Influence of environmental factors on microbial dynamics: assessment on model systems

Ana Beatriz Pina Bastos Rocha

ABSTRACT

Personal care products (PCP) are widely used in order to improve the quality of daily life. In order to assure PCP safety, chemical preservatives are included in their formulation. Preservatives are used at very low levels and usually are present in combination with other preservatives, in order avoid gaps in effectiveness. Challenge testing (CT) and Predictive Microbiology are two tools used to assess microbial safety.

In this work, the efficacy in of several combination of different preservatives and multifunctional ingredients was studied. Three preservatives, A, B and C, and one multifunctional ingredient, Z were tested. These chemicals were tested in the following combinations: triplet ABZ, duo AC and duo CZ, and at different pH values. Challenge testing was performed to this compositions and the results were classified according to the defined acceptance criteria of CT. Two probabilistic growth/no growth models were built, one for ABZ at pH 6 and other for pH 7. Results suggested that the combinations tested were successful in ensuring microbial safety. Also, that the multifunctional ingredient has a real effect of boosting the preservatives activity, and therefore it is possible to reduce the preservatives concentration in formulations. The modelling results suggest that the models can be used in future predictions. In conclusion, the tested combinations were effective in satisfying CT criteria.

Keywords: Personal care products; Microbiological safety; Preservatives; Multifunctional ingredients; Challenge Testing; Predictive Microbiology; Growth/No growth models.

1. INTRODUCTION

Food is “any substance or product, whether processed, partially processed or unprocessed, intended to be, or reasonably expected to be ingested by humans” according to the European Commission’s directive EC 178/2002 (EU, 2002).

Personal care products (PCP) are widely used in high quantities throughout the world in order to improve the quality of daily life. This term refers to a wide variety of items commonly found in health and beauty stores for personal hygiene and beauty, including soaps, shampoos and shower products, sunscreens, skin and hair care products, make-ups, toothpastes, personal hygiene products and many others (Antignac, et al., 2011). In the EU, this category of consumer products is regulated as cosmetics in EC 1223/2009 (EU, 2009). Today’s PCP market is driven by innovation, including new ingredients and unique formulas targeted to specific needs. Products need to be improved constantly in order to stay ahead in a highly competitive market, where more choice and greater efficacy are expected. There is a growing demand for PCP containing natural ingredients as the consumers favor products perceived as healthier or ecological (Antignac, et al., 2011).

When producing or preserving a food or cosmetic product there are two main concerns: quality and safety. The safety of a product is related to the risks and foodborne illness that the product can cause to the consumers when contaminated by microorganisms (Berthele, et al., 2013). The management of microbial food safety is never simple because there are several factors to be considered. Nevertheless, safety is always considered an absolute requirement in the food and personal care product’s industry. Unlike foodstuffs, a much longer shelf-life of PCP is expected. However, formulas of many cosmetics are based on water as the bulk component, which is a decisive factor for microbial multiplication (Lundov, et al., 2009). Additionally, cosmetic products contain enough nutrients, exhibit a neutral pH and are usually stored at ambient temperature. This suitable environment, together with a repeated consumer use, poses a potential risk of microbial contamination (FDA, 2016). The production of PCP does not include heat treatments designed to eradicate any microorganisms likely to occur (Pitt, et al., 2015). Therefore, in this case more intensive reliance is placed on raw material quality and efficacious preservation systems.

Preservatives are static agents used to inhibit the growth of microorganisms in nearly all consumer products, most often in foods and cosmetics (Microchem-Laboratory, 2015). These antimicrobial agents should kill or prevent the growth of microorganisms over a long period of time but must not be toxic to humans (Todar, 2008). The use of combinations of different preservatives in one formulation has several advantages: balanced spectrum of action against bacteria, head-space protection, synergistic effect, reduction of preservative concentration and

prevention of adapted microorganisms (Heydaryinia, et al., 2011; Siegert, 2014).

Challenge testing is a method that consists of challenging a product with a prescribed inoculum of microorganisms and study the ability of the product in killing or reducing the microorganism population. More specifically, first the product is inoculated with the target microorganisms, then stored at a controlled temperature, samples are withdraw at specified intervals of time and the microbial concentration in the samples is counted (Pérez-Rodríguez & Valero, 2013; QACSLab, 2014). There are several areas of application, such as the determination of a product's safety, the establishment of shelf-life period of refrigerated or ambient-stored foods or the formulation of products in terms of intrinsic control factors such as pH and water activity (a_w). When defining a CT method, several steps need to be taken into account: (i) design of CT, (ii) selection of microorganisms, (iii) inoculum level and preparation, (iv) duration of the study and sample analysis, (v) formulation factors and storage conditions and (vi) data interpretation.

Predictive microbiology deals with the development of mathematical models to describe microbial evolution in foods or cosmetics as a function of environmental conditions (Hao Li & Guozhong Xie, 2007). These mathematical models are helpful when understanding the growth or death of microorganisms in relation to their properties and interactions, and also the intrinsic properties of the product that is being tested and the extrinsic factors of the processing environment (Havelaar, et al., 2010; Soboleva, et al., 2000). There are kinetic models: exponential model, Baranyi and Roberts model, Arrhenius type models or cardinal models, for example (Pérez-Rodríguez & Valero, 2013); and probabilistic models such as growth/no growth (G/NG) models (Dang, et al., 2011).

Probability models indicate the probability of growth instead of the growth rate (Fakruddin, et al., 2011). In this models attention is focused on those conditions in which a given microorganism can or cannot grow and their outcome is the probability of growth at the boundary zone of certain tested conditions. The boundary interface between growth and no growth maps is an area of intense physiological interest due to the fact that biological variability increases near the interface (McMeekin & Ross, 2002). These G/NG models have important advantages since they would greatly reduce the need for microbiological tests and enable evaluations of food safety and stability to be carried out quickly and inexpensively (Dang, et al., 2011). In addition, based on the defined no growth regions, food developers can quickly formulate new shelf-stable (additive-free) products (Dang, et al., 2011).

This study was part of a project in cooperation between BioTeC+ research department of Katholieke Universiteit Leuven and Unilever. In this work, the efficacy of different preservative systems was assessed using CT and predictive microbiology as tools.

