resolved</i>	Mass of water resolved	g
<i>mass of emulsion</i>	Mass of emulsion	g
<i>fraction of water in emulsion</i>	Fraction of water in emulsion	

List of Acronyms

BDP	Benzoic acid, Dehydroacetic acid, Phenoxyethanol	GMP	Good Manufacturing Practices
CAGR	Compound Annual Growth Rate	ISO	International Organization for Standardization
CFU	Colony-Forming Units	MI	Methylisothiazolinone
CLP	Classification, Labelling and Packaging	NACDG	North American Contact Dermatitis Group
CMI	Chloromethylisothiazolinone	OD	Optical Density
CMR	Carcinogenic, Mutagenic or Toxic for reproduction	PAO	Period After Opening
COLIPA	Cosmetics Europe	PET	Preservative Effectiveness Test
CPNP	Cosmetic Products Notification Portal	RNA	Ribonucleic Acid
CT	Challenge Test	RT	Room Temperature
CTFA	Cosmetics, Toiletry, and Fragrance Association	RTL	Room Temperature with Light
DHA	Dehydroacetic Acid	SCCS	Scientific Committee on Consumer
DNA	Deoxyribonucleic Acid	SD	Standard Deviation
EC	European Commission	SUE	Serious Undesirable Effects
ECHA	European Chemicals Agency	USA	United States of America
EU	European Union	USP	United States Pharmacopoeia
FDA	Food and Drug Administration	UV	Ultraviolet

1 Project Framework

1.1 Project Presentation

The cosmetics production is required to comply with a set of guidelines that aim to regulate the process as well as the product's market placement in order to ensure quality and safety to consumers. Even though each product is required to demonstrate formulation stability before being placed on the market, the regulations do not specify any protocol to obtain the necessary data and it is the manufacturer's responsibility to achieve these standards. Since cosmetics can be used by a client every single day, the safety and quality regulations are essential to ensure that there is no negative impact being caused on the costumers and the lack of regulation of cosmetics can disproportionately impact people's health.

The stability of a cosmetic product reflects its ability to preserve its physicochemical, microbiological, functional, and aesthetic properties over time within acceptable standards. This makes it a crucial quality assessment parameter that allows the deduction of the recommended period of use, under similar conditions of the product's utilization.

On a different topic, cosmetics are easily degraded by microorganisms due to their organic composition and high-water content and contamination could cause infections or irritation in consumers. Preservatives are substances added to these products to prevent microbial contamination and therefore, enhance their stability. One of the most popular classes of preservatives – isothiazolinones - includes methylisothiazolinone (MI) and chloromethylisothiazolinone (CMI), and have raised some concerns regarding the possibility of causing contact allergy.

The main objectives of this dissertation project are to (1) evaluate the substitution of the preservatives MI and CMI used in liquid soap products from *Castelbel* by a preservative considered safer, that is a mixture of phenoxyethanol, benzoic acid, and dehydroacetic acid (BDP), and (2) perform stability tests in this new formulation, as well as the previously used one, to establish a comparison and confirm its properties and appropriate quality levels.

The work outline includes a description of the investigated product characteristics, the test conditions, the monitoring methods, the preparation of the associated documentation (procedures, data records, stability reports), and equipment and laboratory management activities.

1.2 Presentation of the Company

Castelbel is a Portuguese brand specialized in the production of luxury fragranced body care and home articles. Founded in 1999, the company started by providing handmade soap bars to its first client located in North America and as it grew, the soap production was extended to brands such as Zara Home or Gant Home¹. Eventually, in 2006 its own label was launched as “Castelbel”, a name that reflects two of the main characteristics associated with the company: its location, *Castêlo da Maia*, and beauty which is present in all their products. One year later, the luxury brand designated “Portus Cale” was added to the repertoire and nowadays, their products range from soaps to scented candles, room diffusers, hand and body creams, dish washing soaps and a variety of other personal care, perfumery and interior decoration products². All of them, stand out for their quality, innovative design and handmade packaging².

Through hard work and creative thinking, this company has managed to secure its presence in over 1000 sale points worldwide, reaching more than 50 countries and continues to exceed expectations and thrive year after year².

2 Introduction

2.1 Cosmetic Products

People have taken great care in their appearance and tidiness for quite a long time, which makes the concept of beauty and cosmetics as old as mankind and civilization. Cosmetics have a history covering thousands of years of applying ingredients from plants, animals and mineral sources ³. According to the Council of European Union regulation (Directive 76/768/ EEC) a cosmetic product is defined as: “cosmetic product means any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips, and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition, or correcting body odors” ⁴. In other words, the main purpose of a cosmetic product is to treat and improve the external surface of the human body ⁵ and they can be classified according to their functions and fields of application as ⁶:

- cosmetics for personal cleansing (soaps, deodorants, shampoos);
- cosmetics for the skin, hair, and integument care (toothpastes, products for external intimate care);
- cosmetics for embellishment (perfumes, lip colors);
- protective cosmetics (solar products, anti-wrinkle products);
- corrective cosmetics (beauty masks, hair dyes);
- maintenance cosmetics (shaving cream, moisturizing creams);
- active cosmetics (fluoridated toothpastes, antiseptics).

Presently, cosmetics have become an indispensable feature of modern lifestyle of individuals. In addition, growth in consciousness about external beauty along with individual’s internal intellect has become one of the major driving factors for use of cosmetics in the global market. Nowadays, along with women, there is an increase in the use of cosmetics among men in their daily routine. Hence, these lifestyle changes have led to a massive growth of these product’s usage. This translates into the development of the cosmetic market, which offers a wide range of products to millions of consumers worldwide ⁷. The global cosmetics market size was valued at \$380.2 billion in 2019, and it is projected to reach \$463.5 billion by 2027, registering a Compound Annual Growth Rate (CAGR) of 5.3% from 2021 to 2027 ⁷. The European cosmetics and personal care market is the largest in the world, valued at €76.7 billion at retail sales price in 2020 and bringing in at least €29 billion in added value to the European economy annually and supporting over 2 million jobs ⁸. The retail sales prices of the European market for cosmetic products, €76.7 billion, in each country is represented in Figure 1.

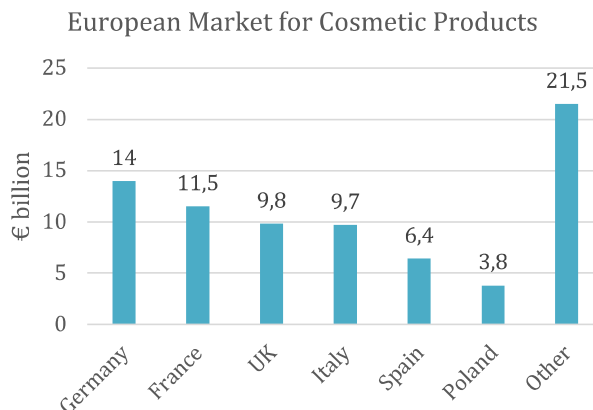


Figure 1. European market for cosmetic products in 2020, adapted from ⁸.

Regarding *Castelbel*, this company produces cosmetics as well as non-cosmetic products intended for personal and home care, respectively (e.g., solid and liquid soaps, creams, shampoos, scented candles, room diffusers, dish washing soaps). This dissertation project focuses on a specific cosmetic product produced by the company, liquid soaps, whose production, distribution, and commercialization need to comply with established guidelines according to the market in which they will be placed.

2.1.1 Regulation on Cosmetic Products

The main regulatory framework for cosmetic products placed on the European Union (EU) market is the European Commission (EC) regulation N° 1223/2009 ⁹. Since *Castelbel* sells cosmetic products in more than 50 countries, most of them belonging to the EU, this regulation applies to it ¹⁰.

This regulation is effective in organizing the framework for all operators in the cosmetic sector and provides increased product safety as well as simplification of procedures. It replaces the revised Directive 76/768/EC, providing a more robust and internationally recognized regime that considers current technological developments and highlights product safety ¹⁰. The main changes established in the cosmetic sector by this regulation are ¹⁰:

- Enhanced safety measures for products that manufacturers need to follow and report before placing a product on the market;
- Presenting the concept of “responsible person”, who is a person designated within the EU to guarantee the product safety assessment;
- Centralized notification of all cosmetic products placed on the EU market via the EU cosmetic products notification portal (CPNP), which is performed by the manufacturers;
- Reporting serious undesirable effects (SUE) to national authorities that will also contact users, healthcare professionals and others to collect information and share with other EU countries;

- New rules prohibiting or restricting the use of certain ingredients such as colorants, preservatives, ultraviolet (UV) filters, nanomaterials. Prohibition of the use of carcinogenic, mutagenic or toxic for reproduction (CMR) substances, apart from exceptional cases;
- Added requirements for packaging and labeling, such as inclusion of name and address of the responsible person, country of origin for imported products, nominal content, pictograms showing the date of minimum durability if less than 30 months (hourglass symbol), or period after opening (PAO) if the minimum shelf-life is more than 30 months (open jar symbol), precautions, batch number reference, function of the cosmetic product, and list of ingredients in descending order down to 1% (under 1% in any order), including the indication of nanomaterials with the word *nano* after the name of the ingredient.

2.2 Liquid Soaps

Soap is considered the oldest cosmetic product to be produced and plays an important role in our daily lives, being one of the most consumed personal care products worldwide, reflecting a highly competitive market ¹¹. The earliest recorded evidence of soap-like materials production dates back to around 2800 BC in ancient Babylon. It is unclear what exactly these products were used for, although their use was probably restricted to washing clothing. During the nineteenth century, the chemistry of soap production was developed and improved with the discovery of the different fatty acids present in neutral fats and oils. This led to the establishment of the fundamental modern-day process, which involves the saponification of neutral fats or fatty acids with the appropriate caustic material ¹¹.

In the late 1970s, liquid soaps were developed and launched on the global market in the U.S.A., and they soon gained consumer acceptance and became increasingly popular due to some advantages offered over conventional bar soaps, such as a having a higher antibacterial effect while bar soaps can also turn slippery and with a slimy consistence in standing water which is unpleasant for consumers and leads to wastage ¹². A typical liquid soap has a mixture of different types of surfactants in its composition to achieve the desired cleaning and foaming properties ¹³. Liquid soaps produced at *Castelbel* contain the type of ingredients mentioned in Table 1 in their formulation.

Table 1. Components of Castelbel's liquid soaps ¹⁴.

Component	Function	Example
Solvent	The solvent is the main component of liquid soaps. <i>Castelbel</i> always uses water.	Water
Surfactant	More than one surfactant is used in liquid soaps and they act as emulsifying and cleaning agents. Having a hydrophilic head (water-soluble) and a hydrophobic tail (water insoluble and soluble in lipids, or oils) is what allows to remove dirt - hydrophilic part binds to water and the hydrophobic to the solid that is meant to be removed.	Cocamidopropyl betaine and Sodium laureth sulfate
Denaturants/ humectants	It gives the skin a moisturizing texture.	Glycerin
Viscosity agent	It provides the liquid soap with the desired and expected viscosity.	Sodium Chloride
Fragrance	Parfum that gives the liquid soap its scent.	Lavender
Antistatic/film-forming agent	This component leaves cohesive covering over the skin when applied to their surface, leaving a smooth feel on skin.	Polyquaternium-7
Preservative	It inhibits the growth of microorganisms capable of causing biologic deterioration that endangers the consumer.	Methylchloroisothiazolinone and Methylisothiazolinone

Within the soap category, the liquid type is gaining popularity and since most of the available products are similar in terms of performance, this is a highly competitive market, which translates into a growing need for companies that provide liquid soap products that offer cosmetic benefits that go beyond cleansing and therapeutic actions ¹⁵.

2.3 Stability Studies

The concept of stability represents the ability of a cosmetic product to maintain its initial and desired physical, chemical, sensorial, microbiological and functional properties from its manufacturing date until the end of the recommended period of use, under the conditions in which it is sold and used ¹⁶. Therefore, to fully evaluate a cosmetic product's performance and quality, the stability of the formulation is a crucial parameter since it demonstrates possible physicochemical changes, as well as microbiological and packaging interactions that may lead to a loss of functional or aesthetic properties and, consequently, impact the shelf-life of the product ^{16,17}. The information provided by these tests is essential for defining the product's formulation (helping in the optimization and manufacturing), choosing the appropriate packaging, defining transportation, storage, and usage conditions and also to estimate shelf-life of products and to ensure customer safety.

An important part of stability studies relies on the product's manufacturer, since it is his responsibility to specify the critical product attributes to be investigated, test conditions (which

encompasses physical, chemical and microbiological parameters), methods for monitoring changes over time, and the degree of change that is acceptable^{16,17}. Since each product can react differently to the same procedure due to its particular chemical composition, packaging, and external conditions, these aspects of a product's stability are obviously established according to the specific formulation of the cosmetic^{16,18}.

The stability of cosmetic products is generally evaluated under accelerated conditions prior to commercialization to predict product shelf-life and behavior of the product formulation over the long term and therefore estimate the actual stability of the product for specified market conditions in a short period of time^{16,18}. To achieve this, the samples are kept under test conditions designed to accelerate changes that may occur.¹⁶ According to the COLIPA (presently denominated as Cosmetics Europe) the product should be stored under different temperature conditions for a certain period of time that allows to infer a product's stability in real-time. Since a cosmetic product is exposed to multiple temperatures and humidity conditions during their life cycle, when testing these products a wide range of conditions should be used^{16,18}. Cosmetic stability guidelines list multiple conditions for accelerated stability testing: i) $(30 \pm 2)^\circ\text{C}$; ii) $(37 \pm 2)^\circ\text{C}$; iii) $(40 \pm 2)^\circ\text{C}$; iv) $(45 \pm 2)^\circ\text{C}$; v) $(50 \pm 2)^\circ\text{C}$. Low temperature conditions are helpful in predicting changes in the product caused during the marketing process, such as transportation. The tests can be performed in a period of one week to three months and generally, eight weeks under accelerated stability correspond to one year of real-time stability. However, this estimation of the real-time stability from the accelerated stability results depends on the test conditions and methods implemented by the manufacturer¹⁸.

Since soaps are considered to be stable products, during real-time monitoring the alterations happen very slowly, that's why accelerated conditions are appealing, they increase the rate of potential degradation in order to shorten the evaluation time to reach results on a product's stability^{16,18}. As mentioned above, the assessment of stability criteria is defined by each company according to the product and packaging types. In the present dissertation project, accelerated stability studies on liquid soaps were implemented and the following sections will describe the parameters under scope in these studies.

2.3.1 Physical stability

When discussing physical stability, it is important to describe its opposite, physical destabilization. It is a phenomena that comprises phase separation, precipitation of ingredients, segregation of particles, color change and weight loss which are alterations that can influence products' stability, as well as affect its aesthetic and ultimately the customer acceptance¹⁶. A key aspect that can introduce physical destabilization in the product is exposure to light which can lead to discoloration¹⁶. For this reason, it is relevant to assure the photostability of a cosmetic product by testing it in daylight or artificial ambient light conditions to mimic the environment to which the products will be exposed during commercialization and in customers' homes. These tests should be performed with clear containers and on finished packaging to determine whether the product requires protective packaging and, if so, to determine whether the packaging under study is suitable for the specified market conditions.

To test a product's vulnerability to destabilization (like phase separation), a centrifugal test is a usual procedure that increases gravity force in order to analyze the products constituents of different densities and its phase separation.

Finally, it is crucial to record a visual observation and sensorial examination of product attributes, including color, odor, appearance, and texture, using for example a numerical or descriptive rating system to objectively describe potential changes in the product. Meaning that, the organoleptic profile of the product should be analyzed since it can also vary over time due to physical and chemical alterations as well as microbiological ones.

2.3.2 Chemical stability

A formulation can become chemically instable when chemical degradation, changes in molecular structures or chemical reactions between its constituents (e.g., oxidation, hydrolysis, and transesterification) occurs. In liquid soaps, these common reactions can cause the breakdown of the stabilizing surfactant, hinder functional polymeric additives, or deactivate the preservatives used to inhibit microbial growth ¹⁶. Besides these obvious drawbacks, chemical reactions can also lead to structural changes that affect the physicochemical properties of the product, including moisture content, alkali content, pH and total fat matter, which is a crucial parameter in defining a product's quality, efficiency, and cleansing properties. An important variable to take into consideration is the pH value since its variation may indicate chemical and microbiological changes in the formulation ¹⁶.

Regarding the chemical destabilization processes:

- oxidation happens when oxygen leads to the formation of free radicals triggering oxidation-reduction reactions and may cause undesirable odors, discoloration, and changes in the organoleptic characteristics of the formulation. Many fragrance ingredients, that are part of the composition of liquid soaps, are sensitive to oxygen exposure and more prone to oxidize, particularly if accelerated by heat or light.
- light alterations may induce photoreactions and UV light may lead to the formation of free radicals, possibly causing changes in organoleptic and physical properties.
- hydrolysis occurs in reactions with water and affect esters and amides and impact the aesthetic or physical attributes of the product (such as loss of cleaning efficacy or becoming irritating to the skin).
- Transesterification results from the reaction between the ester function of one molecule with the hydroxyl group of an alcohol and may alter the odor or taste.
- Chemical interactions between ingredients may lead to discoloration and viscosity alterations amongst others. These reactions are typically triggered by light, heat, oxygen or pH. The presence of oxygen can lead to polymerization, which is a common interaction that affects the product's viscosity.

2.3.3 Microbiological stability

Every cosmetic manufacturer has a responsibility relative to the microbiological safety of its products, having to ensure that they have been produced under hygienic conditions¹⁹. Cosmetic products are not expected to be sterile. However, they shall not contain some types of microorganisms that could affect consumer health¹⁹ and cosmetic manufacturers' may also ensure that microorganisms introduced in every-day use won't affect the quality or safety of the product²⁰. Therefore, the first step would be to perform a microbiological risk assessment of the product to determine if the cosmetic microbiological international standards apply. This microbiological risk assessment is intended to help determine the risk level of the product and what level of testing, if any, is necessary to ensure the product quality²⁰. The manufacturer should follow the Good Manufacturing Practices described in ISO 22716 and take the necessary precautions to limit the introduction of microorganisms from raw materials, processing and packaging¹⁹. The degree of risk depends on the ability of a product to support the growth of microorganisms and on the probability that those microorganisms can cause harm to the user. Since cosmetics usually contain water and nutrients, they present optimal conditions for microbial growth. However, for some cosmetics, the likelihood of microbiological contamination is extremely low (or non-existent) due to product characteristics that create a hostile environment for the survival and growth of microorganisms. These products do not require microbiological testing and manufacturers can decide not to perform them if they can ensure that products meet this standard^{19,20}.

As previously mentioned, water is an important variable when discussing the growth of microorganisms. The metabolism and reproduction of microorganisms require the presence of water in an available form and the most useful measurement of water availability in a product formulation is water activity, which is defined as the ratio of the water vapor pressure of the product to that of pure water at the same temperature²⁰. In the food and cosmetic industry is very common to use acidic pH to avoid bacteria. Alkaline pH may also create a hostile environment and can be used as part of a preservative system. An example of that environment are liquid soaps, which with an alkaline pH (pH 9.0 to 10.0) offer conditions that inhibit the growth of some microorganisms. When these pH values are used in combination with chelating agents, glycols, antioxidants, low water activity and high levels of surfactants, an environment that does not support microbial growth can be created. Another factor that can influence the microbiological risk of a cosmetic product is temperature. In fact, there is an ideal temperature range for microbial growth and values far above or below this range can inhibit the growth of microorganisms in the formulation¹⁹. Two different but temperature-related approaches to microbiological control are the application of an adequate temperature for rapid cell kill, i.e., a thermal shock, or maintaining the temperature above the optimum value for an extended period of time¹⁹.

In summary, the main microbiological parameters for stability testing are microbial count, and preservation efficacy testing (challenge-test), which will be discussed in more detail in Section 2.4.3.

2.4 Preservatives

As mentioned before, liquid cosmetic formulations, due to their organic composition and high-water content, are easily degraded by microorganisms. This microbial contamination in cosmetics constitutes a severe risk for consumer health since contaminated products can cause infections or irritation, mainly when applied to damaged skin, eyes or babies, whose skin is commonly more sensitive²¹. The multiple reported microbial infection, both in costumer's homes and in hospitalized individuals, caused by contaminated cosmetics serves as proof of this reality²². In 1946, the first contamination of cosmetics was reported with several cases of neonatal death by talcum powder containing *Clostridium tetani*. Since the 1960s, opportunist organisms, such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas sp.*, *Serratia sp.* and *Enterobacter sp.*, have all been isolated from cosmetic products²³.

Preservatives are frequently added to cosmetics to prevent this microbial growth. However, they can also be useful to cosmetic products in terms of protection against damage and degradation caused by exposure to oxygen (antioxidant function) or to UV light (photoprotective function)²⁴. Since preservatives are biologically active compounds, they can cause an irritating, toxic or sensitizing effect. For this reason, the safe use of these compounds is always being questioned and scientific evidence of possible harmful effects on consumers' health has resulted in increasing restrictions imposed by various international regulations. In fact, preservatives are among the most common causes of contact dermatitis and have caused several epidemics. In the 1950s and 1960s, a cutaneous exposure to textile finishes and cosmetics that were preserved with formaldehyde caused one. Later, in the 1990s and early 2000s, the use of methyldibromoglutaronitrile resulted in another epidemic, which later led to a ban on the incorporation of this compound in cosmetic products in Europe. Other preservatives, such as iodopropynyl butylcarbamate, are emerging as allergens whose prevalence has been rising over the past few years. Surprisingly, parabens, one of the oldest and still the most frequently used biocide in cosmetics, continue to be far less sensitizing than most of the newer agents²⁵. However, some parabens were already prohibited in this kind of products in the revision of the EU Regulations²⁶ after being linked to reproductive problems²⁷.

The most commonly used chemical classes of antimicrobial preservatives are organic acids, alcohols and phenols, aldehydes and formaldehyde releasers, isothiazolinones, biguanides, quaternary ammonium compounds (QAC), nitrogen compounds, heavy metal derivatives, and inorganic compounds (particularly inorganic sulfites and bisulphites)).

This dissertation project focusses on three different classes – organic acids, alcohols and phenols and isothiazolinones. The organic acids antimicrobial effect depends on its aliphatic structure, that makes the molecule lipophilic. This allows the preservative to penetrate the membranes of microorganisms. Entering the cell, it has the ability to dissociate and acidify the medium, disrupting the balance of ions and salts and making cellular mechanisms and processes unfeasible⁹. Two of the most popular organic acids are benzoic acid and dehydroacetic acid which are represented in Figure 2.

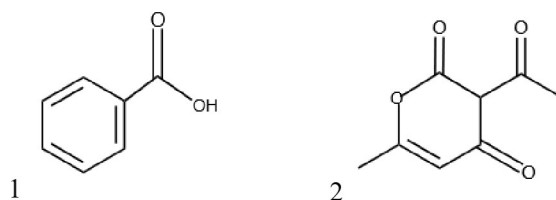


Figure 2. Chemical structure of benzoic acid (1) and dehydroacetic acid (2).

Another mentioned class of preservatives is alcohols and phenols, these can cause membrane damage and rapid denaturation of proteins, with subsequent interference with metabolism and cell lysis²⁸. One of the most commonly used preservatives of this class, which is included on the preservatives list permitted by the EU is phenoxyethanol²⁹, which is represented in Figure 3.

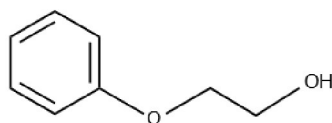


Figure 3. Chemical structure of phenoxyethanol.

The last class of preservatives addressed in this project is isothiazolinones. These compounds can diffuse across the bacterial cell membrane and cell wall of fungi. In the intracellular media, the electron-deficient sulfur of the N-S bond of these compounds can react with the nucleophilic groups of the cellular components, such as the thiols from cysteines of proteins active sites blocking their enzymatic activity and ultimately causing cellular death³⁰. This class of preservatives will be further discussed in the next section. The chemical structure of two preservatives from this class are presented in Figure 3.

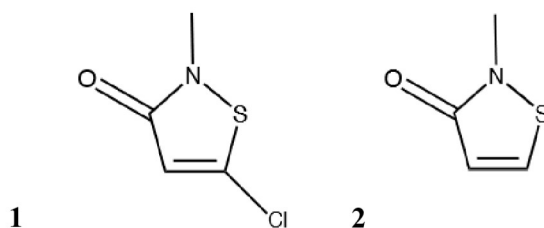


Figure 4. Chemical structure of methylchloroisothiazolinone (1) and methylisothiazolinone (2).

2.4.1 Isothiazolinones

Isothiazolinones are among the most popular preservatives in aqueous-based industrial or domestic products and applications due to their effectiveness against a broad spectrum of bacteria, fungi and yeasts over a wide range of pH levels ²¹.

These compounds are heterocyclic organic chemicals containing vicinal sulfur and nitrogen atoms. More specifically, they are derivatives of 2*H*-isothiazolin-3-one with an active sulfur moiety capable of oxidizing thiol-containing residues, thereby offering powerful preservation activity ³¹.

Two of the most used biocides from this class are the 2-methyl-3-isothiazolinone, also known as methylisothiazolinone (MI) and 5-chloro-2-methyl-3-isothiazolinone (CMI), also known as chloromethylisothiazolinone (Figure 4). They are commonly used as a mixture in the ratio of 3:1 (CMI:MI) and this mixture first appeared in the United States in 1980. Five years later they were introduced in Europe. ³². The use of both MI and CMI has increased dramatically, and has even gained presence at much higher concentrations ³³. After analyzing 2000 cosmetics, The Food and Drug Administration (FDA) registered an increase of the presence of this mixture from 222 products in 1984 to 512 in 1987. These preservatives are powerful biocides that are used in a wide range of daily life products such as detergents, paints and cosmetic products like shampoos, liquid soaps and many others ³².

Nevertheless, these two compounds have attracted attention for the wrong reasons, since their introduction in the cosmetic field resulted in a contact allergy epidemic, leading to the formation of strict regulations regarding its use and the necessity of establishing a maximum allowed concentration for these substances. In fact, these limitations helped to reduce the number of sensitized patients.

However, MI was later introduced as a stand-alone preservative in personal care products, and from 2007 to 2010, the use of MI in cosmetics doubled ³⁴. This originated new cases of contact dermatitis ³⁵. There were cases of contact allergy related to MI in cosmetic products reported by Garcia-Gavin et al. ³⁶ and occupational cases of MI contact allergy described by Isaksson et al. ³⁷ and Thyssen et al. ³⁸ from occupational exposure to wallpaper glue and paint, respectively.

The North American Contact Dermatitis Group (NACDG) created a top seventy allergens listed by the 2009 and 2010 patch test results, in which methylchloroisothiazolinone (CMI)/methylisothiazolinone (MI) ranked sixteenth place ^{39,25}. Recently there has been increasing prevalence of contact dermatitis to MI alone, and in 2013, MI was named the contact allergen of the year by the American Contact Dermatitis Society ⁴⁰. Besides allergens, CMI and MI are also classified as skin sensitizers in the harmonized classification according to (EC) 1272/2008 (Regulation on classification, labelling and packaging of substances and mixtures – CLP regulation) ⁴¹.

As previously mentioned, in the 1960s and 1970s, an epidemic of contact dermatitis led to an increasing awareness of preservatives in cosmetic products, and regulation and legislation was introduced in the EU and the USA ⁴². Since 1976, the Cosmetic Directive is responsible for the EU cosmetic legislation, while the US cosmetic legislation is mostly managed through the 1938 Federal Food, Drug, and Cosmetic Act issued by the US FDA and the US Department of Health and Human Services ²¹. Since the manufacturers add preservatives to their products during use and storage to avoid microbiological

contamination of cosmetics, in both the EU and the USA, all preservatives are safety evaluated by expert committees that confirms the safety of use of the maximum concentration of a preservative requested by the cosmetic industry. Furthermore, both legislations have specific demands as to the microbiological quality of finished products as well as the products' ability to withstand contamination. These demands are based on the requirements of the pharmaceutical industry for non-sterile products, which can be found in the European and US Pharmacopoeia (USP) ²¹.

An important milestone in avoiding the contamination of cosmetic products, was the implementation of the Good Manufacturing Practices (GMP). Which are guidelines from ISO 22716:2007 intended to provide guidance for the cosmetic industry on the management of the human, technical and administrative factors affecting product quality. The main objective is to define the activities that enable you to obtain a product that meets defined characteristics ⁴³. In fact, over the last 30 years, the implementation of these practices has been the foundation for improving industrial quality control analyses, which naturally led to a decrease in the number of intrinsically contaminated cosmetic products.

In regards to the regulations of isothiazolinones, the Cosmetic Ingredient Review in the US and the Scientific Committee on Consumer Safety (SCCS) in the EU established in an early evaluation that a concentration of MI as a stand-alone preservative of 100 ppm would be considered safe. However, after this stand-alone preservative caused new cases of contact dermatitis, as previously stated ³⁶, the EU's SCCS updated its assessment on MI and determined that rinse-off products would be considered safe at a limit of 15 ppm from the viewpoint of the induction of contact allergy (sensitization), but in terms of leave-on cosmetics no safe concentrations had been adequately demonstrated. In terms of legislative demands of the preservative mixture of CMI/MI, the maximum allowed concentration in the EU is of 15 ppm in rinse-off cosmetic products as well. And in the US the mixture is considered safe up to a concentration of 15ppm in rinse-off products and 7.5 ppm in leave-on products ²¹.

Even with these restrictions, scientific experiments suggest that MI may be a neurotoxic biocide after prolonged exposures even at low levels ⁴⁴ and evidence of contact allergy is still reported in cases associated with occupational or domestic exposure to products containing isothiazolinones ⁴⁵. This prevalence of contact allergy still raises concerns, which creates the need for more information on the occurrence and concentrations of these compounds in industrial and consumer products.

2.4.2 Alternative Preservatives

In an effort to substitute the preservatives MI and CMI in liquid soaps formulations, another mixture of preservatives was chosen. This mixture is BDP and was selected due to its efficiency and the fact that one of its components, phenoxyethanol, is already widely used in *Castelbel's* cosmetic products, which makes this decision of switching preservatives convenient and practical for the company.

BDP is a preservative described as a liquid blend of phenoxyethanol and organic acids for gentle preservation ⁴⁶. Besides phenoxyethanol, BDP it contains benzoic acid and dehydroacetic acid and the name stands for its three components, BDP - benzoic acid, dehydroacetic acid and phenoxyethanol. The manufacturer doesn't provide the ratio of each component's concentration but from the initial description

it has been assumed that phenoxyethanol is present in a bigger proportion. This mixture has a density ranging from 1.118 to 1.132 g/cm³ and presents its maximum activity at pH values below 6, when organic acids are in their undissociated form. Benzoic and dehydroacetic acids display a pKa value of 4.20 and 5.12^{47,48}, respectively, and act in a complementary manner, as a bactericide along with phenoxyethanol ⁴⁹. As previously mentioned, benzoic and dehydroacetic acid are inserted in the class of preservatives of organic acids and phenoxyethanol in alcohols and phenols and their antimicrobial mechanism has been described in Section 2.4.

This mixture is recommended for leave-and-rinse applications at a dose of 0.6 to 1% and should be added, under agitation, at the earliest possible stage of the manufacturing process. It tolerates temperatures up to 80 °C and in the case of pH values higher than 6, the production process can also be carried out without causing the degradation of the active ingredients. However, to ensure adequate protection, the pH of the final product must be adjusted below 6 ⁴⁹.

In terms of regulations, the use of phenoxyethanol is allowed in Europe, Japan and USA up to 1%. In the EU, dehydroacetic acid is approved up to 0.6% (as acid) but prohibited in aerosols (sprays). In Japan, dehydroacetic acid and dehydroacetate are allowed up to 0.5% in all kinds of applications. Dehydroacetic acid is also allowed in the USA up to 0.7% ⁴⁹. The European limitations for benzoic acid are 2.5 % for rinse off products and 0.5% for leave-on products. In Japan, benzoic acid is approved with a limitation of 1% and in the USA, benzoic acid is considered safe up to 5%, but without sufficient guarantee when the primary route of exposure is inhalation. To conclude, the use of this preservative mixture is allowed in Europe, USA and Japan up to 1.2% ⁴⁹.

The toxicity of BDP is related to that of its separate ingredients. Phenoxyethanol is a proven cosmetic ingredient, meaning that it is safe when used according to current methods and recommended concentrations of use. Benzoic acid can be found naturally in plants and degraded in the environment. It is well-documented toxicologically and considered to be non-irritating or slightly irritating. An often overlooked side effect of organic acids is that they can cause non-allergic contact urticaria. However it is non-teratogenic, non-mutagenic and non-carcinogenic. Numerous clinical trials show that dehydroacetic acid is practically non-irritating, non-sensitizing, non-photosensitizing and non-phototoxic. Based on the clinical data and available animal tests, it can be concluded that dehydroacetic acid is safe as a cosmetic ingredient used according to the methods and concentrations of use stated ⁴⁹. Fundamentally, BDP is non-irritating based on in vitro tests with reconstructed human skin under the conditions and at the doses of use recommended to users.

As previously mentioned, since there is considerable variation in the composition of cosmetic products, it is always important to assess these preservative's efficacy in the final product through appropriate microbiological efficacy tests (Challenge Test) ⁴⁹.

- Phenoxyethanol safety

This section is focused on the main component of the preservative mixture BDP, which is phenoxyethanol and its safety towards consumers in cosmetic products.

Phenoxyethanol is an ether and aromatic alcohol, also recognized as 2-phenoxyethanol, ethylene glycol monophenyl ether, phenoxytol, 1-hydroxy-2-phenoxyethane and (2-hydroxyethoxy) benzene and in terms of antimicrobial activity, this substance acts in a very large spectrum being effective towards multiple bacteria⁵⁰, as well as against yeasts⁵¹. It can serve as a bactericidal agent due to its ability to increase the permeability of the cell membrane to potassium ions and inhibit the microbial DNA and RNA synthesis⁵¹. It is important to state that phenoxyethanol can display a less strong inhibitory effect on normal skin-resident bacteria in comparison to some cosmetic chemical preservatives such as methylisothiazolinone, but is widely used in an ample span of rinse-off and leave-on cosmetic products⁵². It comes as no surprise that it has been used as a preservative for decades in medicines, hand disinfecting biocidal products, products for toddlers, and many others and can be found in 23.9% of cosmetic products marketed in the USA, according to the American Contact Dermatitis Society database – the Contact Allergen Management Program⁵². Moreover, a recent study conducted in Spain reported that 43.09% of cosmetics sold exclusively in pharmacies, 23.29% of cosmetics sold in supermarkets and 14.1% of cosmetics from herbal shops, contained phenoxyethanol⁵².