2. MATERIALS AND METHODS

2.1. Experimental procedure

Microorganisms and inoculum preparation. Three different bacterial strains, chosen and provided by Unilever, were used as the target microorganisms in this study and are to remain confidential. The strains were stored in cryovials (Microbrank™ Vials with Cryopreservative, Pro-lab diagnostics, UK) at -80ºC in an ultra-low temperature freezer (U101 Innova®, New Brunswick, UK). The inoculum was prepared transferring one cryobead of stock culture of each strain to 3 mL of autoclaved Tryptic Soy Broth (TSB) medium (30 g/L TSB, VWR Chemicals, India). After incubation (ED 240 Incubator, Binder, Germany) at 30ºC for 24 h, 100 µL of inoculated medium were transferred into 100 mL of fresh TSB, which represents the second preculture, and incubated again in the same conditions for another 24 h. After incubation, the microbial culture had to become concentrated to achieve the microbial level of 10^9 CFU/mL used for the inoculation of the compositions in challenge testing. In order to concentrate, 40 mL were removed from the second culture into a 50 mL falcon tube and centrifuged at 12.000 rpm (Eppendorf centrifuge 5810R, VWR, Germany) for 10 minutes. The supernatant was discarded and the pellet was resuspended in a certain volume of fresh TSB, according to each microorganism (in the range from 4 mL to 10 mL). The optical density (OD) was assessed at 600 nm (FilterMax F5, Molecular devices, USA).

This resulted in a set of three pure cultures of different strains with the microbial level of 10^9 CFU/mL. This level was chosen as, in the inoculation step, the inoculum is going to be diluted in the factor of 2, meaning that the microbial level in the sample will be in the order of 10^7 CFU/mL. The cultures were mixed in the same volumetric proportion 1:1:1 and the cocktail of the three strains was used to inoculate the different compositions.

***Tested compositions*.** In this study, several combinations of preservatives were tested in order to study the magnitude of their antimicrobial activity, i.e., the level of microbial decrease in these media. The tested compositions were composed by mixing different preservatives and multifunctional ingredients, which, in the case of this study, are compounds that enhance the activity of preservatives. Three different preservatives: A, B, and C; and one multifunctional ingredient Z were tested for antimicrobial activity, at different pH values. All these chemicals were decided and supplied by Unilever and are to remain confidential. In this work, the following conditions were tested: (i) the triplet ABZ, composed by preservatives A

and B and multifunctional ingredient Z, at pH 6 and 7, (ii) the duo AC, composed by preservatives A and C, at pH 5 and 6, (iii) the duo CZ at pH 5, composed by preservative C and multifunctional ingredient Z and (iv) variations in the detergent concentration. The detergent is one of the compounds of the dilution medium, explained in the next section.

Compositions preparation. In order to prepare the triplets or duos that were used in challenge testing, stock solutions of each individual preservative/multifunctional ingredient in a specific concentration and pH were prepared individually. The necessary amount of each preservative or multifunctional ingredient was dissolved in Dilution Medium (30 g/L of TSB, 0.05% (w/v) of chelating agent and 12% (v/v) of detergent). The chelating agent and the detergent are to remain confidential. The function of the dilution medium is to mimic the characteristic conditions and compounds present in most personal care products. For the pH correction, a pH meter (S220 SevenCompact™ pH/Ion, Mettler Toledo, UK) and concentrated solutions of Hydrochloric acid (5 M) and Sodium hydroxide (1 M) were used. Afterwards, the individual stock solutions of each preservative were mixed in the right proportions to prepare the triplets or duos that were used in challenge testing.

Method of Challenge Testing. For each composition 3 or 5 replicates were processed, number of replicates depending on the composition in question, each one with 30 mL volume of preservative media. First, each one of the compositions was inoculated with 300 µL of the inoculum previously described. Immediately after inoculation, the time 0 h was sampled: 1 mL of inoculated composition was transferred to 9 mL of sterile neutralizer Dey/Engley (D/E), (39 g/L D/E Neutralizing broth, Difco™, Becton Dickinson). The neutralizer was used to stop the action of the preservatives and multifunctional ingredients. After waiting 10 minutes in order for the neutralizer to have the required effect, the sample was plated. For each sample, the neat sample (S) and the dilutions 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were plated using the EasySpiral Dilute (Easy Spiral Dilute®, Interscience, France). The dilution medium used to dilute the sample was sterile saline solution of 0.90% (w/v) NaCl (Sigma-Aldrich, Germany). General Brain Heart Infusion Agar (BHIA) plating medium was used (Composition (g/L): 12.5 brain extract; 5.0 hear extract; 10.0 proteose peptone; 5.0 sodium chloride; 2.5 di-sodium phosphate; 2.0 Dextrose; 15.0 Agar; VWR Chemicals, Leuven, Belgium). The plates were then incubated at 30ºC for 48h. The compositions were incubated (BD 115 Incubater, Binder, Germany) at 25ºC until the next sampling time point. For each other time points of 5 h, 10 h, 24 h, 34 h, 48 h, 7 d and 22 d the procedure was the same: transfer 1 mL of one sample to a tube of 9 mL of neutralizer, wait for 10 minutes, and plating in BHIA. After incubation, at 30ºC for 48h, the total number of colonies of each plate was counted using an automatic counter (Scan 300®, Interscience, France) and the software Scan Interscience. Three controls were processed in each challenge testing, in order to evaluate the normal growth of the microorganisms thought time. The growth media used in the controls was TSB medium.

Acceptance criteria. For this specific research project, two criteria were defined in collaboration with Unilever for assessing the efficacy of the preservation system (Table 1). This way, for a composition to satisfy criterion 1, there must be a 3 log reduction in the microbial population at 2 days. Criterion 2 is fulfilled if a composition presents no observable counts at 7 and 22 days. A composition is considered to 'pass' challenge testing if both criteria are satisfied.
Based on these criteria of CT, experimental conditions were classified into 3 classes (Table 2). There are 3 classes to which a composition may belong. The class 0 includes the compositions that fail both criteria 1 and 2. Class 1 is for the compositions that only meet criterion 2. Finally the class 2 consists of the compositions that meet both criteria 1 and 2. Results from challenge testing give information about the antimicrobial susceptibility towards the cocktail of species.

Table 1: Acceptance criteria of CT defined by Unilever. *ND stands for not detectable counts.

CT criteria based on log reductions of the microbial population	
Criterion 1	3 log reduction after 2 days
Criterion 2	No observable counts after 7 and 22 days

Table 2: Comparison between the original criteria for challenge testing and the modelling criteria.

Original criteria	**Classes**
Log reductions	3 classes: 0, 1 and 2
Criterion 1: 2 days: 3 log reduc. **Criterion 2:** 7 days: ND 22 days: ND	**0** → **X** Fails criterion 1 and 2
	1 → **X** Fails criterion 1 ✓ Meets criterion 2
	2 → ✓ Meets criteria 1 and 2

*ND stands for no observable counts.

2.2. Modelling approach

A probabilistic growth/no growth model was chosen, opposed to a kinetic model, due to the typical applications of these types of models. In this work, instead of mentioning growth or no growth of microorganisms, the pass or no passing of challenge testing criteria was considered, which is also correlated with growth or not of microorganisms under certain conditions. The goal of the model was to predict, for a certain

composition of specific concentrations of preservative and/or multifunctional ingredient, the microbial decrease through time and therefore, whether the compositions pass the challenge test criteria or not. For implementation and simulations MATLAB R2012a was used.

An ordinal multinomial model was chosen since the results are going to be separated in classes and the order of the classes is important. The three previously defined classes of 0, 1 and 2 were the ones used in these models. An assumption was made, as this model was considered as linear.

These models took into account each composition of ABZ and the class to which composition belonged, and estimated parameters for the model equation (Equation 1). The output of the model is the probability of one composition belonging in one of the three defined classes.

This equation is a typical growth/no growth model equation. The *logit* function was used and it can be explained as the *log* of the probability of one composition belonging to one class opposed to the probability of not belonging to that class. In this Equation 1, π_n, n = 0, 1 or 2, represents the probability of a composition belonging in a certain class of 0, 1 or 2, and the π_n value varies between 0 and 1. The parameters that were estimated are represented by α_0, …, α_n and $\alpha_0^{'}$, …, $\alpha_n^{'}$ and it is important to notice that they are not the same in both equations. Finally, A, B and Z represent the concentration of preservative A, preservative B and multifunctional ingredient Z, respectively, all in percentage (%).

$$\begin{cases} ln\left(\frac{\pi_0}{\pi_1+\pi_2}\right) = \alpha_0 + \alpha_1 \cdot A + \alpha_2 \cdot B + \alpha_3 \cdot Z \\ ln\left(\frac{\pi_0+\pi_1}{\pi_2}\right) = \alpha_0' + \alpha_1' \cdot A + \alpha_2' \cdot B + \alpha_3' \cdot Z \end{cases} \quad (1)$$

3. Results and Discussion

3.1. Experimental results

A summary of all the results is represented in Figures 1 to 11. Each title and the respective axis captions provide information about the tested compositions represented in each graph. A system of colors was used in order to easily classify the results. Each color represents one class: (i) blue for the compositions which belong to class 0, (ii) orange for the compositions that belong to class 1 and (iii) black for compositions that meet class 2 criteria. The concentrations will be expressed throughout this work in percentage. It can be volumetric (v/v)% or weigh/volume percentage (w/v)%, depending on the chemical.

A (%) / B (%) / Z (%) expresses the concentration of each composition, in the same order of the preservatives in the condition name. So one composition of 0.05 / 0.25 / 0.10 (%), for example, represents the composition that has an A concentration of 0.05%, B concentration of 0.25% and Z concentration of 0.10%.

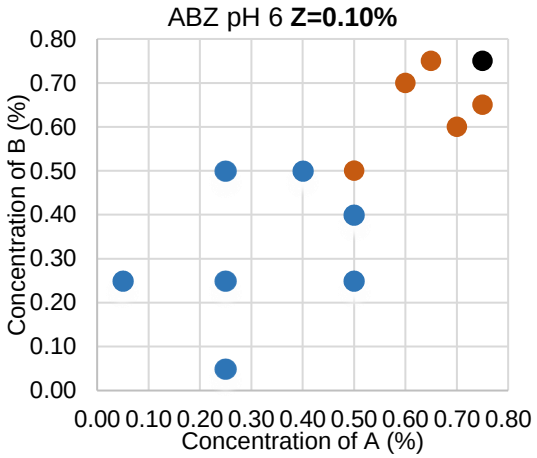

Figure 1: Tested compositions of the triplet ABZ at pH 6, with 0.10% concentration of Z. Each dot represents one tested composition. The axis give information regarding A and B concentration of each composition. (i) Blue for class 0 compositions, (ii) orange for class 1 and (iii) black for class 2.

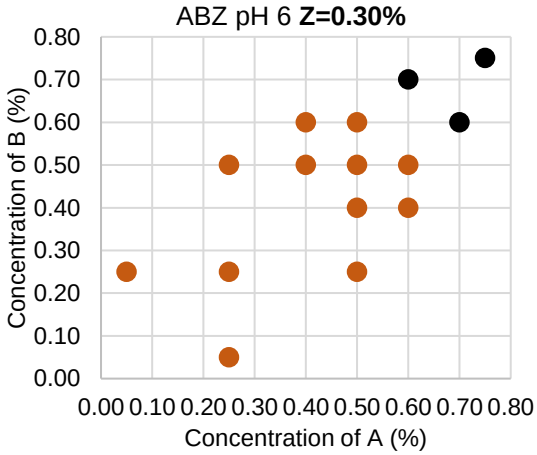

Figure 2: Tested compositions of the triplet ABZ at pH 6, with 0.30% concentration of Z. Each dot represents one tested composition. The axis give information regarding A and B concentration of each composition. (i) Blue for class 0 compositions, (ii) orange for class 1 and (iii) black for class 2.

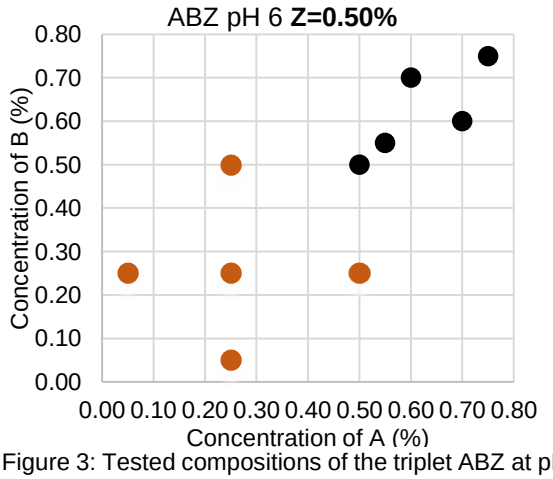

Figure 3: Tested compositions of the triplet ABZ at pH 6, with 0.50% concentration of Z. Each dot represents one tested composition. The axis give information regarding A and B concentration of each composition. (i) Blue for class 0 compositions, (ii) orange for class 1 and (iii) black for class 2.