As aforementioned, the European Scientific Committee on Consumer Safety designated phenoxyethanol as a safe preservative to be used in cosmetics for all costumers, children included, at a maximum concentration of 1%⁵³. It is considered one of the most well-tolerated cosmetic's preservatives with an extensive utilization and it has not been classified as a skin sensitizer by the European Chemicals Agency (ECHA)²⁹ nor a reproductive toxicant in (EC) No 1272/2008^{41,54}.

There have been controversial claims stating that phenoxyethanol affects the blood and liver and disrupts endocrine activity. However, those claims were unfounded, having in June 2015, the Endocrine Disruptor Screening Program of the U.S. Environmental Protection Agency (US-EPA) declared that phenoxyethanol had no oestrogenic activity⁵³.

In order to review this substance, multiple studies and tests will be explored according to the data collected by the SCCS, from peer-reviewed scientific journals and safety data from unpublished studies carried out by cosmetic product manufacturers and other published scientific literature.

In regards to the dermal absorption, in an unpublished study by Vincent CM and Marty JP cited by the SCCS⁵¹ it was demonstrated that *vitro* dermal absorption of phenoxyethanol in humans was high, fast and independent of its concentration in the formulation test. Following a 24h exposure, only small amounts of less than 0.1% remained in the skin's layers and most of the phenoxyethanol was recovered in the receptor fluid, showing that it doesn't bind to or accumulate in the skin. After 24h, the dermal absorption of phenoxyethanol, meaning the full amount of it in the receptor fluid, dermis and epidermis, for a product containing 1% of phenoxyethanol was $(37 \pm 10)\%$ for the rinse-off products and $(78 \pm 7)\%$ for the leave-on products.

In terms of skin irritation, an undiluted sample of phenoxyethanol was used and resulted in mild-to-moderate irritation in animals studies conducted in rabbits and guinea pigs⁵¹. However, in humans there were no reported effects after using phenoxyethanol on injured skin⁵⁵ even in a study where an aqueous solution containing 2% phenoxyethanol was used in premature neonatal infants⁵⁶.

2.4.3 Evaluation of Antimicrobial Preservation

The Challenge Test (CT, also known as a preservative effectiveness test, PET, or antimicrobial effectiveness test) is a procedure to determine whether a formulated cosmetic, pharmaceutical or other type of product is adequately preserved to prevent contamination from raw products and during consumer use⁵⁷. Even though the chemical, physical and microbiological properties of a preservative should be taken into consideration, these characteristics are not enough to evaluate if a product will be adequately preserved. In addition, the action of a preservative can be affected by several factors that must be considered when choosing an antimicrobial agent. Some of these factors are presented on Table 2.

*Table 2. Effect of various factors on preservative efficacy*⁵⁷.

Factor	Effect on preservative activity
Concentration	Increases activity
pH	A rise in pH may increase or decrease activity depending on the preservative
Temperature	Activity increases with a rise in temperature
Containers	Can possibly cause interactions or adsorption of the preservative
Microorganisms	The effect depends on the type and nature of the microorganism
Organic matter	Activity may be reduced
Formulation constituents	May reduce or improve activity

Briefly, a CT is designed to establish whether a product is adequately preserved over its stipulated shelf-life. A CT may follow the instructions of ISO 11930 or other methods such as the ones from SCCS, the EU and US pharmacopoeia (USP) or the test from the Cosmetics, Toiletry, and Fragrance Association (CTFA) Microbiology Guidelines amongst others. These may diverge in some aspects, like the test microorganism and acceptance criteria, but all thrive to reach the same goal - to evaluate the effectiveness of antimicrobial preservation. In this dissertation project, the guidelines of ISO 11930 will be followed, which is a standard for evaluating the antimicrobial stabilization of a cosmetic product⁵⁸.

As mentioned before, the adequate level of preservation of a cosmetic product should be maintained throughout the shelf-life of the product and through the consumer's use of it. During use, a product may be contaminated with multiple microorganisms and to ensure that it is adequately preserved, CT are conducted on batch samples at the beginning, during and at the end of the shelf-life of that product. A suitable CT involves the deliberate addition of bacteria or fungi to a test product to ensure that adequate microbial inactivation provided by the action of the product's preservative occurs⁵⁷.

The choice of test organism when conducting a CT is based on those who are likely to be present during product use. For cosmetic products, the ISO 11930 specified the microorganisms *P. aeruginosa*, *Staphylococcus aureus*, *Escherichia Coli*, *Candida albicans* and *Aspergillus brasiliensis* as the recommended for the test ^{58,54}. The CTPA specifies a few other microorganisms for water-miscible cosmetics that are displayed in Table 3.

Table 3. Choice of test organisms for CT on water-miscible cosmetics ⁵⁷.

Gram-positive bacteria	Gram-negative bacteria	Mould	Yeast
<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>Aspergillus niger</i>	<i>C. albicans</i>
<i>Staphylococcus epidermidis</i>	<i>K. pneumoniae</i>		
	<i>E. coli</i>		
	<i>Enterobacter gergoviae</i>		
	<i>Burkholderia cepacian</i>		
	<i>Acinetobacter baumannii</i>		

Regarding the inoculation of test organisms, the most common procedure and used in this dissertation project is the single challenge, in which the microbial counts of inoculum should be 10^7 to 10^8 Colony-Forming Units (CFU)/mL of bacteria and 10^6 to 10^7 CFU/mL of fungi; then, the microbial counts in the cosmetic product should be 10^5 to 10^6 CFU/mL of bacteria and 10^4 to 10^5 CFU/mL of fungi. To prevent dilution of the product/preservative, the volume of microbial suspension added should be not more than 1% of the volume of the product ⁵⁴. In terms of sampling procedures, at defined periods of time, the samples should be removed and serially diluted to enumerate bacterial colonies after incubation of culture agar for appropriate periods of time at certain temperatures. From the colony counting method, it is possible to calculate the log reductions from zero (starting time point) to a given time.

In the criteria of acceptance of antimicrobial preservation efficacy, there are two levels for bacteria and fungi, namely an A level (the recommended efficacy to be achieved) and a B level (to be met only if there are strong reasons why the A criteria cannot be satisfied) (Table 4). In other words, if the formulation meets criteria A, the microbiological risk is considered to be tolerable (the cosmetic product is protected against microbial proliferation that may present a potential risk for the user) and the cosmetic product is deemed to meet the requirements of this International Standard without additional rationale. 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 provides a higher

level of protection than a jar. This would be considered a protective device for risk reduction ⁵⁴. The criteria for evaluation of antimicrobial activity for bacteria and fungi are given in Table 4.

Table 4. Acceptance criteria for evaluation of antimicrobial activity of cutaneous formulations for bacteria and fungi ⁵⁸. NI: no increase in number of viable microorganisms compared to the previous reading.

	Level	Log reduction			
		2 days	7 days	14 days	28 days
Bacteria	A	2	3	-	NI
	B	-	-	3	NI
Fungi	A	-	-	2	NI
	B	-	-	1	NI

Focusing on the criteria for bacteria, this is at least 2 log reduction from initial count to 2 days after or 3 log reduction until 7 days after, and no increase after 28 days of assay ⁵⁷.

3 State of the Art

3.1 Contamination on cosmetics

Although cosmetic products are not sterile, they must be free of pathogenic microorganisms (such as *S. aureus*, *E. coli*, *P. aeruginosa*) and the total aerobic microbial count must be low. The presence of these organisms as well as high levels of other microorganisms in cosmetics can cause spoilage, i.e. physical deterioration of the product, which can be a health hazard for consumers ⁵⁹.

There have been many studies over the years that prove how easily cosmetic products can be contaminated. Tan *et al.* ⁵⁹ tested ninety-three cosmetic products that were commercially available (of those, five were liquid soaps). The cosmetics were microbiologically analyzed to detect the presence of bacteria and fungi. The samples were plated and incubated and, at the end of the incubation, the emergent colonies were counted and the numbers of colony forming units (CFU/mL) were determined. The samples were also plated on selective mediums to determine the presence of *S. aureus*, *P. aeruginosa*, *E. coli* and *Salmonella sp.*, the microbial species suggested for investigation in the United States Pharmacopeia ⁶⁰. The results showed that even though some cosmetic categories did not appear to be contaminated, 20% of shaving products, 57.1% of moisturizing products, 42.8% of face care products, 11.1% of depilatories, 9.09% of make-up removers and 50% of toothpastes yielded contaminated results. The highest bacterial contamination (1.4×10^5 CFU/mL) was observed in a moisturizing product and the lowest (1.5×10^2 CFU/mL) in an eye make-up remover. And *S. aureus*, which is a common skin organism that can cause boils, impetigo, conjunctivitis, folliculitis and food poisoning ⁶¹, was the most frequently isolated contaminant from products. Gram-negative bacteria as *P. aeruginosa* are usually the most frequently found contaminant in cosmetics and can infect wounds and burns as well as cause pneumonia in immunosuppressed patients ⁶², was also detected in one product. According to microbiological standards of cosmetic products, they must be free of high virulence microbial pathogens like *S. aureus* and *P. aeruginosa*; however, this study reveals that these microorganisms can be found in unused cosmetic products ⁵⁹.

In related experiments, Hopfer *et al.* ⁶³ reported infections and one death due to a shampoo used by immunosuppressed patients, which was contaminated with *P. aeruginosa*. Other studies have also divulged several cases of eye infections and even loss of vision were also caused by cosmetic products contaminated with the same microorganism ⁶⁴. Also, a survey on recalls of microbiologically contaminated cosmetics in Europe between 2005 and 2008 reports that 24 different cosmetic products have been contaminated with *P. aeruginosa* as well as *B. cepacia*, *S. aureus*, *Enterococcus sp.*, etc. ²¹. Okeke and Lamikanra ⁶⁵ recovered *E. coli*, *Pseudomonas sp.*, *Staphylococcus sp.* and *Bacillus sp.* after analyzing the bacteriological quality of skin moisturizing creams and lotions that were purchased at a market ⁶⁵. Similar results were shown by Hugbo *et al.* ⁶⁶, after having evaluated the microbiological content of ten commercially available cosmetic creams and lotions. Another example is the study of 127 used and unused cosmetic products

accomplished by Çarıkçı *et al.*⁶⁷, in which was reported *P. aeruginosa*, *K. pneumoniae*, *Pseudomonas putida*, and other bacterial strains were found in unused samples. Boynukara *et al.*⁶⁸ also isolated *A. niger* from an imported commercial skin cream.

As shown, microbial contamination is still one of the major causes of product recalls and spoilage. This reinforces the importance of improving the preservatives system in order to prevent the growth of contaminating microorganisms during manufacturing, storage and use by consumers⁵⁹.

3.2 Efficacy of preservatives in cosmetics

As previously mentioned, contaminating microorganisms in cosmetics may cause a spoilage of the product and, when pathogenic, they represent a serious health risk for consumers worldwide. Despite the guidelines, microbial contamination is still one of the major causes of product recalls in the world, in particular in developing tropical countries⁵⁹. Therefore, it is important to evaluate and improve the preservative systems. In Section 2.4.3 it was clarified how the efficacy of preservatives is commonly tested.

To analyze the effectiveness of a mix of preservatives in two cosmetic products, Campana *et al.*⁶⁹ performed a CT following the CTFA procedure with two reference strains, *S. aureus* and *P. aeruginosa*, and two isolates from the analyzed products, *Pseudomonas putida* and *S. epidermidis*. The results showed a 99.99% reduction in bacterial cell number after 7 days in the two tested cosmetic products. This means that the preservative systems of the two tested cosmetics (a combination of methylparaben and propylparaben) showed long-lasting antimicrobial activity, satisfying the CTFA criteria. Based on this data, supported by the use of a neutralizing solution, which avoided the eventual presence of false positives, the tested cosmetic products were not considered a risk for the consumer by the authors.

In a similar study, Tan *et al.*⁵⁹ investigated the efficacy of antimicrobial preservation of 93 cosmetic products performing a CT as suggested by the USP. The used microorganisms were *P. aeruginosa*, *E. coli*, *S. aureus*, *C. albicans*, and *A. niger*. The results showed that 14 products did not comply with the USP recommended antimicrobial preservative activity criteria and that there was microbiological contamination in 14 cosmetics as well⁵⁹. These results suggest how easily cosmetic products can be contaminated and consequently, how important it is to take precautions, comply with GMP standards during production and add preservatives to the formulations as determined by regulations.

3.3 Stability Studies in Cosmetics

In this section, stability studies performed on cosmetic products are reviewed.

Smaoui *et al.*⁷⁰ aimed to formulate and examine a topical skin-care cream containing 3% virgin olive oil and compare it to the oil-free cream base. Both the cream base and the formulation were stored at 8, 25, 40 and 55 °C and observed at various time intervals for four weeks (24 h, 7, 14, 21, and 28 days) to investigate their stability and the effect of these storage conditions. This investigation focused on the color, smell, phase separation or liquefaction, pH, and microbiological stability of the formulations. The results

revealed that the only color changes were detected for the emulsions using olive oil at 40 and 55 °C from the 21st day and persisted up to the 28th day presumably due to the oily phase separation, which was promoted at higher temperatures. In terms of phase separation, tested by centrifugation assays, there was a slight phase separation only in the base sample from the 21st day until the 28th day of observation in the samples kept at 40 and 55 °C. The multiple emulsions prepared in this work had a pH value of 7.15, which is close to the neutral pH. Moreover, it was observed that the pH of the several emulsion samples kept at different storage conditions suffered a continuous decrease until the 28th day of observation, reaching values from 4.0 to 5.2. This decrease in pH was presumably due to the presence of fatty acids. In terms of microbiological analysis, a colony count test was performed and afterwards, selective isolation media were used to identify the presence of *P. aeruginosa* and *S. aureus*. The microbial evaluation indicated that the formulations with olive oil were efficient against the proliferation of various spoilage microorganisms, such as *P. aeruginosa* and *S. aureus*. Thus, the authors concluded that the organoleptic stability of the creams was achieved for four weeks, as well as physicochemical and microbiological stability. The newly formulated cream of virgin olive oil proved to exhibit promising properties and attributes ⁷⁰.

In a similar study, Rodriguez *et al.* ⁷¹ analyzed the inclusion of coffee silverskin, a natural extract with established antioxidant activity, in hand cream. They also evaluated the physical and microbiological stabilities, and antioxidant activity of a formulation containing 2.5% (w/w) of the extract upon production and after 6 months of shelf-life. Since it is a long-term stability evaluation, the sample's physical stability was evaluated by submitting the formulation to storage for 6 months, at two different conditions: 25 °C with a 65% relative humidity and 40 °C with 75% relative humidity; the properties were evaluated at the initial time and after 30, 60, 90, and 180 days of storage. To test its physical, chemical and microbiological stability, centrifugation, viscosity, pH, color, contamination count, and CT procedures were performed according to ISO 11930:2012. Regarding the results, the authors stated that there was no phase separation after centrifugation, which means that all samples were stable. Apparent viscosity decreased with increasing shear rate, the pH values showed minimal changes, and all formulations showed stable organoleptic characteristics during the time and conditions of storage. Specifically for the color, as the extract color varies between green and brown, the final formulation had a different reddish tone because, when the extract ages, sugar and amino acid elements produce melanoidin, a dark-colored substance that may be responsible for these changes ⁷². The total colony count did not reveal signs of microbial contamination, being an indicator of the use of a proper preservative system. CT results showed the efficacy of the preservative system used as no growth was observed and the product was classified as class A for all the tested microorganisms recommended in ISO 11930:2012. Lastly, the study concluded that the formulation containing 2.5% coffee silverskin extract maintained the physical and microbiological stability, independently of the storage conditions, meaning that it could be recommended ⁷².

To test the stability of a formulation containing a new emulsifier (Sepigel 305), Anchisi *et al.* ⁷³ also performed centrifugation, organoleptic, pH, viscosity and microbiological tests in samples submitted to low (5 °C), room, high (42 °C) and freezing temperatures (-10 °C) for two weeks. The formulation gave positive stability results for centrifugation, minimal changes in color and pH, displayed viscosity ranging

between high and medium consistency and the microbiological tests provided good results. This shows that the preservative system used is adequate for good microbiological quality and that Sepigel 305 can stabilize emulsions as an emulsifier. Another example is the investigation of a moisturizing cosmetic emulsion with phenoxyethanol as a preservative by Silva *et al.*⁷⁴ where accelerated stability assays (pH, viscosity, and centrifugation) with the cosmetic emulsion being kept at different temperatures (2 °C, 25 °C, and 40 °C) and time durations (24 hours, 7 days, 15 days, and 30 days) were performed. This formulation with phenoxyethanol revealed no phase separation or physical instability in the centrifugation test and pH values in agreement with those found for human skin. In terms of viscosity, there were different rotation speeds used to observe its effect in the emulsion, which displayed a viscosity decrease with an increase of speed. This is the typical thixotropic behavior of a cosmetic emulsion, which becomes more fluid when subjected to external pressure, spreading easily and recovering the initial viscosity after application, which prevents the product from dripping. The accelerated stability tests concluded that this formulation containing phenoxyethanol was stable⁷⁴.