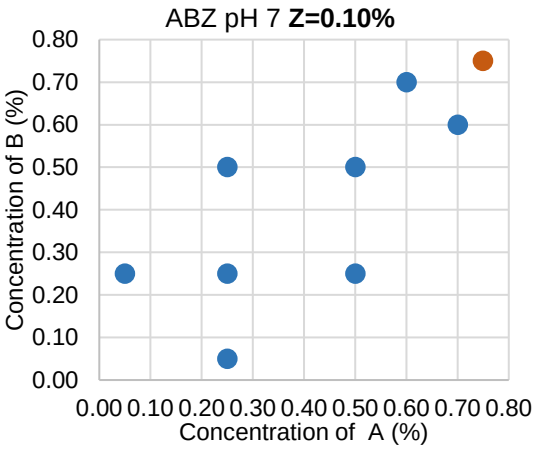

Figure 4: Tested compositions of the triplet ABZ at pH 7, with 0.10% concentration of Z. Each dot represents one tested composition. The axis give information regarding A and B concentration of each composition. (i) Blue for class 0 compositions, (ii) orange for class 1 and (iii) black for class 2.

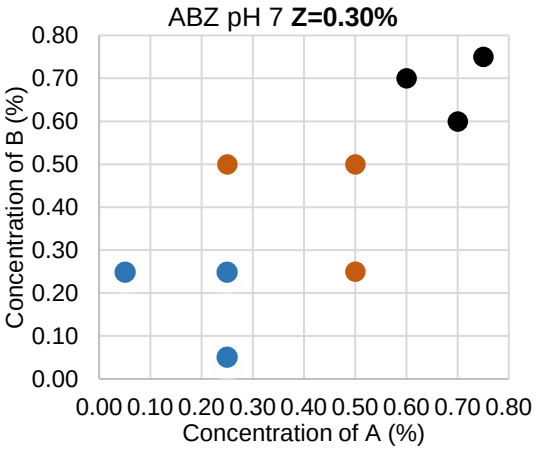

Figure 6: Tested compositions of the triplet ABZ at pH 7, with 0.30% concentration of Z. Each dot represents one tested composition. The axis give information regarding A and B concentration of each composition. (i) Blue for class 0 compositions, (ii) orange for class 1 and (iii) black for class 2.

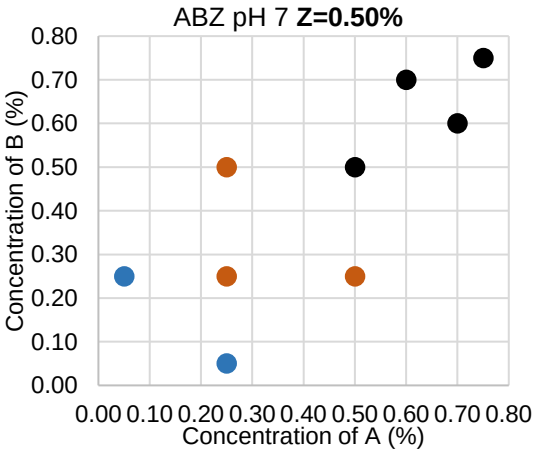

Figure 7: Tested compositions of the triplet ABZ at pH 7, with 0.50% concentration of Z. Each dot represents one tested composition. The axis give information regarding A and B concentration of each composition. (i) Blue for class 0 compositions, (ii) orange for class 1 and (iii) black for class 2.

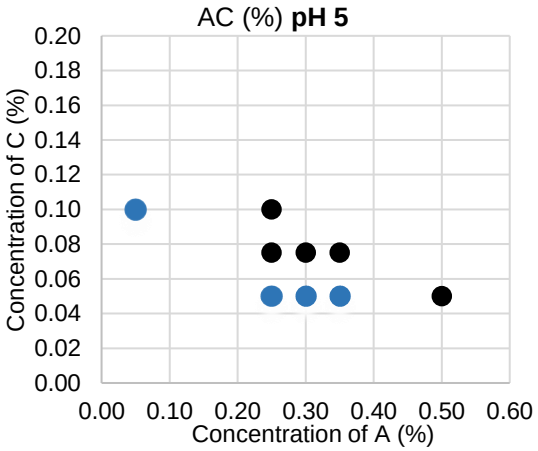

Figure 8: Tested compositions of the duo AC at pH 5. Each dot represents one tested composition. The axis give information regarding A and C concentration of each composition. (i) Blue for class 0 compositions, (ii) orange for class 1 and (iii) black for class 2.

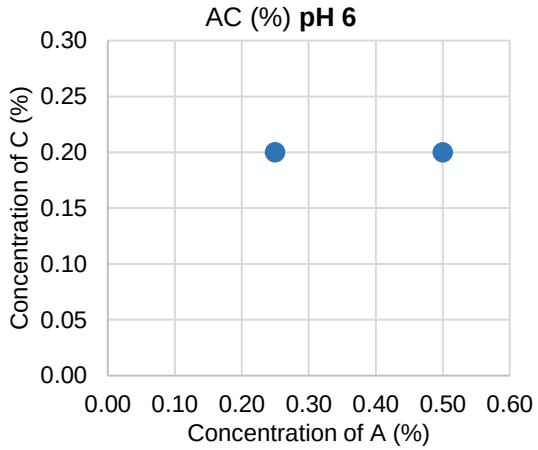

Figure 9: Tested compositions of the duo AC at pH 6. Each dot represents one tested composition. The axis give information regarding A and C concentration of each composition. (i) Blue for class 0 compositions, (ii) orange for class 1 and (iii) black for class 2.

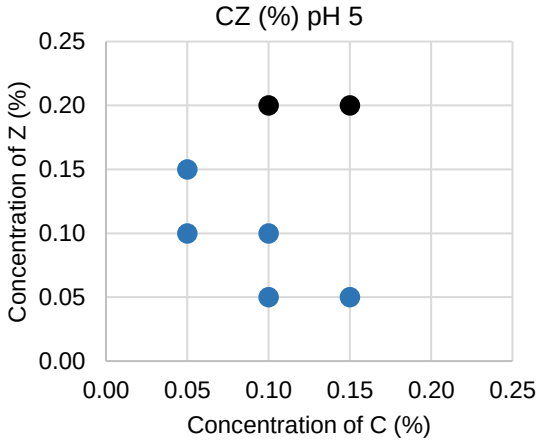

Figure 10: Tested compositions of the duo CZ at pH 5. Each dot represents one tested composition. The axis give information regarding C and Z concentration of each composition. (i) Blue for class 0 compositions, (ii) orange for class 1 and (iii) black for class 2.

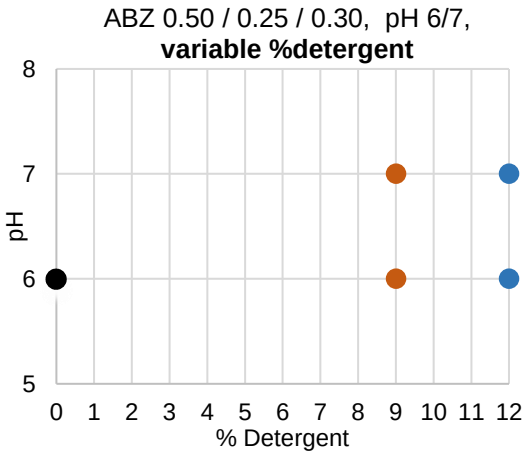

Figure 11: Tested compositions of detergent variations with the triplet ABZ 0.50 / 0.25 / 0.30 (%), at different pH. The axis give information regarding detergent concentration and pH of each composition. (i) Blue for class 0 compositions, (ii) orange for class 1 and (iii) black for class 2.

Triplet ABZ. For the triplet ABZ at pH 6, 38 compositions were tested and for pH 7, 27 compositions. For each pH, three different concentrations of multifunctional ingredient were tested: 0.10%, 0.30% and 0.50%. In both cases of pH 6 and 7, the multifunctional ingredient effect is very clear. Comparing the three graphs for each pH it is possible to see the multifunctional ingredient effect, of boosting the preservative's activity, as with the increase of Z concentration the compositions increase in order of class. Considering the multifunctional ingredient effect in the triplet at pH 6, there is only one condition that passes CT criteria, belonging to class 2, at the lowest concentration of Z (0.10%), 0.75 / 0.75 / 0.10 (%) (Figure 1). In contrast, for the intermediate concentration of Z, 0.30%, three conditions belong to class 2 (Figure 2). For the highest multifunctional ingredient concentration of 0.50%, 5 of the tested compositions passed all the criteria (Figure 3). As for the multifunctional ingredient effect at pH 7, at the lowest Z concentration no composition passes the criteria of CT (Figure 4). For the intermediate concentration of 0.30% three compositions belong to class 2, the same three compositions that passed all CT criteria at pH 6 (Figure 5). With the highest concentration of multifunctional ingredient already four compositions belonged to class 2 (Figure 6). It is obvious that the multifunctional ingredient has a significant influence on enhancing preservative activity. Synergistic preservative systems, created by the presence of a multifunctional ingredient, have been reported to demonstrate adequate anti-microbial efficacy without the use of harsher preservative systems, such as formaldehyde donors or alcohols (bibliographic reference from 2007, not indicated due to confidentiality). The multifunctional ingredient activity has a major significance as it allows to reduce the preservative concentration in products and design products with lower concentrations of preservative chemicals that also satisfy CT criteria, due to the multifunctional ingredient presence in the composition.

Secondly, it is also possible to conclude that the antimicrobial effect of the preservative compositions tested increases as a function of preservatives concentration. One composition has a bigger probability of passing CT if A and B concentrations are high. It is also possible to see that the results are symmetric. Imagining the line that represents A concentration equal to B concentration ($x=y$), it is possible to see that, in most of the cases, both sides of the line are symmetric. This leads us to conclude that both A and B have the same weight in microbial reduction. According to the Cosmetics Regulation (EC) No. 1223/2009, both A and B are limited to the same maximum concentration in cosmetics (EU, 2009). In this way, in order to assure safety and the law requirements, it is safer to use A and B in similar proportions (bibliographic reference from 2009, not indicated due to confidentiality). Otherwise one preservative had to be present in a very high concentration, in order to compensate for the lowest concentration preservative and to assure the microbial requirements, therefore risking a concentration close to the maximum limit allowed by regulation.

It is possible to observe the differences between the results of pH 6 and the ones of pH 7. For the lowest Z concentration, 0.10%, the compositions were more effective in achieving microbial reduction at pH 6 than at pH 7 (Figures 1 and 4). The same can be concluded for the compositions with a 0.30% concentration of Z: pH 6 compositions were more effective than pH 7. However, in this case, the difference of results is focused in the range of low concentrations of A and B. More specifically, the compositions 0.05 / 0.25 / 0.30, 0.25 / 0.05 / 0.30 and 0.25 / 0.25 / 0.30 (%), belong to class 0 in the pH 7 results while in pH 6 they belong to class 1. For the 0.50% concentration of Z, for low A and B concentrations, the compositions 0.05 / 0.25 / 0.50 and 0.25 / 0.05 / 0.50 (%) at pH 7, still belong to class 0, opposite to the same pH 6 compositions that belong to class 1 (Figures 1 to 6). This means that, for Z=0.50% and for low preservatives concentration, the antimicrobial compositions are more effective at pH 6 than at pH 7. However, this difference was not reported for higher concentrations of preservatives.

In summary, it is not possible to indicate that the compositions tested at pH 6 were more effective than at pH 7 or otherwise, as it depends on the multifunctional ingredient concentration also. For low multifunctional ingredient concentrations, the pH 6 compositions were clearly more effective than at pH 7 (Figures 1 to 6). As for intermediate and high multifunctional ingredient concentrations (0.30 and 0.50%), compositions with lower A and B concentrations, were more effective at pH 6 than at

pH 7. Yet, for this same range of Z, and for intermediate and high A and B concentrations, there was not a big different between the results of the compositions at pH 6 or at pH 7 (Figures 1 to 6).
The main conclusion is that the triplet ABZ is a successful combination of chemicals in reducing microbial levels of the tested pathogens. Multifunctional ingredients are gaining more recognition nowadays and can be the solution for all the personal care products that contain aggressive preservatives in their formulations, as formaldehyde donors or ethanol (bibliographic reference from 2007, not indicated due to confidentiality).