In another stability study, Stephane *et al.*⁷⁵ paid special attention to the concentration value of preservatives during the investigation period. Two of the studied preservatives were phenoxyethanol and benzoic acid. Twelve cosmetic products were stored at 25 and 45 °C for three months to, once again, compare the condition that causes accelerated aging (45 °C) to the normal one (25 °C), and chemical and physical parameters were evaluated. In addition to the typical organoleptic, centrifugal, pH and viscosity analysis, the author quantified the mentioned preservatives by liquid chromatography and the water activity evaluation, which is the measurement of available water in a product formulation¹⁹. The organoleptic results showed no modifications at 25 °C. However, at 45 °C, the color changed for most products, having suffered a yellowing transition. Some products displayed phase separation after centrifugation, and the pH and viscosity modified in a few samples. In terms of water activity, there was a decrease of at least 5% in the samples stored at 45 °C due to water evaporation accelerated by temperature; according to standard ISO 29621:2012, the reported value of water activity (0.6) corresponds to a hostile medium to microbial growth¹⁹. In regards to the quantification of preservatives, benzoic acid showed a decrease of 10% in both temperature conditions, while phenoxyethanol maintained its concentration. This decrease can end up causing skin infections.

From these studies, it can be concluded that cosmetic stability studies are essential for guaranteeing the quality of the product and to assure the efficiency of the preservative system, and consumer safety. These evaluation assessments usually follow a common set of procedures that include organoleptic, centrifugation, pH, viscosity, colony count and Challenge Test, amongst others.

4 Materials and Methods

In this chapter, the laboratory procedures and experimental set-up used throughout this dissertation will be presented. Each of the methods applied to assess the stability of liquid soaps by accelerated stability tests and evaluate the preservatives will be explained in different sub-sections.

4.1 Tested Samples

Two different liquid soaps were tested: i) Sample A, which is the base soap from *Castelbel*, used to produce all liquid soaps, containing only the preservative MI and CMI; ii) Sample B, which has the same soap base with a different preservative, the BDP (mixture of benzoic acid, dehydroacetic acid and phenoxyethanol).

To produce the sample B, 10 L of base soap without preservative were manually mixed with 80 g of BDP preservative. The pH of the soap was measured before and after the addition of the preservative to verify that it was in accordance to the manufacturer's instructions (pH close to 6).

Both soap bulks were divided in two independent samples; one of the samples was used to perform the organoleptic and weight analysis; the other was meant for the remaining stability and preservative evaluation tests.

4.2 Accelerated stability assessment

To perform an accelerated stability assessment (see Section 2.3), both soaps A and B were placed under different conditions and analyzed at different time intervals for a period of 2 months (60 days). Ideally, the study should last up to 3 months (90 days), but the dissertation timeframe did not allow it. During this period, the samples were evaluated at three different moments, using the tests detailed in the next sub-sections: t_0 representing the initial moment of the study; t_1 which represents 1 month later (30 days); and t_2 which indicates the final moment of the study, after 2 months (60 days).

Following the guidelines from ISO 18811¹⁶, the liquid soap samples were placed in four different conditions: i) protected from light by placement in a fridge at 4 °C (± 3 °C); ii) protected from light by being placed inside a cupboard at room temperature (RT; 23 °C (± 3 °C)); iii) exposed to daylight at RT; iv) protected from light by being placed in an incubator at 50 °C (± 3 °C). This last option was chosen due to the high temperatures to which these products can be subjected during transportation.

The performed tests can be divided into physical and chemical properties evaluation tests (e.g., organoleptic evaluation, weight, pH, density, viscosity, foaming capacity, foam stability, and centrifugation analysis) and microbiological tests (e.g., contamination level evaluation). These tests carried out at 20- 25 °C on samples subject to the conditions mentioned above, will make it possible to assess whether the liquid soaps produced maintain their properties throughout the entire process. All experiments with the exception

of organoleptic analysis were performed in three technical replicates ($n=3$) for each assessment time-point and storage condition to ensure statistical relevance of the results.

4.2.1 Organoleptic analysis

This examination for each condition was performed under natural light and room temperature conditions and in a room exempt from intense odors or fragrance, following the company's internal protocol. The final product's organoleptic properties analyzed were the color, odor and appearance and a descriptive evaluation was selected to define possible changes in soap samples ^{76,77,78}.

4.2.2 Weight analysis

To record the possible weight loss over time, the samples were weighted with an analytical scale (EW1500-2M, Kern) and then subtracted the previously measured empty container's weight.

4.2.3 pH analysis

The pH measurements were performed with a potentiometer (MU6100L, VWR) directly in each liquid soap sample ⁷⁹.

4.2.4 Density Analysis

To study the liquid soap's density, 20 mL of sample was placed in 250 mL beakers and measured with the proper instrument (DensitoPro, Mettler Toledo) that pumped the sample into a measuring cell through a filling tube and automatically provided a density value. Afterwards the equipment was washed with distilled water.

4.2.5 Viscosity Analysis

The viscosity measurement was performed using appropriate equipment (Rotational Viscometer, Fungilab) with a disc-shaped spindle R2 and velocities of 100 rpm for the liquid soap A and 30 rpm for the soap B, which were chosen according to the equipment's manual ⁸⁰. Having previously measured the density of each soap, this value is a parameter used in the viscosity equipment. A total volume of 350 mL of each sample was placed in 600 mL beakers. After setting the parameters, the spindle connected to equipment was stationed in the middle of the beakers and after it started rotating, the instrument provided a viscosity value.

4.2.6 Foaming Capacity and Foam Stability Analysis

In order to analyze the foam's stability, an amount of 40 mL of 0.5% soap solution was homogenized for 1 min in a 100 mL graduated cylinder using a dispersion equipment (Ultra Turrax, IKA) at 10.4 rpm.

The foam and solution volume were measured in the 100 mL graduated cylinder after mixing and then, after 30 min. The foaming capacity and stability were calculated using the equation 1 and 2 ⁸¹:

$$\% \text{ foaming capacity} = \frac{V_{\text{foam after homogenization}} - V_{\text{initial solution}}}{V_{\text{initial solution}}} \times 100 \quad (1)$$

$$\% \text{ foam stability} = \frac{V_{\text{foam after 30min}}}{V_{\text{foam after homogenization}}} \times 100 \quad (2)$$

4.2.7 Centrifugation Analysis

To analyze a possible phase separation in the samples, they were submitted to a centrifugation test at 3000 rpm for 30 min, in which 8 mL of the liquid soap sample was placed in 15 mL polypropylene Falcon tubes ⁷³. With the occurrence of phase separation, the water was recovered with a glass pipette and transferred to a pre-weighted container in order to obtain the weight of the resolved water. The product stability assessed from the mass of resolved water, as show in Equation 3 ⁸².

$$\% \text{ water recovered} = \frac{\text{mass water resolved}}{\text{mass of emulsion}} \times \text{fraction of water in emulsion} \times 100 \quad (3)$$

4.2.8 Contamination Level Analysis

100 µL of each soap sample was plated on Plate Count Agar (PCA; VWR, Belgium), and incubated at 37 °C for 48 hours (Agitorb 200 Incubator, Aralab, Portugal). After incubation, the culturable cells were enumerated to assess if the soaps were contaminated.

4.3 Preservative's Evaluation

4.3.1 Antimicrobial Activity Analysis

To evaluate the antimicrobial activity of both soaps by the agar-well diffusion method, the same model strains were used: *S. aureus* (ATCC 25923), *S. epidermis* (ATCC 35984) and *E. coli* (ATCC 25922). Single colonies of each strain were collected from PCA plates, inoculated in 50 mL of LB broth, and incubated overnight at 37 °C. The bacterial strains were then harvested by centrifugation at 3202 g for 10 min at 18 °C. Then, cell concentration was adjusted to OD of 0.2 at 610 nm in LB in order to obtain a final concentration of approximately 10⁷ CFU/mL. Subsequently, PCA plates were inoculated by spreading the bacterial suspension over the agar surface. Then, wells with a diameter of 6 to 8 mm were punched with a sterile tip instrument, 50 µL of soap solution was added into it, and the plates were incubated at 37 °C for 24 h. Control assays were performed with distilled water ⁸³.

4.3.2 Challenge Test

As previously stated, this test follows the indications of ISO 11930⁵⁸ and aims to evaluate the efficacy of antimicrobial preservation and consists of challenging the product, with a prescribed inoculum of suitable micro-organisms, storing the inoculated preparation at a prescribed temperature, withdrawing samples from the container at specified intervals of time and counting the organisms in the samples removed.

The bacteria used were *S. aureus* (ATCC 25923), *S. epidermis* (ATCC 35984) and *E. coli* (ATCC 25922), these were not the ones indicated in ISO 11930 since those were not easily available. However, these chosen strains are frequently found on the human skin⁸⁴ and also listed on Table 3 on Section 2.4.3 as a recommended microorganism for this procedure. Firstly, single colonies of each strain were collected from PCA plates and used to inoculate 50 mL of Luria-Bertani Broth (LB Broth; Thermo Fisher Scientific, USA);. The cell density of each overnight culture was then adjusted with fresh LB medium to an optical density (OD) of 0.20 at 610 nm (corresponding to approximately 10^7 CFU/mL) in a spectrophotometer (V-1200, VWR, Belgium). From this inoculum, 0.2×10^{-2} mL were placed in a 50 mL recipient with 20 mL of each soap sample. The inoculated samples were immediately diluted (ten-fold series dilutions) to an appropriate cell density to yield > 10 and < 100 CFU per 10 μ L-drop plated on solid growth medium (PCA; VWR, Belgium) using the pendant drop method⁸⁵. The number of CFU/mL of sample was determined after plate incubation for 24 h at 37 °C. The inoculated liquid soap samples were incubated for 14 days at 25 °C⁵⁸. After 7 and 14 days, the inoculated samples were plated again in order to determine the reduction in bacterial cell counts compared to the initial assay sample (before incubation)⁵⁸.

The preservative properties of the preparation are adequate if, in the conditions of the test, there is a significant fall or no increase in the number of microorganisms in the inoculated preparation after the times and at the temperatures prescribed. The acceptance criteria, in terms of decrease in the number of microorganisms with time, varies for different types of preparations according to the degree of protection intended (see Section 2.4.3).

The ISO 11930 states that if there's previous proof that the product being studied has an antimicrobial component, it is essential to quench or negate the activity of this component in order to make a viable count⁵⁸. This removal of activity may be achieved by chemical neutralization with an appropriate neutralizer. Since in the beginning of this procedure the results of the antimicrobial action weren't available, this test was primarily performed without a neutralizer. After obtaining the results proving the antimicrobial capacity of the formulations, the test was adjusted to include the addition of a neutralizer to the samples, in order to confirm the results obtained in the first attempt in the most adequate conditions. The neutralizer used was an universal neutralizer containing L-histidin (1 g/L), lecithin (3 g/L), sodium thiosulphate (5 g/L), saponin (30 g/L), Tween 80 (30 g/L), phosphate buffer (0.25N) and distilled water⁸⁶, and its purpose was to cancel the effects of any antimicrobial component in the samples weather, such as the preservative.

4.4 Statistical Analysis

In order to evaluate and better compare the obtained results in the several conditions and timestamps, a statistical analysis of a Student's t-test was performed using Excel. This statistical method was used to test the null hypothesis, that says that the average of the samples is the same between two tests. That being said, one can reject the null hypothesis when the significance (p-values) obtained in the test are smaller than 0.05 (95% confidence interval).

The results obtained for each evaluated parameter will be analyzed under three different angles: (1) Comparison between the three storing timestamps, namely comparing both t1 and t2 against t0, which is the control essay; (2) Comparison between the different storage conditions within the same timestamp, mainly against RT which is the control essay in this case and (3) Comparison between both formulations A and B, specifically looking for differences in behavior or scale for the same timestamp and condition. The statistical analysis (1) will be visible along Section 5, while analysis (2) and (3) will be highlighted and discussed when relevant. Additional analysis may also be discussed sporadically, when relevant.

5 Results and discussion

5.1 Accelerated Stability Studies

In accelerated stability tests, a product is stored at elevated stress conditions (such as temperature, light etc.). As mentioned before, these results were obtained at three assessment points: i) t_0 , where the multiple samples were analyzed before being submitted to the four storage conditions. This timestamp is used as a control; ii) t_1 , where the samples have been submitted for 1 month to the different conditions; t_2 , where the samples have been submitted for 2 months to the different conditions. The four storage conditions used were: i) room temperature protected from the light ($RT = 23 \pm 3^\circ\text{C}$); ii) room temperature exposed to the sunlight (RTL); iii) $50 \pm 3^\circ\text{C}$ protected from the light; iv) $4 \pm 3^\circ\text{C}$ protected from the light. Analyzing these factors, it was possible not only to compare formulation A (containing isothiazolinones as preservatives) and formulation B (containing the BDP preservative), ensuring that the liquid soap with the new preservative maintains its characteristics and function, but also to examine the effect of temperature and light on each formulation, as well as their stability for two months under these conditions.

5.1.1 Physical, Chemical and Microbiological Properties Evaluation Tests

5.1.1.1 Organoleptic Evaluation

To examine the physical properties and predict potential changes in the products during transportation, storage and commercialization, an organoleptic evaluation under different temperature and light conditions was performed. The results are presented in Table 5.

Table 5. Organoleptic analysis of Formulation A and B.

Temperature Condition	Assessment time	Formulation A			Formulation B					
		Color	Odor	Appearance	Color	Odor	Appearance			
RT	t ₀	Transparent	None	Clear	Transparent					
	t ₁									
	t ₂									
RTL	t ₀				Transparent					
	t ₁				Transparent					
	t ₂									
50 °C	t ₀				Transparent	None	Clear	Transparent		
	t ₁							Light yellow		
	t ₂							Intense yellow		
4 °C	t ₀							Transparent		
	t ₁									
	t ₂									

The results showed that there were no changes in odor and appearance in any of the formulations, regardless of the storage conditions. They also barely demonstrated no differences in terms of color, except for formulation B at 50°C , which showed a drastic transformation, turning more and more yellow over

time. This transformation can be considered relevant to the aesthetic of this specific product from a commercial standpoint. These results are presented in Figure 5.



Figure 5. Formulation B at 50 °C.

Since this color change only occurred in formulation B, it suggests that it may be associated with the addition of the preservative BDP, which contains dehydroacetic acid (DHA) and phenoxyethanol as preservative agents, amongst other components. The yellowing transformation is usually related to oxidizing processes and has been proven during thermal aging^{87,88}. In fact, it is known that formulation containing dehydroacetic acid tend to show discoloration or yellowing with time, temperature and/or reaction with other formulation components. DHA is a particularly unstable molecule and suffers hydrolysis when in protic (non-aqueous solutions) or polar (aqueous solutions) solvents, like ethanol or water⁸⁹. The extent of the yellowing process is dependent on heat exposure during storage, which accelerates the hydrolysis reaction, being coherent with the results shown in Figure 5. This reaction produces free radicals that may suffer coupling, generating chromophoric molecules that are responsible for the yellow tonality in the solutions⁸⁹. Additionally, these free radicals together with the reactive oxygen species (ROS) created by exposing phenoxyethanol to elevated temperatures⁹⁰ may react with the other chemical components on the formulation leading to accelerated oxidative damage and degradation. Some components of the analyzed formulation are known to be prone to oxidation when in contact with strong oxidizing agents (such as ROS), namely glycerin, which is known to degrade into ketones and aldehydes while acquiring a yellowish color⁹¹ which degrade into smaller chain molecules when oxidized, creating a darker solution^{92,93}. This strongly suggests that, in the present work, the possible cause for the yellowing of formulation B, could be the hydrolysis of DHA and/or the action of thermal-aging of phenoxyethanol that led to oxidation.

DHA is usually avoided when the formulation is not pigmented due to the yellowing effect, which, influences the aesthetics and commercial value of end-user products. However, in order to reduce the oxidation extent and prevent product degradation from occurring, an antioxidant should be added to the formulation. Antioxidants, as the name suggests, are substances added to protect oxygen-sensitive formulation ingredients from oxidation, such as the surfactants. They are also used in cosmetic formulations to counter free radicals that damage lipids and proteins. Examples of commonly used antioxidants in cosmetics are butylated hydroxytoluene, butylated hydroxyanisole, retinol, ascorbic acid, and isoflavones; polymeric antioxidants include açai oil, coenzyme Q-10, and polyphenols⁹⁴.

5.1.1.2 Weight loss analysis

In parallel with the organoleptic examination, a weight analysis was accomplished. The results are summarized in Figure 6.

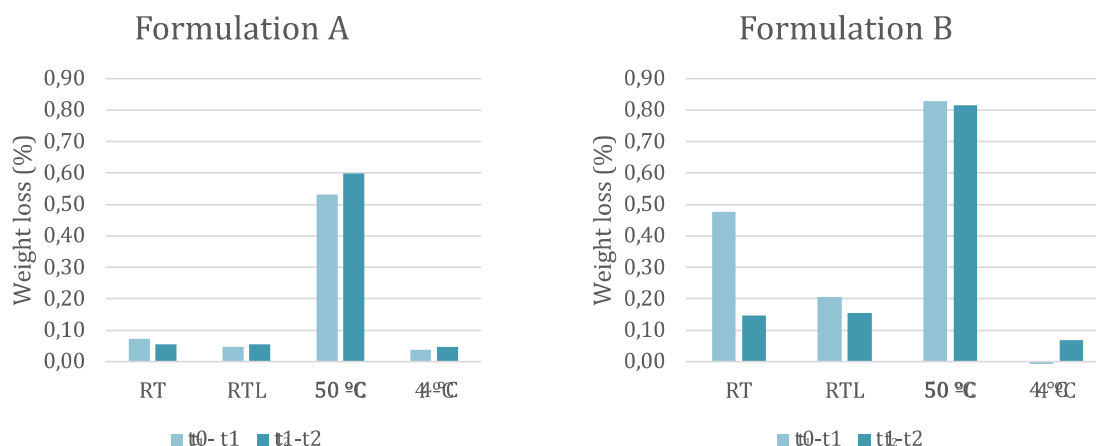


Figure 6. Weight loss (%) of formulations A and B during the accelerated stability evaluation. Since the organoleptic analysis was performed only on one sample the standard deviations are not presented.