Duo AC. For the duo AC, 9 compositions were tested at pH 5 and only 2 for pH 6 (Figures 8 and 9). Four of the compositions tested at pH 5 belong to class 0 while the other five compositions belong to class 2, satisfying all of the challenge test criteria. It is interesting to note that a small variation of C could make the results change from class 0 to class 2, specifically in the region of preservative A with range between 0.25% and 0.35%. In the mentioned A range, a small increase of C, from 0.05% to 0.075%, translates in very different results – the compositions with 0.05% of C belong to class 0, while the ones with 0.08% of C to class 2 (Figure 8). Therefore, in this range, the C concentration is of main importance. There is a strong synergy between preservatives A and C for pH 5, while for pH 6 there is indifference between the preservatives (based on results of preliminary studies made previously to this work).
However, this strong synergy is not reflected in the brother A concentration range. For the lowest A concentration, 0.05%, despite having a high concentration of 0.10% of C, the composition still did not fulfill any of the original CT criteria. Opposite to this, for the highest concentration of A of 0.50% and lowest C concentration of 0.05%, the composition already belongs to class 2. It is possible to conclude that the concentration of preservative A is also very important for the outcome of the results. For the duo AC further studies should be performed in order to have enough information to build a model.

Duo CZ. In the duo CZ at pH 5, both the preservative C and the multifunctional ingredient Z were tested in the same range of concentrations. In the 7 tested compositions, 5 of them belonged to class 0 and did not pass any CT criteria (Figure 10). Two compositions, 0.10 / 0.20 and 0.15 / 0.20 (%), passed all the criteria of CT and belong to class 2. It is possible to define a boundary of growth/no growth between the class 0 range and the class 2 range, where the class 0 range is represented by the compositions in blue, and class 2 by the compositions in black. For the same C range, between 0.10 and 0.15%, just by varying the Z concentration between 0.05% and 0.20%, the compositions pass from class 0 to class 2 (Figure 10). This is caused by the multifunctional ingredient effect, as for the same C values, an increase in the multifunctional ingredient concentrations enhances preservative C activity and the results change (as for example, comparing 0.10 / 0.05 with 0.10 / 0.20 (%)).

Detergent variations. The influence of the detergent concentration on the preservative activity was also studied. The referred detergent is one of the three compounds of the dilution media. In this work, the same triplet ABZ 0.50 / 0.25 / 0.30 (%) was tested at pH 6 and 7, with dilution medium containing different concentrations of detergent. The triplet was tested at three detergent concentrations: (i) 12% at both pH 6 and 7 – the same concentration used in all CT –, (ii) 9% at pH 6 and 7, and (iii) 0% tested at pH 6.
Considering the results, the detergent had an influence on the preservative activity (Figure 11). Firstly, for the same detergent concentration, the results were very similar for both pH values. Both compositions tested with a 12% of detergent belonged to class 0. For the compositions with a lower concentration detergent at 9%, the results are classified as belonging to class 1. The composition tested at 0% of detergent, with no detergent in the dilution medium, belonged to class 2 and passes all CT criteria. Therefore, these results indicate that a higher concentration of detergent causes a loss of preservative effect.
This detergent belongs to a group of anionic surfactants and therefore, may affect the activity of a chemical. It has been reported that when the surfactant concentration exceeds the critical micelle concentration, the rate of penetration of drugs in the microorganisms decreases a lot (Mishra, et al., 2009). In a micellar medium, a chemical is divided between the micelles and the aqueous phase. Only the chemical present in the aqueous phase can interact with cells. Therefore the effective concentration of preservatives in the free media is lower than the reported concentration in the composition. The critical micelle concentration, meaning the minimum concentration of the surfactant necessary to create micelles, of other very similar detergent to the one used, is in the order of 0.20% (v/v) in water at 25ºC (bibliographic reference from 1955, not indicated due to confidentiality). There are not reported values for the critical micelle concentration of the specific detergent studied in this work. However, in this work it is possible to consider this value as the critical micelle concentration, considering that both detergents are very similar, and considering that the low concentration of chelating agent in the dilution medium does not affect this value. Therefore it is possible to conclude that, except in the case of 0% of detergent, all the other compositions were tested in conditions with

micelles dispersed in the medium. This can be the explanation to why the increase of detergent diminishes the preservative activity.
Considering the experimental results, it was concluded that a higher detergent concentration translates in lower preservatives activity. Therefore the detergent concentration could be a factor to microbial inactivation. In the end, a deeper study on the detergent parameters, would allow to optimize more parameters on PCP design.

3.2. Modelling results

Only the results of the triplet ABZ at pH 6 and pH 7 were used in the modelling. Two separate models were build, one for each pH value tested.
Using Matlab it was possible to calculate the parameters for the two equations (Equation 1). The solution of these equations, for one composition, was the probability of belonging to each class. The multinomial logistic regression (*mnrfit*) routine was used to estimate the model parameters based on the dataset. The multinomial logistic regression values (*mnrval*) routine, was also used to give model predictions based on the model parameters. In the growth/no growth models, the parameters have no biological interpretation. Due to this and to confidentiality, the obtained parameters for each equation will not be presented.
In order to be possible to have a 2D graph view of the results, A and B were considered as equal in all the compositions represented (Figures 12 and 13). The value read in the *x* axis represents the A concentration and B concentration (not the sum of them). In the graphs, the ranges of color represent the probability values. The yellow stands for 100% of probability and the dark blue represents 0%. Therefore, the range of compositions present in the yellow region, have a 100% of probability of belonging to the class considered in that area. The compositions present in the dark blue range have a 0% probability of belonging to the class represented in that area. The colors of transition represent an area of uncertainty were it is not possible to predict if those compositions belong to that class or not (Figures 12 and 13).