The analysis of Figure 6 suggests that the 50 °C temperature condition in both formulations caused a greater decrease in the weight value of the product, compared to the other conditions tested. This might be due to the evaporation of water, which is the main component of both liquid soap formulations, caused by the higher temperatures. As previously observed in Section 3.4 this is a fairly common occurrence in cosmetic products at high temperatures ⁷⁵.

Regarding the conditions evaluated at room temperature (RT and RTL), especially from t_0 to t_1 , a greater weight reduction could be observed for formulation B - 0.47% at RT and 0.21% at RTL - when compared to formulation A - 0.07% at RT and 0.05% at RTL. However, in all the presented cases, the decrease in sample weight is very low and is considered acceptable by the criterium of the supplier, which states that up to a 10% of weight loss is admissible ⁹⁵.

Another point that proves how irrelevant these weight variations are is the fact that viscosity typically increases dramatically with loss of water availability ⁹⁶ which means that, if the heaviest reduction of water through evaporation at 50 °C was substantial, there would be a visible rise in viscosity in the same samples. However, when observing the viscosity results at 50 °C there was no variation of viscosity when compared to samples in other conditions, meaning that even the biggest weight loss registered didn't have a significant impact (Section 4.2.5).

5.1.1.3 pH analysis

As far as the effectiveness of a liquid soap is concerned, pH is considered a very important parameter. The pH of human skin generally ranges from 4.5 to 6.0, and 5.5 is considered the average pH of human skin ⁹⁷. Therefore, for a formulation to be accepted for industrial application, it must have a pH close to this range ⁹⁷. According to the company specifications, the pH values of liquid soaps should be in the range of

5.5 to 6.5⁹⁵, which is a particularly high pH when taking into consideration the skin range. The pH values determined for the two formulations are presented in Figure 7.

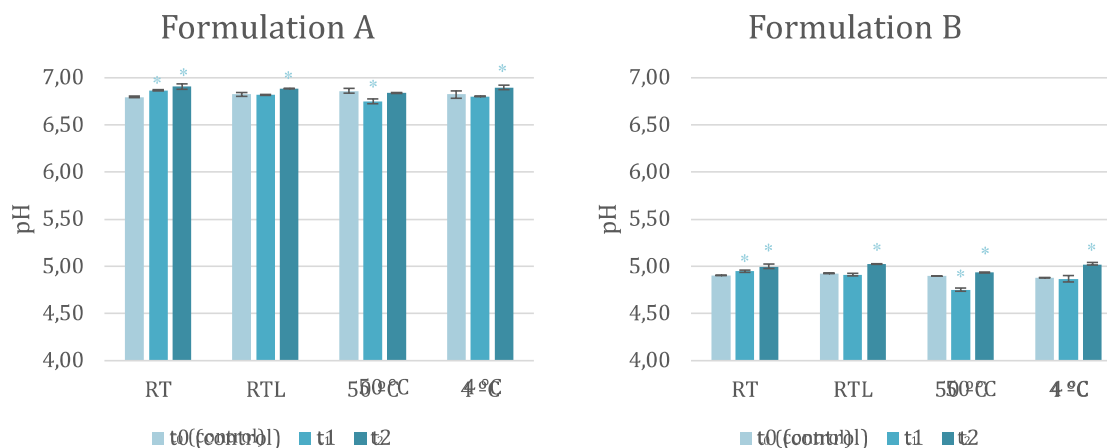


Figure 7. pH measurements of formulations A and B during the accelerated stability evaluation. The means $\pm$ SD are presented and the symbol * indicate statistically different values for $p < 0.05$.

Liquid soap A, showed pH values higher than the manufacturer's specifications, which means that it would be necessary to adjust the pH in the final stage of production. Being the one currently produced by the company, formulation A demonstrates the standard behavior to which B will be compared to. In fact, formulation A presents a pH value of 6.80 ± 0.01 when measured at room temperature and considering the initial timestamp t_0 . When measured at t_2 under the same conditions, the recorded pH value showed a slight rise to 6.91 ± 0.03 , with statistically significant difference ($p < 0.05$). This behavior is evident in most temperature conditions for formulation A. The same small increase was also registered for formulation B, showing a statistically significant increase from t_0 to t_2 in all tested conditions ($p < 0.05$). However, these changes are virtually neglectable considering their magnitude, as well as the effect of this increase in cosmetic products (e.g., for formulation B at RT, from t_0 to t_2 , the variation represents only 1.85%).

Regarding the changes in temperature and light conditions in the three assessment time points, a behavior similar to the one described above was found. In fact, the slight variations are not relevant when analyzing the usability of a cosmetic product.

The main difference reported is that formulation B has a considerably lower pH than formulation A, ranging from 4.75 ± 0.01 to 5.03 ± 0.01 . This may be due to the acidity of the constituents of the BDP preservative, which includes phenoxyethanol (mildly acidic compound) and two acids, dehydroacetic acid and benzoic acid. According to the manufacturer's indications, a liquid soap containing the BDP preservative should have a pH value lower than 6, as the preservative has its maximum activity below this value (see Section 2.4.2). However, this value is slightly lower than the one established by *Castelbel* for its formulations – pH between 5.5 and 6.5. This range of pH was established taking into consideration the

properties of the preservative that was substituted which, as the results from formulation A show, would increase the liquid soap's pH. So, it is expected that the formulation with a new preservative would not match this limit. Nevertheless, all the results obtained from formulation B are inside the skin pH interval, 4.5 to 6.0, which makes them acceptable for a liquid soap and actually provide skin friendlier pH values than formulation A. This means that, contrary to A, B would not need an adjustment in pH at the final stage of production which is an advantage in terms of time, resources and chemicals added to the liquid soap.

5.1.1.4 Density evaluation

The density of each formulation is an important physicochemical parameter to evaluate when characterizing a liquid soap. These products from *Castelbel* usually present a density of 0.950 to 1.200 g/cm³⁹⁵. The density measurements of both formulations are presented in Figure 8.

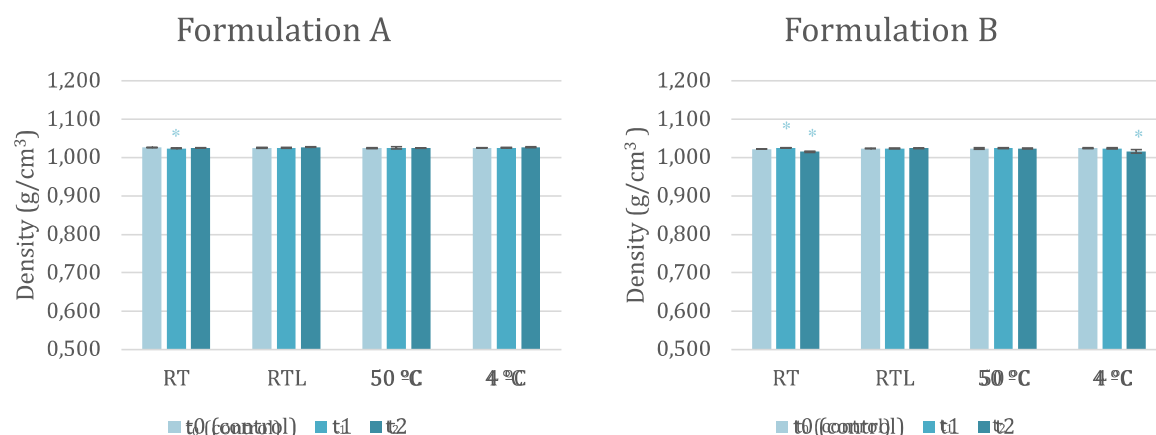


Figure 8. Density measurements of formulations A and B during the accelerated stability evaluation. The means $\pm$ SD are presented and the symbol * indicate statistically different values for $p < 0.05$.

When comparing the density values of formulations A and B, it is clear that both samples demonstrate very similar values - formulation A varying from 1.024 ± 0.001 g/cm³ to 1.027 ± 0.001 g/cm³ and formulation B from 1.015 ± 0.002 g/cm³ to 1.025 ± 0.001 g/cm³.

Focusing on formulation A, at t_0 and RT (control), the obtained density was 1.026 ± 0.001 g/cm³ which suffered a decrease of 0.19% to 1.024 ± 0.001 g/cm³ at t_1 . This variation has statistical relevance ($p < 0.05$) but, as the decrease percentage shows, it is not a pertinent change when analyzing the usability of a cosmetic product.

On the other hand, formulation B showed statistically significant results ($p < 0.05$), namely at RT and 4 °C from t_0 to t_2 , where a density decrease of 0.82% occurred. Focusing on the results at RT, there was an initial density of 1.022 ± 0.003 g/cm³ that increased 0.25% to 1.025 ± 0.001 g/cm³ at t_1 and decreased 0.73% to 1.015 ± 0.002 g/cm³ from t_0 to t_2 ($p < 0.05$). However, given once again the low percentage of these alterations, they do not represent a problem for the stability or usability of the liquid soap.

Comparing the density values of formulation A and B, the results are very similar, with few statistically significant variations. In fact, formulation B shows values according to what is stipulated by the manufacturer, given the typical density of liquid soaps in *Castelbel* that is between 0.950 to 1.200g/cm⁹⁵.

5.1.1.5 Viscosity evaluation

Viscosity is an important aspect when determining the quality of hand washing products. In these products, customers associate a higher viscosity with a greater content of active compounds and thus, with increased efficiency and better performance. On the other hand, these cosmetics are not expected to have an extremely high viscosity as they are commonly applied from pump dispensers for hand washing and that would difficult the process⁹⁸. The Brazilian Health Regulatory Agency reports that limit values of viscosity should be stipulated by the producer considering the costumer's preference⁹⁹. Liquid soaps sold by *Castelbel* should have a viscosity close to 3000 cP to 4000 cP⁹⁵. The results of viscosity analysis are shown in Figure 9.

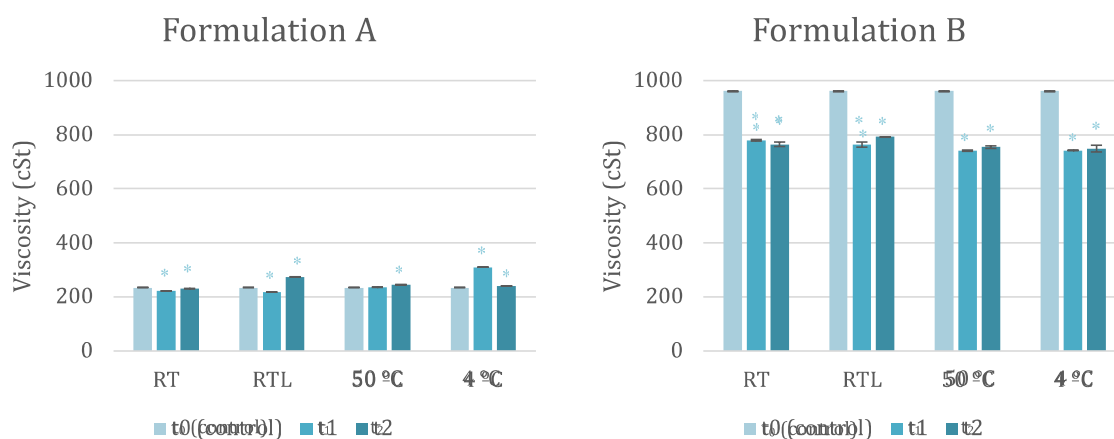


Figure 9. Viscosity measurements of formulations A and B during the accelerated stability evaluation. The means $\pm$ SD are presented and the symbol * indicate statistically different values for $p < 0.05$.

Looking at Figure 9, it is clear that formulation A has a much lower apparent viscosity than formulation B, at all timestamp and conditions (A: $217.93 \pm 0.06 - 309.80 \pm 0.27$ cP; B: $741.03 \pm 2.32 - 960.60 \pm 2.15$ cP). Both formulations reveal values lower than expected (3000 cP to 4000 cP), especially A that even from an organoleptic perspective, does not correspond to the consumers anticipated appearance of a liquid soap, seaming too fluid for the standard. To fix this, a higher concentration of viscosity agent could be implemented, such as sodium chloride (usually used by *Castelbel*). Despite having an extremely low viscosity, formulation A maintained this property throughout the experimental period: before being submitted to any storage condition (t_0) the viscosity value was 234.27 ± 0.45 cP; the highest viscosity was 309.80 ± 0.27 cP found at 4 °C and t_1 (increase of 32%) and the lowest of 217.93 ± 0.06 cP at RTL and t_1 (decrease of 7%). Although these variations are considered statistically relevant ($p < 0.05$), they do not constitute an obstacle to the product's stability, contrary to its low viscosity value, which raises some concerns.

On the other hand, the formulation B has a higher overall value of viscosity, but showed a significant decrease ($p < 0.05$) from t_0 both to t_1 and t_2 in every storage conditions. In the RT tests, the viscosity went from 960.60 ± 2.15 cP at t_0 to 779.47 ± 3.05 cP at t_1 (a decrease of 19%). This behavior, which is similar for all storage conditions, suggests that this relevant difference is probably not related to the storage conditions nor time elapsed. Instead, it suggests that some process occurred in t_0 is responsible for the sharp decrease, followed by a stabilizing phase. Taking into account that formulation B was prepared on site by adding the preservative to the base, through a process of manual mixing, there could have been some extent of aeration and formation of bubbles in the final mix. On the other hand, formulation A did not undergo this process since it was already mixed with the preservative when acquired from the producer. That being said, the resting period between preparation (mixing) and viscosity measurement might not have been enough for the solution to recover its properties. It is known that aeration and the presence of bubbles in liquids is responsible for an increase in kinematic viscosity, or even reduce the measurement quality of the viscosimeter¹⁰⁰. Moreover, as explained in Section 5.1.1.1, the reactions between the preservative components and the other formulation components, as well as the lower pH, higher ionic strength induced by the acidic preservative and its inherent chemical structure may be responsible for the overall higher value of viscosity, due to the formation of cross-links between molecules and structural changes in the other components. Once again, the reduced resting time between preparation and measurement in t_0 might have played a role in artificially increasing viscosity values, due to reaction onset. The time elapsed from t_0 to t_1 allowed for the formulation to recover its properties and for the entrapped air to escape the liquid.

5.1.1.6 Foam evaluation

Another parameter evaluated was the foaming properties, which are one of the main assessment indicators of quality in body wash cosmetics. Foams are described in the European Pharmacopoeia as “formulation, consisting of a large amount of gas dispersed in a liquid phase”¹⁰¹, they are colloids composed of two or three distinct phases: normally a hydrophilic liquid continuous phase with a foaming agent, in which a gaseous dispersion phase is distributed¹⁰².

To provide consumers with a feeling of high efficiency, these products are expected to form a large amount of thick, stable and velvety foam during the use. However, foam is created by the presence of a foaming agent, which is an amphiphilic substance whose hydrophilic part is responsible for their solubility and when in contact with water, the hydrophobic parts of the molecule arrange themselves in a way to minimize the area of contact with it. This leads to their specific orientation in which the hydrophilic part is in contact with the water and the hydrophobic part with the air, causing the formation of micelles in the bulk of liquid phase¹⁰². That being said, it is important to notice how this process is not related to the product's cleaning abilities and that foaming is not connected with the quality of the cleaning process and merely a cosmetic attribute. Even so, in search of a greater sale appeal, manufacturers continue to give their products a good foaming quality, in spite of the fact that the newly developed synthetic detergents create a good washing quality without foam generation¹⁰³. On the other hand, an excessive volume of foam can

cause difficulties in removing the cosmetic from the skin of the hands when rinsing with water in the final washing step⁹⁸.

In this dissertation project, two foaming parameters were evaluated: foaming capacity, which is characterized as the interfacial area that can be created by the foam¹⁰⁴ and foam stability which is defined as the time that foam will maintain its initial properties as generated and remain stable despite gravitational or mechanical forces that causes liquid drainage from the colloidal mixture that composes foam¹⁰⁵.

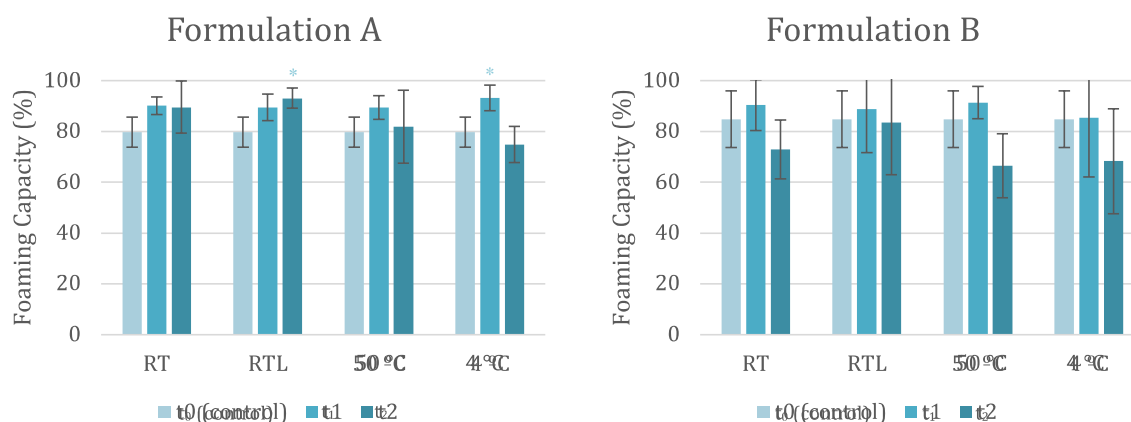


Figure 10. Foaming Capacity measurements of formulations A and B during the accelerated stability evaluation. The means $\pm$ SD are presented and the symbol * indicate statistically different values for $p < 0.05$.

Regarding the foam capacity, both formulations show very similar values, having almost no variations with statistical relevance ($p > 0.05$). This immediately confirms that this parameter for formulation B is satisfactory, since it matches the results obtain from A, the formulation currently sold by the company. The results of formulation A, from all timestamps and conditions, range from $(75 \pm 7)\%$ to $(93 \pm 5)\%$ and formulation B from $(67 \pm 13)\%$ to $(91 \pm 6)\%$, which are acceptable values since they clearly show that both formulations are capable of forming the foam that consumers expect. According to statistical analysis, the results of each temperature and light condition are similar, denying any reaction caused by these factors.

In terms of foam stability, Figure 11 clearly shows that both samples display very close values that are not affected by the different conditions or evaluation period.

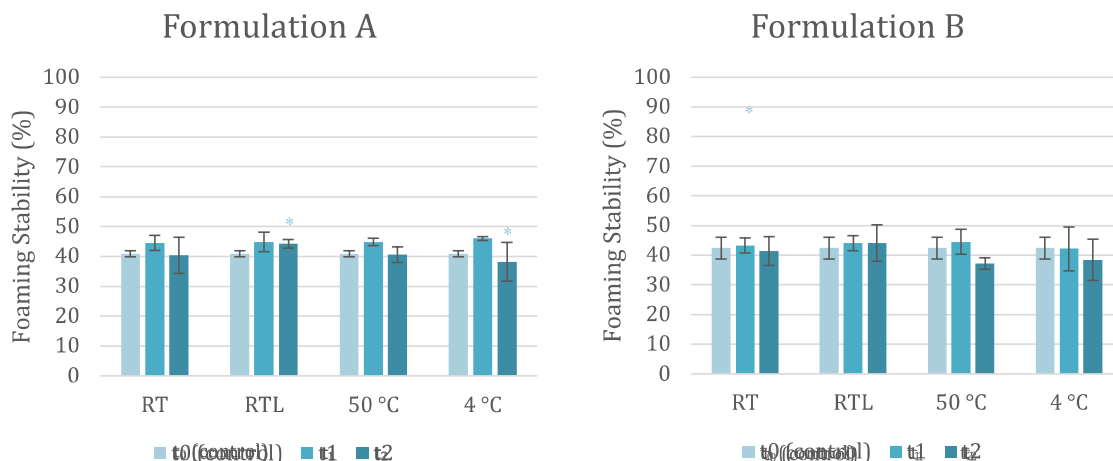


Figure 11. Foaming Stability measurements of formulations A and B during the accelerated stability evaluation. The means $\pm$ SD are presented and the symbol * indicate statistically different values for $p < 0.05$.

In all timestamps and conditions tested, foaming stability values ranged from $(37 \pm 2)\%$ to $(45 \pm 4)\%$ and from $(38 \pm 6)\%$ to $(46 \pm 1)\%$ for formulation A and B, respectively. Since the results between the two formulations don't display any differences with statistical importance ($p > 0.05$) which means that B shows a similar foaming stability to the presently used formulation, A, we can assume that the foam stability of the formulation B is acceptable for a marketable liquid soap. Even so, a factor that can impact this stability is the formulation's viscosity. An increase in viscosity can prolong the relaxation time of foam gravity unloading thus improving foaming stability¹⁰⁶. Meaning that, the adjustment in the viscosity properties of formulation B that are suggested in Section 4.2.5, would also better this parameter.

5.1.1.7 Centrifugation analysis

A centrifugation test is based on the principle of using centrifugal force to separate two or more substances of different densities, such as two different liquids or a liquid and a solid, and is a useful tool for assessing and predicting the shelf life of the solutions⁷⁰.

In this dissertation project, a centrifugation test was performed at the timeframes t_0 , t_1 and t_2 and taking into consideration the different storage conditions, to evaluate the thermostability properties of each formulation (Figure 12). With the stated method, the main purpose was to observe if any of the samples suffered phase separation and if so, the solution stability would be calculated from the volume of water resolved (Equation 1 in Section 4).

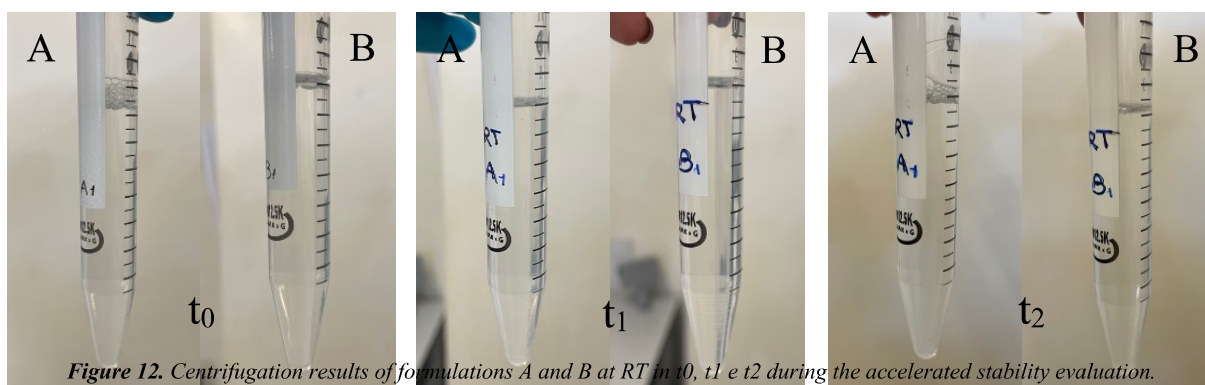


Figure 12. Centrifugation results of formulations A and B at RT in t0, t1 e t2 during the accelerated stability evaluation.

As can be observed in Figure 12, there was no phase separation after centrifugation for any storage condition during the entire study period, which means that both formulations are stable under all conditions for at least two months. This indicates that there was a proper homogenization speed during formulation, which may have prevented the formulations from breaking during the tests ⁷⁰.

5.1.1.8 Contamination level analysis

The level of microbial contamination was analyzed at the three time points (t0, t1 and t2), the first one working as control and the other two corresponding to samples that have been stored under different temperature conditions. The results obtained from the contamination level assay are represented in Figure 13.

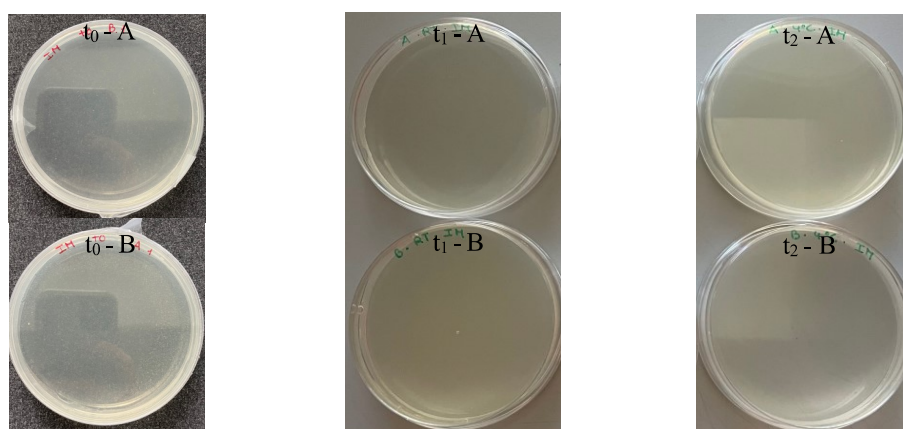


Figure 13. Contamination levels results of formulations A and B in the accelerated stability evaluation.

Looking at the representative images of agar plates, there was no sign of microbial contamination by bacteria or fungi in any soap sample (0 CFU/mL). This is an indicator of the use of a proper preservative system, as well as proof that the physicochemical properties of the liquid soap, such as the presence of surfactants capable of dissolving cellular membranes ¹⁰⁷, create an environment that seem to not support

microbial growth at least for two months. Regarding formulation A, both CMI and MI are not only responsible for the preservation capacity against bacteria (both gram-negative and gram-positive), but also against fungi ¹⁰⁸. The results obtained for formulation A were expected since it is a stable and commercially available formulation. Similarly, regarding formulation B it is known that the phenoxyethanol present in the preservative induce cell lysis by damaging cell membrane, as well as oxidative damage in the cellular structures and proton gradient dissipation through membrane permeabilization ⁹⁰⁶. This last effect causes the uncoupling of respiration from the oxidative phosphorylation, affecting cell metabolism ¹⁰⁹. Moreover, phenoxyethanol is known to be volatile, which also enables it to protect the headspace of the packaging against bacteria ¹⁰⁸. In fact, phenoxyethanol is very effective on protecting the formulation mostly against gram-negative bacteria ^{93,110}. On the other hand, benzoic acid (another preservative component) confers strong protection against gram-positive bacteria, as well as against fungi ^{108,111}. The last preservative component, DHA, reinforces the bactericidal strength, in a similar way as the benzoic acid ^{108,112}.

5.2 Preservative's Evaluation

5.2.1 Antimicrobial activity analysis– Agar-well diffusion method

The agar-well diffusion method is widely used to evaluate antimicrobial activity ⁸³. The agar plate surface is inoculated by spreading a volume of the microbial inoculum, then a hole punched aseptically, and a volume of the antimicrobial agent is introduced into the well. Typically, after incubation, it is possible to observe that the antimicrobial agent diffused in the agar medium and inhibited the growth of the microbial strain in the form of an circular inhibition halo ⁸³.

In the present work, this method was used to assess the antimicrobial activity of the liquid soaps against three reference bacterial strains resident in the human skin, *S. aureus*, *S. epidermidis* and *E. coli*. The inhibition halos obtained for each bacterial strain are presented in the Table 6.

Table 6. Inhibition halo (diameter values in millimeter) obtained for each bacterial strain with formulations A and B.

Formulation	Strain	Inibition halo (mm)	Formulation	Strain	Inibition halo (mm)
A	<i>E. coli</i>	14.15 ± 0.13	B	<i>E. coli</i>	15.13 ± 0.38
	<i>S. aureus</i>	25.07 ± 0.16		<i>S. aureus</i>	21.50 ± 0.10
	<i>S. epidermidis</i>	25.01 ± 0.13		<i>S. epidermidis</i>	23.05 ± 0.10

The results that both formulations were effective at inhibiting bacterial growth when compared to the control (distilled water; no inhibition halos were formed). *S. epidermidis* revealed to be the more sensitive strain to both soap formulations, followed by *S. aureus*, and finally by *E. coli*, which was the only Gram-negative bacteria tested and the least sensitive to the antimicrobial agents present in both liquid soaps solutions ($p < 0.05$). This means that *E. coli* is the most resistant to the preservative activity, which might be due to the fact that, in the preservative mixture, two out of three components, benzoic acid and DHA,

act on gram-positive bacteria like *S. epidermidis* and *S. aureus*^{108,111,112} while for gram-negative bacteria, such as *E. coli*, the protection is assured by only one of the three components, phenoxyethanol^{93,110}. This indicates that, by containing a majority of preservatives that act on gram-positive bacteria, BDP has a bigger antimicrobial impact on these bacteria.

5.2.2 Challenge Test

This test aims to demonstrate the efficacy of preservatives in both formulations. A sample of liquid soap was contaminated purposely with bacteria (that can be found on the skin flora) at the beginning of the experimental period. The preservative efficacy is shown by a reduction in the number of cell colonies in the sample after a certain period of time, in this case, after 7, 14 and 28 days. Therefore, this reduction is measurable and if it respects the defined acceptance criteria in terms of a decrease in the number of microorganisms with time, the preservative properties of the formulation are considered adequate⁵⁸.

The obtained number of colonies per mL of liquid soap in an independent experiment (with two technical replicas) without adding neutralizer are presented in Table 7.

Table 7. CT results (CFU/mL).

Formulation	Time point (days)	Bacterial strains (CFU/mL)		
		<i>E. coli</i>	<i>S. aureus</i>	<i>S. epidermidis</i>
A	0	$(2.23 \pm 1.50) \times 10^3$	$(1.40 \pm 0.42) \times 10^4$	$(2.30 \pm 0.86) \times 10^5$
	7	0	0	0
	28	0	0	0
B	0	$1.00 \times 10^2 \pm (0)$	$(8.50 \pm 4.51) \times 10^3$	$(2.82 \pm 1.00) \times 10^4$
	7	0	0	0
	28	0	0	0

The results obtained showed that there were no longer microorganisms in the liquid soaps samples after 7 days. This means that both formulations were able to reduce the initial amount of microorganisms in a period of 7 days. Since the acceptance criteria defined a reduction of at least a 3 Log on day 7 (see Section 2.4.3), the results exceeded this limit, which proves the efficacy of both preservatives used in formulations A and B. This was confirmed when the samples were plated again 28 days after the initial time point, and once again no bacteria was detected. This met the acceptance criteria that establishes that there should not be any increase in the number of viable microorganisms compared to the previous readings⁵⁸.

As previously mentioned in Section 4, this test was repeated with the addition of a neutralizer that aims to annul the microbial activity of preservatives or any other compound in the cosmetic sample. In microbiological quality control of cosmetic, the neutralization of the antimicrobial effect of the

preservatives is essential for the safety of the tests and performed according to the international standards used in microbiological analyses of cosmetics. These neutralizers should be added according to the antimicrobial preservative group. For isothiazolinones some of the recommended neutralizers are lecithin and saponin. For phenoxyethanol some suggested neutralizers are lecithin as well, and polysorbate 80 amongst others. For this reason, a universal neutralizer was used. The results displaying the number of colonies are presented in Table 8:

Table 8. CT with neutralizer results (CFU/mL). un: uncountable.

Formulation	Time point (days)	Bacterial strains (CFU/mL)		
		<i>E. coli</i>	<i>S. aureus</i>	<i>S. epidermidis</i>
A	0	$(4.04 \pm 0.75) \times 10^5$	$(3.57 \pm 0.30) \times 10^5$	$(5.62 \pm 0.12) \times 10^5$
	7	0	0	0
	28	0	0	0
B	0	2.39×10^5	un	3.96×10^5
	7	0	0	0
	28	0	0	0

After 7 days, the samples were plated once again to record the bacteria reduction, and, as observed for the assay without neutralizer, a total cell inactivation was registered for both formulations with any strain. The samples were plated once again after 28 days to confirm this total reduction and that there was no additional microorganisms growth. To guarantee accurate viable counts, it is essential to quench or otherwise negate the activity of preservatives⁵⁸. A suitable study method starts with an initial CT performed on the tested product, if the results show that the growth is inhibited with a reduction by a factor greater than 2, the procedure is modified to ensure the validity of results and a neutralizing agent is added. If the neutralizer can't fully annul the antimicrobial activity, the information serves to indicate that the product in itself is not likely to be contaminated with the given species of the microorganisms used due to its microbicidal activity¹¹³, which is what the results in this case demonstrate. Even with the presence of a neutralizer, the preservative was still active and its activity was effective confirming that this product provides an environment not appropriate for the growth of the tested microorganisms.

6 Conclusion

In this dissertation project, the preservatives MI and CMI, that can cause allergic reaction, used in liquid soaps from *Castelbel*, were substituted for a preservative mixture containing phenoxyethanol, benzoic acid, and dehydroacetic acid (BDP) which is considered safer. In order to evaluate the formulation containing the new preservative system, the stability of both liquid soaps was assessed through accelerated conditions and the preservative's antimicrobial activity was tested through microbiological studies. The accelerated stability evaluation involved the assessment of the physical and chemical attributes and microbiological safety of the products during 8 weeks with assessment time-points at 0 (t0), 4 (t1) and 8 (t2) weeks. This period under accelerated stability corresponds to 1 year of real-time stability.

The accelerated conditions in the new formulation introduced relevant changes in the aesthetics of the products, mainly in the color that changed to yellow. Regarding the physical and chemical evaluation, the pH, density and foam capacity and stability were in accordance with the manufacturer or supplier's specifications from the beginning to the end of the assessment period. The centrifugation assessment also confirmed the stability of both formulations. However, the viscosity of both formulation presented lower values than the manufacturer's specifications, which suggests the need to adjust the evaluation method or the concentration of the viscosity agent. In the microbiological assessment, both formulations presented no contamination. Overall, it can be considered that the physical, chemical and microbiological properties remained stable during the testing period. In regards to the preservative's evaluation, both the agar-well diffusion method and the challenge test confirmed each formulation's antimicrobial capacity against *E. coli*, *S. aureus* and *S. epidermidis*, three bacterial strains commonly found on the skin. These antimicrobial properties were maintained during the CT period, 28 days. According to all results obtained, even though the new formulation might need a few adjustments in terms of viscosity and aesthetic parameters, the physical, chemical and microbiological properties of the liquid soaps tested are considered stable from the beginning to the end of the assessment period and the new preservative- BDP- has proven its antimicrobial capacity, showing that the new formulation is an adequate substitute.