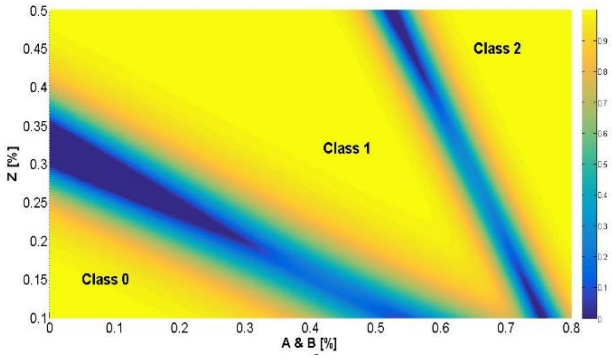

Figure 12: Results of the model established for data at pH 6. The dark blue stands for the compositions which have 0% of probability of belonging to one class while the yellow for the compositions that have 100% probability of belonging to the correspondent class (as indicated in the color bar).

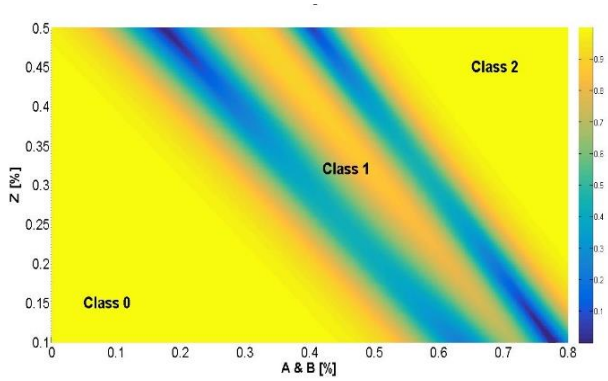

Figure 13: Results of the model established for data at pH 7. The dark blue stands for the compositions which have 0% of probability of belonging to one class while the yellow for the compositions that have 100% probability of belonging to the correspondent class (as indicated in the color bar).

Figure 12 and 13 represent the results overlapped in one graph, with 97.5% certainty. In both figures it is possible to distinguish three separate regions, represented in yellow: the region of class 0, the one of class 1 and the region of class 2. The region of each class represents the compositions that have a 100% probability of belonging to that class.
For pH 6, the range of compositions belonging to class 0 is much smaller than at pH 7. The area of class 1 reduces considerably with pH increase. Class 1 occupies the majority of the considered area at pH 6, opposite to what happens at pH 7, where the area of class 1 is very small. However, the range of compositions at pH 7 that belong to class 2 is bigger than at pH 6. Concluding, considering that the goal is to assure safety using compositions with A concentration equal to B concentration, both pH 6 and pH 7 are suitable. Regardless, even with high and intermediate Z concentrations, there is a chance that a composition will belong to class 0, if A and B concentrations are low, for pH 7 compositions. As for pH 6, multifunctional ingredient concentrations equal or lower than 0.25% should be avoided as these compositions require high A and B concentrations (between 0.70% and 0.80%) to pass CT criteria. Consequently, this demands the use of A and B concentrations close to the maximum allowed by law. In both pH values, compositions with high Z concentrations (over 0.40%) and A/B concentrations over 0.60% satisfy CT criteria. However Z is an expensive chemical so it is not in the best interest to consider compositions with high Z concentrations.
In conclusion, there is not a clear answer whether compositions at pH 6 are considered more effective than at pH 7 in assuring microbial decrease. The results of this models are interesting to gain knowledge about the different classes regions. However, there is not an obvious conclusion to which composition is the best, as it depends if the

manufacturer prefers to increase on multifunctional ingredient concentration or preservatives concentration in the product of study. Most likely this choice also requires adaptation to each product and to how the product's chemicals interact with each one of these three chemicals used in the preservation system.

After constructing a model it is necessary to validate it. Validation considers two steps: internal validation and external validation (Fakruddin, et al., 2011). In this case it is possible to perform internal validation, using the results of the dataset that was used to build the model. For the model at pH 6, the accuracy obtained was of 89.13%, while for pH 7 it was 89.36%. This values of accuracy are considered low, as they are lower than 90%. A reason for this is that some of the data points belong to the boundary of uncertainty of predictions. Another reason is how the model is built. This model does not consider (A*Z) and (B*Z) interactions. However, one of the terms of the model depends on the Z concentration. Therefore, the influence of Z was considered linear. However not many different Z concentrations were tested and this relationship does not appear linear when considering the experimental results.

External validation compares model predictions with microbial responses in actual personal care products. The purpose of this validation is to show model limitations. Therefore this second step of validation should be performed by Unilever, considering real products.

4. Conclusions and Future Perspectives

The goal of this work was to optimize personal care products preservation systems through the study of different antimicrobial formulations and the development of predictive models.

The PCP's industry has been studying for many years synergistic combinations of preservatives (Varvaresou, et al., 2009). However, almost none information is available regarding predictive microbiology applied to PCP. Modelling growth/no growth of undesired microorganisms in foods has been studied for several years in foods (Dang 2010), (Baranyi, 1994), yet not on PCP. Ghalleb et al. and Berthele et al. are authors to some of the new and few studies relative to predictive microbiology of cosmetics and it is possible to notice that this work is mostly relative to pH or a_w as factors of study. The G/NG models allow to estimate quickly the influence of minor changes in product formulas on the microbial stability of products, instead of time-consuming shelf-life studies or CT (Dang, et al., 2010).

Up to now, the choice of optimal preservatives mixture was based on empirical testing and often viewed as an art rather than a science (Prevot, et al., 2000). This work represents highly ground-breaking knowledge on preservative systems of PCP. Modelling antimicrobial systems' activity is the future. Firstly, it represents a massive economic advantage for companies, as it allows to reduce on the quantities of preservatives used. Secondly, it allows to satisfy consumer's demands for products with less concentration of preservatives. It definitely generates a competitive advantage for companies. Therefore, an interesting application of this study would be to easily predict successful compositions in reducing microbial level, so that they could be incorporated in a product's formulation, in order to guarantee that the product is not spoiled during shelf-life time.

5. References

Antignac, E. et al., 2011. Safety of botanical ingredients in personal care products/cosmetics. *Food and Chemical Toxicology,* Volume 49, pp. 324-341.

Baranyi, J. & Roberts, T. A., 1994. A dynamic approach to predicting bacterial growth in food. *International Jounal of Food Microbiology,* pp. 277-294.

Belda-Galbis, C. M., Leufvén, A., Martínez, A. & Rodrigo, D., 2014. Predictive microbiology quantification of the antimicrobial effect of carvacrol. *Journal of Food Engineering,* Volume 141, pp. 37-43.

Berthele, H., Sella, O. & Lavard, M., 2013. Determination of the influence of factors (ethanol, pH and aw) on the preservation of cosmetics using experimental design. *International Journal of Cosmetic Science,* pp. 54-61.