7 Future Work

In terms of future work, new or improved stability testing with procedures such as the study of water availability, can be implemented in the company, expanding the stability evaluation to other cosmetic formulations, such as hand creams. The formula can be analyzed with the addition of an antioxidant to investigate the change in color that has a strong aesthetic impact and an adjustment in viscosity agent to comply with the manufacturer's specification. Additionally, the liquid soap or even the preservative in itself can be studied in terms of safety for consumers through a toxicological matrix correlating the toxicity level and exposure, considering the endpoints of skin sensitization, skin irritation, and carcinogenicity. Isolated data may not be adequate for safety assessment, ideally, an estimation of the level of risk of an adverse health outcome should be based on the concentration in a product, frequency of use, type of product, route of exposure, body surface location, and target population.

8 References

1. Pinheiro, S. Aquiles Barros é o engenheiro químico que criou a Castelbel. *Público* (2018). Available at: <https://www.publico.pt/2018/03/30/impar/noticia/aquilos-barros-e-o-engenheiro-quimico-que-criou-a-castelbel-1808568>.
2. Castelbel. Available at: <https://castelbel.com/>. (Accessed: 13th November 2022)
3. Chaudhri, S. K. & Jain, N. K. History of cosmetics. *Asian J. Pharm.* **3**, 164–167 (2009).
4. Council Directive 76/768/EEC. Cosmetics legislation. *Methods* **2**, (1999).
5. Jungermann, E. & Sonntag, N. O. V. Glycerine: A Key Cosmetic Ingredient. Routledge. (2018).
6. Halla, N., Fernandes, I. P., Heleno, S. A., Costa, P. & Boucherit-Otmani, Z. Cosmetics preservation: A review on present strategies. *Molecules* **23**, 1–41 (2018).
7. Chouhan, N., Vig, H. & Deshmukh, R. Cosmetics Market by Category. (2021). Available at: <https://www.alliedmarketresearch.com/cosmetics-market>. (Accessed: 20th December 2022)
8. Cosmetics Europe. Cosmetics and personal care industry overviews. *Cosmetics Europe* (2020). Available at: <https://cosmeticseurope.eu/cosmetics-industry/>. (Accessed: 21st December 2022)
9. European Parliament. Regulation (EC) No 1223/2009. *Off. J. Eur. Union* (2009).
10. European Commission. Legislation. *Internal Market, Industry, Entrepreneurship and SMEs* Available at: https://ec.europa.eu/growth/sectors/cosmetics/legislation_en. (Accessed: 22nd December 2022)
11. Nazdrajic, S. & Bratovic, A. the Role of Surfactants in Liquid Soaps and Its Antimicrobial Properties. *Int. J. Adv. Res.* **7**, 501–507 (2019).
12. Perencevich, E. N., Wong, M. T. & Harris, A. D. National and regional assessment of the antibacterial soap market: A step toward determining the impact of prevalent antibacterial soaps. *Am. J. Infect. Control* **29**, 281–283 (2001).
13. Willcox, M. 454 Poucher's Perfumes, Cosmetics and Soaps. 453–465 (2000).
14. Wang, L.-F., Wang, L.-L., Li, W.-W., He, D.-Q. & Jiang, H. Surfactant-mediated settleability and dewaterability of activated sludge. *Chem. Eng. Sci.* **116**, 228–234 (2014).
15. Parente, E. & Ares, G. How do appearance and fragrance influence expectations and conceptual associations of cosmetic products? An exploratory case study with liquid hand soap. *J. Sens. Stud.* **36**, (2021).
16. ISO 18811. Cosmetics — Guidelines on the stability testing of cosmetic products. **2018**, (2018).
17. Magdassi, S. & Touitou, E. Novel Cosmetic Delivery Systems. *CRC Press* (1998).
18. Cosmetics Europe. Guidelines on Stability Testing of Cosmetic Products. *Cosmet. Toilet. Fragr. Assoc. Eur. Cosmet. Toilet. Perfum. Assoc.* 1–8 (2004).
19. ISO 29621. Cosmetics — Microbiology — Guidelines for the risk assessment and identification of microbiologically. (2017).
20. ISO 17516. Cosmetics — Microbiology — Microbiological limits. **44**, (2015).
21. Lundov, M. D., Moesby, L., Zachariae, C. & Johansen, J. D. Contamination versus preservation of cosmetics: A review on legislation, usage, infections, and contact allergy. *Contact Dermatitis* **60**, 70–78 (2009).
22. Álvarez-Lerma, F., Maull, E., Terradas, R., Segura, C. & Planells, I. Moisturizing body milk as a reservoir of *Burkholderia cepacia*: outbreak of nosocomial infection in a multidisciplinary intensive care unit. *Crit. Care* **12**, R10 (2008).
23. Neza, E. & Centini, M. Microbiologically Contaminated and Over-Preserved Cosmetic Products According Rapex 2008–2014. *Cosmetics* **3**, (2016).
24. Alvarez-Rivera, G., Llopart, M., Lores, M. & Garcia-Jares, C. *Preservatives in Cosmetics: Regulatory Aspects and Analytical Methods. Analysis of Cosmetic Products: Second Edition* (Elsevier B.V., 2018). doi:10.1016/B978-0-444-63508-2.00009-6
25. Yim, E., Nole, K. L. B. & Tosti, A. Contact dermatitis caused by preservatives. *Dermatitis* **25**, 215–231 (2014).

26. Manteghi, M. European Cosmetics Industry: Main Aspects and Regulation. *SSRN Electron. J.* 1–21 (2017). doi:10.2139/ssrn.3082290
27. Oishi, S. Effects of propyl paraben on the male reproductive system. *Food Chem. Toxicol.* **40**, 1807–1813 (2002).
28. McDonnell, G. & Russell, A. D. Antiseptics and disinfectants: activity, action, and resistance. *Clin. Microbiol. Rev.* **12**, 147–179 (1999).
29. ECHA. Cosmetic Products Regulation, Annex V - Allowed Preservatives. Available at: <https://echa.europa.eu/cosmetics-preservatives>. (Accessed: 13th January 1BC)
30. Silva, V., Silva, C., Soares, P. & Garrido, E. M. Isothiazolinone Biocides: Chemistry, Biological, and Toxicity Profiles. *Molecules* **25**, 991 (2020).
31. Collier, P. J., Ramsey, A., Waigh, R. D., Douglas, K. T. & Austin, P. Chemical reactivity of some isothiazole biocides. *J. Appl. Bacteriol.* **69**, 578–584 (1990).
32. Fewings, J. & Menné, T. An update of the risk assessment for methylchloroisothiazolinone/methylisothiazolinone (MCI/MI) with focus on rinse-off products. *Contact Dermatitis* **41**, 1–13 (1999).
33. Bester, K. & Lamani, X. Determination of biocides as well as some biocide metabolites from facade run-off waters by solid phase extraction and high performance liquid chromatographic separation and tandem mass spectrometry detection. *J. Chromatogr. A* **1217**, 5204–5214 (2010).
34. Schnuch, A., Lessmann, H., Geier, J. & Uter, W. Contact allergy to preservatives. Analysis of IVDK data 1996–2009. *Br. J. Dermatol.* **164**, 1316–1325 (2011).
35. Schwensen, J. F., Lundov, M. D., Bossi, R., Banerjee, P. & Gimenez-Arnau, E. Methylisothiazolinone and benzisothiazolinone are widely used in paint: A multicentre study of paints from five European countries. *Contact Dermatitis* **72**, 127–138 (2015).
36. García-Gavín, J., Vansina, S., Kerre, S., Naert, A. & Goossens, A. Methylisothiazolinone, an emerging allergen in cosmetics? *Contact Dermatitis* **63**, 96–101 (2010).
37. Isaksson M, Gruvberger B, B. M. Occupational contact allergy and dermatitis from methylisothiazolinone after contact with wallcovering glue and after a chemical burn from a biocide. *Dermatitis* (2004).
38. Thyssen, J. P., Sederberg-Olsen, N., Thomsen, J. F. & Menné, T. Contact dermatitis from methylisothiazolinone in a paint factory. *Contact Dermatitis* **54**, 322–324 (2006).
39. Warshaw, E. M., Belsito, D. V., Taylor, J. S., Sasseville, D. & DeKoven, J. G. North American Contact Dermatitis Group patch test results: 2009 to 2010. *Dermatitis* **24**, 50–59 (2013).
40. Castaneda-Tardana, M. P. & Zug, K. A. Methylisothiazolinone. *Dermatitis* **24**, 2–6 (2013).
41. European Chemicals Agency. *Guidance on the application of the CLP criteria : guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures*. (2017).
42. Brannan D K. Cosmetic preservation. *J Soc Cosmet Chem* **46**, 199–220 (1995).
43. ISO 21388. ISO 21388 - Good Manufacturing Practices (GMP). 61010-1 © Iec2001 **2014**, 13 (2014).
44. Xiao, G., Déziel, E., He, J., Lépine, F. & Lesic, B. MvfR, a key *Pseudomonas aeruginosa* pathogenicity LTTR-class regulatory protein, has dual ligands. *Mol. Microbiol.* **62**, 1689–1699 (2006).
45. Aalto-Korte, K., Alanko, K., Kuuliala, O. & Jolanki, R. Methacrylate and acrylate allergy in dental personnel. *Contact Dermatitis* **57**, 324–330 (2007).
46. Microcare preservatives. Available at: <https://www.thorpersonalcare.com/preservatives.html>. (Accessed: 10th January 2022)
47. Selimoğlu, F., Ünal, N., Ertekin, Z. C. & Dinç, E. PARAFAC and MCR-ALS approaches to the pKa determination of benzoic acid and its derivatives. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **248**, 119253 (2021).
48. Manku, G. C. S. The formation constants of some metal complexes with 3-Acetyl-4-hydroxycoumarin, dehydroacetic acid, and their oximes. *Aust. J. Chem.* **24**, 925–934 (1971).
49. THOR. *Microcare BDP: Descripción general*.
50. Puschmann, J., Herbig, M. E. & Müller-Goymann, C. C. Correlation of antimicrobial effects of phenoxyethanol with its free concentration in the water phase of o/w-emulsion gels. *Eur. J. Pharm.*

- Biopharm.* **131**, 152–161 (2018).
51. SCCS. Opinion of the Scientific Committee on Consumer Safety on Phenoxyethanol. *Scs* (2016).
 52. Pastor-Nieto, M. A., Alcántara-Nicolás, F., Melgar-Molero, V., Pérez-Mesonero, R. & Vergara-Sánchez, A. Conservantes en productos de higiene y cosméticos, medicamentos tópicos y productos de limpieza doméstica en España. *Actas Dermosifiliogr.* **108**, 758–770 (2017).
 53. US EPA United States Environmental Protection Agency. Endocrine Disruptor Screening Program (EDSP) Estrogen Receptor Bioactivity. (2015).
 54. Siegert, W. ISO 11930_A Comparison to other Methods to Evaluate the Efficacy of Antimicrobial. *Sofw* **138**, 44–53 (2012).
 55. Lawrence, J. C., Cason, S. & Kidson, A. Phenoxetol-chlorhexidine cream as a prophylactic antibacterial agent in burns. *Lancet* 1037–1040 (1980).
 56. Bühner, C., Bahr, S., Siebert, J., Wettstein, R. & Geffers, C. Use of 2% 2-phenoxyethanol and 0.1% octenidine as antiseptic in premature newborn infants of 23–26 weeks gestation. *J. Hosp. Infect.* **51**, 305–307 (2002).
 57. Russell, A. D. Challenge testing: Principles and practice. *Int. J. Cosmet. Sci.* **25**, 147–153 (2003).
 58. ISO 11930. ISO 11930 - Cosmetics — Microbiology — Evaluation of the antimicrobial protection of a cosmetic product. (2019).
 59. Tan, A. S. B., Tüysüz, M. & Ötük, G. Investigation of preservative efficacy and microbiological content of some cosmetics found on the market. *Pak. J. Pharm. Sci.* **26**, 153–157 (2013).
 60. USP, C. The United States Pharmacopeia. *Natl. Formul.* **14**, (2008).
 61. Eldesoukey, R. M. M. & S Alqhtani, B. Comparative Microbiological Study between Traditional and Modern Cosmetics in Saudi Arabia. *Enzym. Eng.* **05**, (2016).
 62. Morrison Jr., A. J. & Wenzel, R. P. Epidemiology of Infections Due to *Pseudomonas aeruginosa*. *Rev. Infect. Dis.* **6**, S627–S642 (1984).
 63. Gonzales, J., Latorre, X., Soriano, E. & Hopfer, R. Hair Clipping : Another Hazard for for granulocytopenic patients. 655–656 (1987).
 64. Birteksoztan, A., Hacıoglu, M. & Otuk, G. Investigation of preservative efficacy and microbiological content of some cosmetics found on the market. *Pak. J. Pharm. Sci.* **26**, 153–157 (2013).
 65. Okeke, I. N. & Lamikanra, A. Bacteriological quality of skin-moisturizing creams and lotions distributed in a tropical developing country. *J. Appl. Microbiol.* **91**, 922–928 (2001).
 66. Hugbo, P., Onyekweli, A. & Igwe, I. Microbial contamination and preservative capacity of some brands of cosmetic creams. *Trop. J. Pharm. Res.* **2**, (2005).
 67. Çarıkçı, A. İ. Y., Uçar, F. & Yalçın, H. T. Kozmetik Ürünlerde Bakteriyal ve Fungal Kompozisyonun Klasik Yöntemler ve PCR Yöntemi Kullanılarak Saptanması 1. *Elektron. Mikrobiyoloji Derg. TR* **6**, 1–16 (2008).
 68. Boynukara, B., İlhan, Z. & Gulhan, T. *Aspergillus niger* isolation from an imported cosmetic skin cream. **13**, 41–43 (2002).
 69. Campana, R., Scesa, C., Patrone, V., Vittoria, E. & Baffone, W. Microbiological study of cosmetic products during their use by consumers: Health risk and efficacy of preservative systems. *Lett. Appl. Microbiol.* **43**, 301–306 (2006).
 70. Smaoui, S. Cosmetic emulsion from virgin olive oil: Formulation and bio-physical evaluation. *African J. Biotechnol.* **11**, 9664–9671 (2012).
 71. Rodrigues, F., Gaspar, C., Palmeira-De-Oliveira, A., Sarmiento, B. & Amaral, M. H. Application of Coffee Silverskin in cosmetic formulations: Physical/antioxidant stability studies and cytotoxicity effects. *Drug Dev. Ind. Pharm.* **42**, 99–106 (2016).
 72. Vignoli, J. A., Bassoli, D. G. & Benassi, M. T. Antioxidant activity, polyphenols, caffeine and melanoidins in soluble coffee: The influence of processing conditions and raw material. *Food Chem.* **124**, 863–868 (2011).
 73. Anchisi, C., Maccioni, A. M., Sinico, C. & Valenti, D. Stability studies of new cosmetic formulations with vegetable extracts as functional agents. *Farmaco* **56**, 427–431 (2001).

74. Silva, M. J. F., Rodrigues, A. M., Vieira, I. R. S., Neves, G. D. A. & Menezes, R. R. Development and characterization of a babassu nut oil-based moisturizing cosmetic emulsion with a high sun protection factor. *RSC Adv.* **10**, 26268–26276 (2020).
75. Stephane, P. Stability of Cosmetic Formulations Containing UV Filters and Preservatives, Based on Physical and Chemical Parameters. *MOJ Toxicol.* **1**, 1–11 (2015).
76. Castelbel. Odor evaluation internal protocol (MTI003). (2021).
77. Castelbel. Color evaluation internal protocol (MTI002). (2021).
78. Castelbel. Appearance evaluation internal protocol (MTI001). (2021).
79. Castelbel. pH measurements internal protocol (MTI004). (2021).
80. Fungilab. Rotational Viscometer Instruction Manual.
81. Ese, M. H. & Kilpatrick, P. K. Stabilization of water-in-oil emulsions by naphthenic acids and their salts: Model compounds, role of pH, and soap: Acid ratio. *J. Dispers. Sci. Technol.* **25**, 253–261 (2004).
82. Antonic, B., Dordevic, D., Jancikova, S., Tremlova, B. & Nejezchlebova, M. Reused plant fried oil: A case study with home-made soaps. *Processes* **9**, 1–12 (2021).
83. Balouiri, M., Sadiki, M. & Ibnsouda, S. K. Methods for in vitro evaluating antimicrobial activity: A review. *J. Pharm. Anal.* **6**, 71–79 (2016).
84. Chen, Y. E., Fischbach, M. A. & Belkaid, Y. Skin microbiota-host interactions. *Nature* **553**, 427–436 (2018).
85. Reed, R. W. & Reed, G. B. ‘Drop Plate’ Method of Counting Viable Bacteria. *Can. J. Res.* **26e**, 317–326 (1948).
86. Abu-Elala, N. M., Attia, M. M., Abd-Elsalam, R. M., Gamal, A. & Younis, N. A. Peracetic acid treatment of *Ichthyophthirius multifiliis* (Ciliophora: Ichthyophthiriidae) and *Trichodina* spp. reduces the infection by *Aeromonas hydrophila* and improves survival in Nile tilapia (*Oreochromis niloticus*). *Aquaculture* **538**, 736591 (2021).
87. Fromageot, D., Roger, A. & Lemaire, J. Thermooxidation yellowing of aliphatic polyamides. *Die Angew. Makromol. Chemie* **170**, 71–85 (1989).
88. Krauklis, A. E. & Echtermeyer, A. T. Mechanism of yellowing: Carbonyl formation during hygrothermal aging in a common amine epoxy. *Polymers (Basel)*. **10**, (2018).
89. Premachandran, R., Mehta, K., Gupta, J. & Hakimi, N. H. Synergistic compositions of dehydroacetic acid and methods for reducing yellowing in various end-user compositions. WO2016154511A1 (2016).
90. Akgündüz, M. Ç., Çavuşoğlu, K. & Yalçın, E. The Potential Risk Assessment of Phenoxyethanol with a Versatile Model System. *Sci. Rep.* **10**, 1209 (2020).
91. National Center for Biotechnology Information. PubChem Compound Summary for Glycerol. *PubChem* Available at: <https://pubchem.ncbi.nlm.nih.gov/compound/Glycerol>. (Accessed: 29th January 2022)
92. IFC Consulting. Sodium Lauryl Sulfate Crops. *Tech. Eval. sodium lauryl sulfate* 1–10 (2006).
93. Preservative Reviews. Available at: <https://makingskincare.com/preservatives/>. (Accessed: 12th February 2022)
94. Kulkarni, V. S. & Shaw, C. Use of Polymers and Thickeners in Semisolid and Liquid Formulations. *Essent. Chem. Formul. Semisolid Liq. Dosages* 43–69 (2016). doi:10.1016/b978-0-12-801024-2.00005-4
95. Castelbel. Supplier Information. (2021).
96. Bones, D. L., Reid, J. P., Lienhard, D. M. & Krieger, U. K. Comparing the mechanism of water condensation and evaporation in glassy aerosol. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 11613–11618 (2012).
97. Matousek, J. L., Campbell, K. L., Kakoma, I., Solter, P. F. & Schaeffer, D. J. Evaluation of the effect of pH on in vitro growth of *Malassezia pachydermatis*. *Can. J. Vet. Res.* **67**, 56–59 (2003).
98. Seweryn, A., Wasilewski, T. & Bocho-Janiszewska, A. Correlation between Sequestrant Type and Properties of Mild Soap-Based Hand Washing Products. *Ind. Eng. Chem. Res.* **57**, 12683–12688 (2018).
99. Pena, D. W. P., Tonoli, G. H. D., Protásio, T., Souza, T. M. & Ferreira, G. C. Exfoliating Agents for Skincare Soaps Obtained from the Crabwood Waste Bagasse, a Natural Abrasive from Amazonia. *Waste and Biomass Valorization* **12**, 4441–4461 (2021).