Brannan, D. K., Dille, J. C. & Kaufman, D. J., 1987. Correlation of In Vitro Challenge Testing with Consumer Use Testing for Cosmetic Products. *Applied and Environmental Microbiology,* Volume 53, pp. 1827-1832.

Brul, S., Mensonides, F. I. C., Hellingwerf, K. J. & Teixeira de Mattos, M. J., 2008. Microbial Systems Biology: New frontiers open to predictive microbiology. *International Journal of Food Microbiology,* pp. 16-21.

Campana, R. et al., 2006. Microbiological study of cosmetic products during their use by consumers: health risk and efficacy of preservative systems. *Letters in Applied Microbiology,* Volume 43, pp. 301-306.

CTPA, 2014. *The Cosmetic, Toiletry & Perfumery Association: Key facts about the cosmetic industry.* [Online]
Available at:
http://www.ctpa.org.uk/content.aspx?pageid=295
[Accessed 03 2016].

Dang, T. D. T. et al., 2011. The importance of expressing antimicrobial agents on water basis in growth/no growth interface models: A case study for Zygosaccharomyces bailii. *International Journal of Food Microbiology,* pp. 258-266.

Dang, T. et al., 2010. Modelling the growth/no growth boundary of Zygosaccharomyces bailii in acidic conditions: A contribution to the alternative method to preserve foods without using chemical preservatives. *International Journal of Food Microbiology,* pp. 1-12.

EU, 2002. *Regulation (EC) No 178/2002 of the European Parliament and of the Council: general principles and requirements of food law.* [Online]

Available at: http://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32002R0178&from=EN
[Accessed 03 2016].

EU, 2009. *Regulation (EC) No 1223/2009 of the European Parliament and of the Council.* [Online]
Available at: http://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:02009R1223-20150416&from=EN
[Accessed 03 2016].

EU, 2009. Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. *Official Journal of the European Union,* Volume L 342/59.

EU, 2015. *EU - Agricultural Markets Briefs: Key facts and figures on the food supply chain in the European Union.* [Online]
Available at: http://ec.europa.eu/agriculture/markets-and-prices/market-briefs/pdf/04_en.pdf
[Accessed 03 2016].

Fakruddin, M., Mazumder, R. M. & Mannan, K. S. B. M., 2011. Predictive microbiology: Modeling microbial responses in food. *Ceylon Journal of Science,* Volume 40, pp. 121-131.

FDA, 2015. *Food and Drug Administration: Evaluation and Definition of Potentially Hazardous Foods - Chapter 6. Microbiological Challenge Testing.* [Online]
Available at: http://www.fda.gov/Food/FoodScienceResearch/SafePracticesforFoodProcesses/ucm094154.htm
[Accessed 02 2016].

FDA, 2016. *Food and Drug Administration: Microbiological Safety and Cosmetics.* [Online]
Available at: http://www.fda.gov/Cosmetics/ProductsIngredients/PotentialContaminants/ucm433748.htm
[Accessed 02 2016].

Ferreira, M. et al., 2014. An innovative challenge test for solid cosmetics using freeze-dried microorganisms and electrical methods. *Journal of Microbiological Methods,* Volume 106, pp. 104-109.

Hao Li & Guozhong Xie, 2007. Evolution and limitations of primary mathemtical models in predictive microbiology. *British Food Journal,* pp. 608-626.

Havelaar, A. H., Brul, S., Jong, A. & Jonge, R., 2010. Future challenges to microbial food safety. *International Jounal of Food Microbiology,* pp. 579-594.

Kornacki, J. L., 2010. *Principles of Microbiological Troubleshooting in the Industrial Food Processing Environment.* London: Springer.

Lundov, M. D., Moesby, L., Zachariae, C. & Johansen, J. D., 2009. Contamination versus preservation of cosmetics: a reivew on legislation, usage, infections, and contact allergy. *Contact Dermatitis,* Volume 60, pp. 70-78.

McCann, D. et al., 2007. Food additives and hyperactive behavious in 3-year-old and 8/9-year-old children in the community: a randomised, double-blinded, placebo-controlled trial.. *Lancet,* Volume 370, pp. 1560-1567.

McMeekin, T., Olley, J., Ratkowsky, D. & Ross, T., 2002. Predictive microbiology: towards the interface and beyond. *International Journal of Food Microbiology,* Volume 73, pp. 395-407.

McMeekin, T., Presser, K., Ratkowsky, D. & Ross, T., 2000. Quantifying the hurdle concept by modelling the bacterial growth/no growth interface. *International Journal of Food Microbiology,* Volume 55, pp. 93-98.

McMeekin, T. & Ross, T., 2002. Predictive microbiology: providing a knowledge-based framework for change management. *International Journal of Food Microbiology,* Volume 78, pp. 133-153.

Microchem-Laboratory, 2015. *Introduction to Preservatives and Challenge Testing.* [Online]
Available at: http://microchemlab.com/Information_About_Preservatives
[Accessed 02 2016].

Mishra, M., Muthuprasanna, P., Prabha, K. S. & Rani, P., 2009. Basics and Potential Applications of Surfactants - A Review. *International Journal of PharmTech Research,* pp. 1354-1365.

Pérez-Rodríguez, F. & Valero, A., 2013. *Predictive Microbiology in Foods.* London: Springer.

Pitt, T. et al., 2015. Bacillus cereus in personal care products: risk to consumers. *International Journal of Cosmetic Science,* Volume 37, pp. 165-174.

Prevot, A. B. et al., 2000. Determination of micelle/water partition coefficients of cosmetic preservatives - Optimisation of the capillary electrophoretic method. *Analytica Chimica Acta,* Volume 412, pp. 141-148.

Todar, K., 2008. *Todar's Online Textbook of Bacteriology - Control of Microbial Growth.* [Online]
Available at: http://textbookofbacteriology.net/control_3.html
[Accessed 02 2016].

Varvaresou, A. et al., 2009. Self-preserving cosmetics. *International Journal of Cosmetic Science,* Volume 31, pp. 163-175.

WHO & Food-safety, 2015. *World Health Organization - Fact sheet Nº399 on Food safety - December 2015.* [Online]
Available at: http://www.who.int/mediacentre/factsheets/fs399/en/
[Accessed 02 2016].