100. Tindal, C. H. & Mason, J. B. Effect of Aeration on the Viscosity of Water. *Am. J. Phys.* **19**, 382 (1951).
101. EDQM - European Directorate for the Quality of Medicines. European Pharmacopoeia (Ph. Eur.) 10th Edition.
102. Wilson, A. J. Foams: Physics, Chemistry and. *Springer-Verlag* (1989).
103. Arzhavitina, A. & Steckel, H. Foams for pharmaceutical and cosmetic application. *Int. J. Pharm.* **394**, 1–17 (2010).
104. Kempka, A. P., Horvath, F. J., Fagundes, P. & Prestes, R. C. Foaming and emulsifying capacity, foam and emulsion stability of proteins of porcine blood. *Rev. Bras. Tecnol. Agroindustrial* **9**, 1797–1809 (2015).
105. Choudhury, A. K. R. Process control in finishing of textiles. in *Woodhead Publishing Series in Textiles* (eds. Majumdar, A., Das, A., Alagirusamy, R. & Kothari, V. K. B. T.-P. C. in T. M.) 363–427 (Woodhead Publishing, 2013). doi:<https://doi.org/10.1533/9780857095633.3.363>
106. Renpu, W. Measures for Putting a Well into Production. in 417–523 (Gulf Professional Publishing, 2011). doi:<https://doi.org/10.1016/B978-0-12-385868-9.00011-7>
107. Aguirre-Ramírez, M., Silva-Jiménez, H., Banat, I. M. & Díaz De Rienzo, M. A. Surfactants: physicochemical interactions with biological macromolecules. *Biotechnol. Lett.* **43**, 523–535 (2021).
108. Siegert, W. *Approved Preservatives for Cosmetics: A Review of Actives Listed in Regulation (EC) No 1223/2009 on Cosmetic Products - Annex V.* (2014).
109. Dréno, B., Zuberbier, T., Gelmetti, C., Gontijo, G. & Marinovich, M. Safety review of phenoxyethanol when used as a preservative in cosmetics. *J. Eur. Acad. Dermatology Venereol.* **33**, 15–24 (2019).
110. Mitchell, P., Powles, R., Rege, K., Treleaven, J. & Catovsky, D. Phenoxyethanol is effective topical therapy of Gram-negative cellulitis in neutropenic patients. *J. Hosp. Infect.* **25**, 53–56 (1993).
111. Andersen, F. A. Final report on the safety assessment of Benzyl Alcohol, Benzoic Acid, and Sodium Benzoate. *Int. J. Toxicol.* **20**, 23–50 (2001).
112. Baldwin, A. G., Bevan, J., Brough, D., Ledder, R. & Freeman, S. Synthesis and antibacterial activities of enamine derivatives of dehydroacetic acid. *Med. Chem. Res.* **27**, 884–889 (2018).
113. Cengiz, G. & Yapar, E. A. Neutralizants for Antimicrobial Effective Microbiological Analysis in Cosmetic Products. 117–119 (2018).

9 Appendices

Appendice 1 - Foaming Capacity and Foam Stability Photographic Record

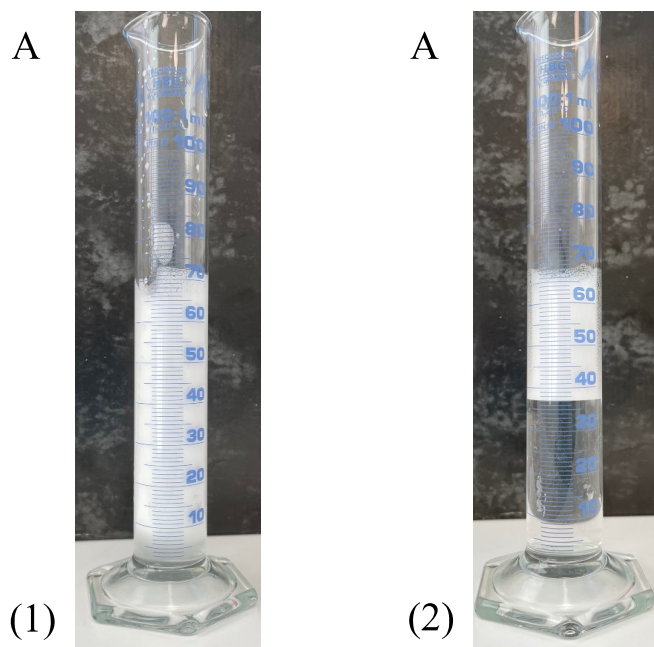


Figure A1. Photographic record of formulation A in the foaming capacity and foam stability analysis immediately after homogenization (1) and after 30 minutes (2).

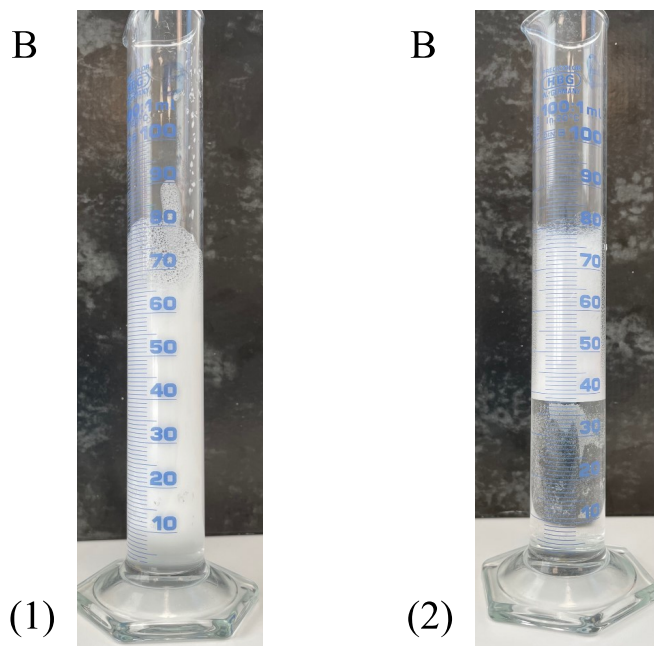


Figure A2. Photographic record of formulation B in the foaming capacity and foam stability analysis immediately after homogenization (1) and after 30 minutes (2).

Appendice 2 – Photographic Record of Antimicrobial activity analysis – Agar-well diffusion method

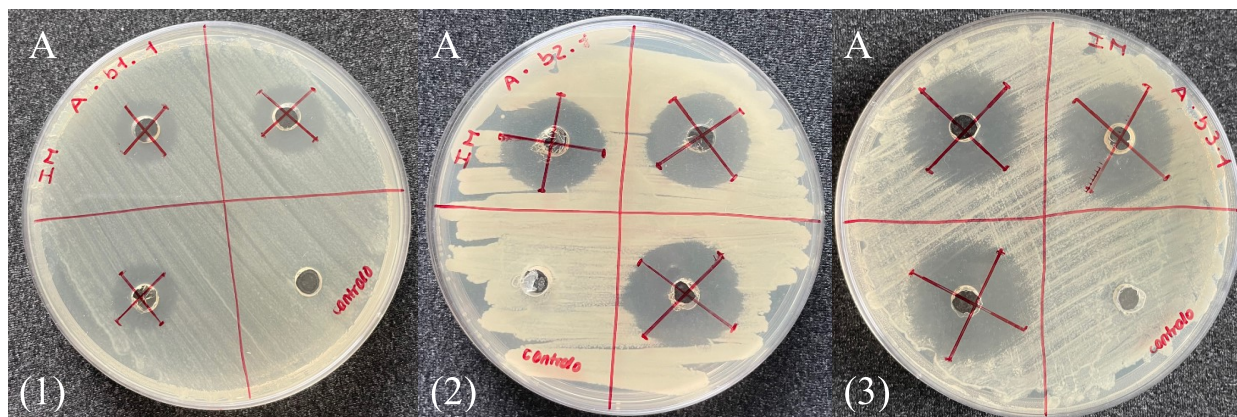


Figure A3. Photographic record of the inhibition halos of formulation A for *E. coli* (1), *S. aureus* (2) and *S. epidermidis* (3).

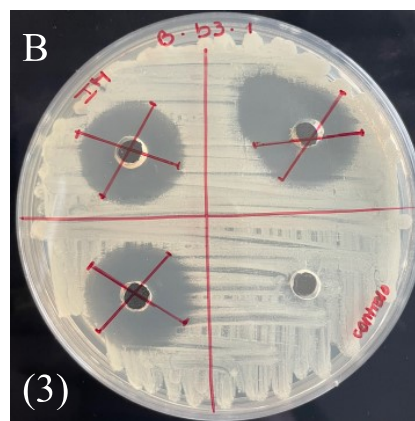
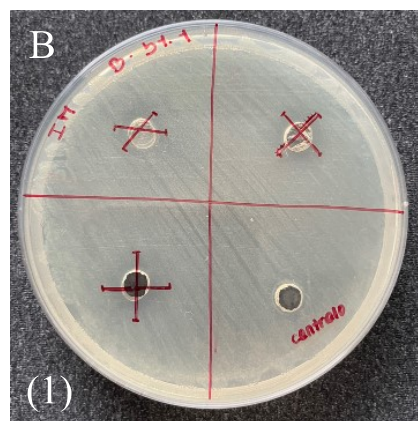


Figure A4. Photographic record of the inhibition halos of formulation B for *E. coli* (1), *S. aureus* (2) and *S. epidermidis* (3).

Appendice 3 – Photographic Record of the Challenge Test

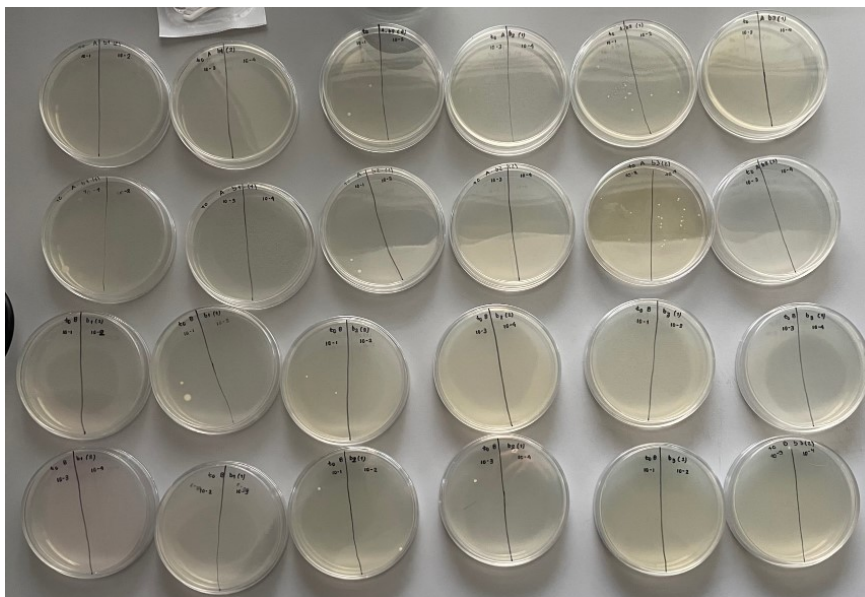


Figure A5. Photographic record of the CT in the first attempt.

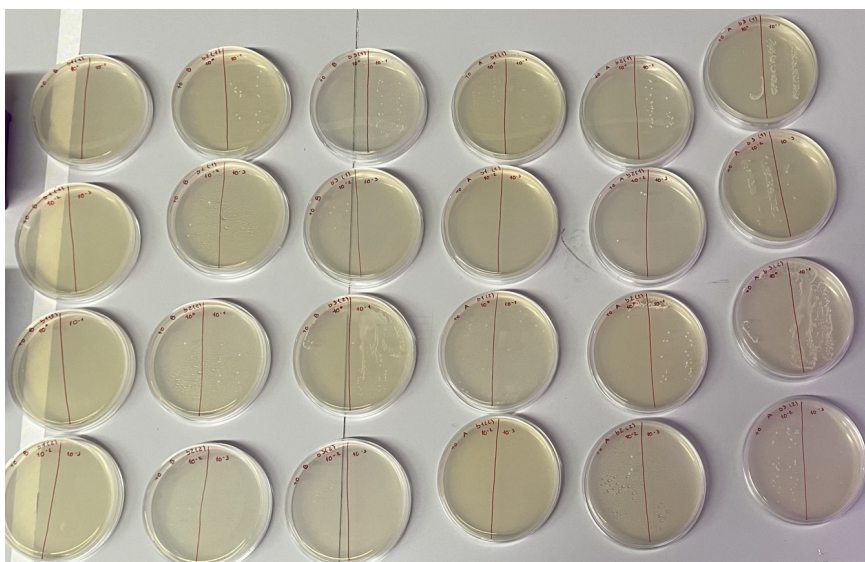


Figure A6. Photographic record of the CT in the second attempt.

Appendice 4 – Graphic Representation of the Challenge Test

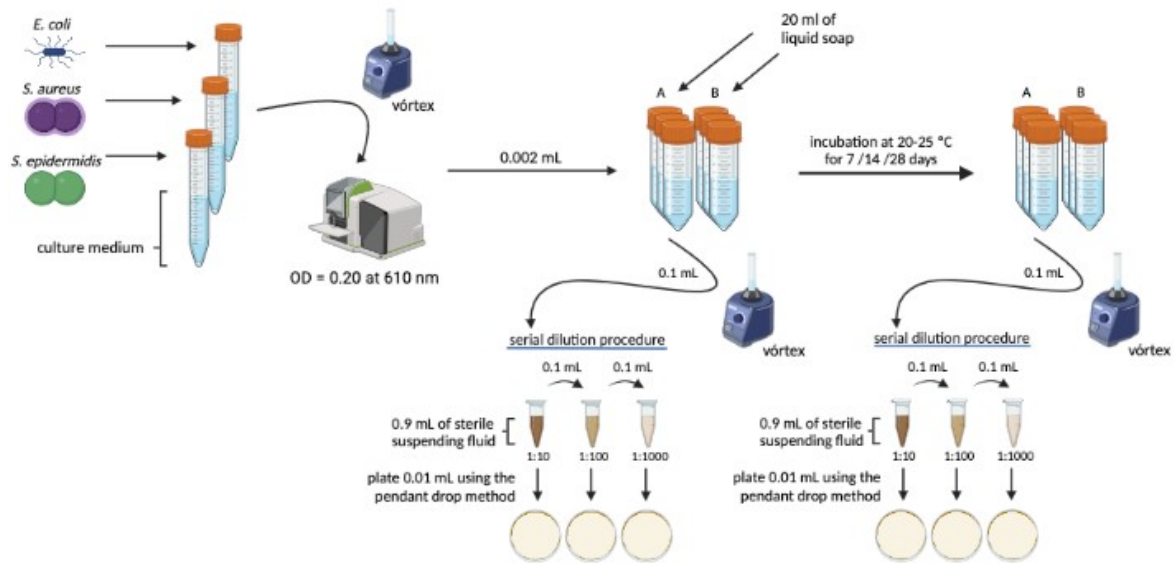


Figure A7. Graphic representation of the Challenge Test.

