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# Rapid assessment and prediction of the efficiency of two preservatives against *S. aureus* in cosmetic products using High Content Screening-Confocal Laser Scanning Microscopy

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1 Rapid assessment and prediction of the efficiency of two preservatives  
2 against *S. aureus* in cosmetic products using High Content Screening  
3 – Confocal Laser Scanning Microscopy  
4

5 **Running title:** Assessment and prediction of preservative efficiency by HCS-CLSM  
6

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17

18 **Keywords:** bacterial inactivation; model; cosmetic matrix; preservative; fluorescence;  
19 CLSM.  
20

21 **Abstract**

22 Most cosmetic products are susceptible to microbiological spoilage due to contaminations that  
23 could happen during fabrication or by consumer's repetitive manipulation. The composition of

24 cosmetic products must guarantee efficient bacterial inactivation all along with the product shelf  
25 life, which is usually assessed by challenge-tests. A challenge-test consists in inoculating  
26 specific bacteria, i.e. *Staphylococcus aureus*, in the formula and then investigating the bacterial  
27 log reduction over time. The main limitation of this method is relative to the time-consuming  
28 protocol, where 30 days are needed to obtain results. In this study, we have proposed a rapid  
29 alternative method coupling High Content Screening - Confocal Laser Scanning Microscopy  
30 (HCS-CLSM), image analysis and modeling. It consists in acquiring real-time *S. aureus*  
31 inactivation kinetics on short-time periods (typically 4h) and in predicting the efficiency of  
32 preservatives on longer scale periods (up to 7 days). The action of two preservatives,  
33 chlorphenesin and benzyl alcohol, was evaluated against *S. aureus* at several concentrations in  
34 a cosmetic matrix. From these datasets, we compared two secondary models to determine the  
35 logarithm reduction time (Dc) for each preservative concentration. Afterwards, we used two  
36 primary inactivation models to predict log reductions for up to 7 days and we compared them  
37 to observed log reductions. The IQ model better fits datasets and the Q value gives information  
38 about the matrix level of interference.

39

## 40 **Introduction**

41 Each year around the world, official authorities in Europe (Rapid Alert System for Non-Food  
42 Products) or USA (US Consumer Product Safety Commission) notify many recalls for cosmetic  
43 products due to microbiological contamination [1-3]. Cosmetic formulas are complex and are  
44 susceptible to microbiological spoilage due to their composition, containing water and nutrients  
45 such as lipids, polysaccharides, proteins...[4]. Contamination of cosmetic products could  
46 happen during their fabrication but also by consumer's repetitive manipulations [5, 6]. The main  
47 pathogens frequently found in cosmetic formulas are *Pseudomonas aeruginosa*, *Escherichia*  
48 *coli*, *Burkholderia cepacia*, *Candida albicans*, *Klebsiella oxytoca*, *Enterobacter gergoviae*,

49 *Serratia marcescens* and *Staphylococcus aureus* [2, 7-9]. *S. aureus* has been found in various  
50 cosmetic products such as shaving cream, moisturizing cream, face care cream and depilatory  
51 cream [10-12]. It is a Gram-positive bacterium present on human skin and mucous membranes  
52 in 30% of the population [13]. Many *S. aureus* strains produce exfoliative toxins secreted on  
53 the skin that cause a wide range of clinical infections, including abscesses, furuncles or impetigo  
54 [14-17].

55 Each cosmetic product has a different level of microbiological risk according to the standard  
56 ISO 29621:2017, which depends on several parameters such as the formula composition  
57 (preservative, ethanol,  $A_w$ , pH) or the type of packaging (unidose, airless pump, pots) [6, 18].  
58 Preservatives that can be used in cosmetic products are listed in Annex V of the European  
59 Regulation No. 1223/2009. Among them are listed chlorphenesin and benzyl alcohol, which  
60 have been tested in this study. Chlorphenesin or 3-(4-chlorophenoxy)-1,2-propanediol is an  
61 antifungal and antibacterial agent (active against both Gram-positive and Gram-negative  
62 bacteria). It can be used at a maximum concentration of 0.32% in rinse-off products and up to  
63 0.30 % in leave-on products [19]. Benzyl alcohol can be used in various cosmetic formulations  
64 as a preservative, but also as a solvent, a fragrance or a viscosity-controlling agent. Its maximum  
65 in-use concentration is 1% [20].

66 The preservation efficiency of a given product is evaluated by proceeding to a challenge-test,  
67 as defined in the European standard EN ISO 11930:2019. During this procedure, specific  
68 microorganisms, including *S. aureus*, are inoculated in the product at a final concentration  
69 between  $1.10^5$  and  $1.10^6$  CFU.ml<sup>-1</sup> for bacteria, and  $1.10^4$  and  $1.10^5$  CFU.ml<sup>-1</sup> for molds or yeast.  
70 The microbial population is evaluated at defined time intervals by enumerating the survivors at  
71 7, 14 and 28 days after inoculation. A preservative system is considered as efficient against  
72 bacteria if the formula composition leads to a bacterial logarithm reduction  $\geq 3$  seven days after  
73 inoculation and without the growth of bacteria after 14 and 28 days. Challenge-test, as described

74 in the European standard involves several steps including sampling, neutralization, serial  
75 dilutions, bacterial plating in duplicate, incubation time and colony counting [21]. The  
76 reliability of challenge-tests depends on several parameters such as the manipulation errors  
77 (pipetting and serial dilutions) [22], the type of plating method (spiral or pour plating), the level  
78 of bacterial enumeration [23], and on the ability of stressed microorganisms to recover and  
79 grow on agar plates [24]. It also relies on the efficiency of the neutralization step which consists  
80 of stopping the antimicrobial activity of preservatives by diluting the surviving population in a  
81 quenching solution [25]. The main limitation of the challenge-test procedure is relative to the  
82 time-consuming protocol (inoculation, sampling, counting) and to the duration of the whole test  
83 process (last sample analyzed on day 28).

84 Confocal Laser Scanning Microscopy (CLSM) allows *in-situ* 3-D visualization of microbial  
85 consortia thanks to various fluorescent markers. It is commonly used to investigate complex  
86 microbial spatial organizations such as biofilms [26], to analyze interactions between bacteria  
87 and oil droplets [27] or to evaluate bacterial distribution in food systems [28-30]. Moreover,  
88 CLSM was previously used to study the spatiotemporal action of biocide in biofilms [26, 31-  
89 34]. This method enables a real-time and *in situ* visualization of the bacterial inactivation  
90 kinetics after biocide addition. Typically, living cells are stained with an esterase viability  
91 marker, such as cFDA or calcein-AM, and after subsequent biocide addition, the fluorescence  
92 is lost due to the leakage of the fluorescent marker out of the cell when the cell membrane is  
93 permeabilized.

94 In this study, we used CLSM and image analysis for acquiring datasets of bacterial inactivation  
95 kinetics upon short periods in model cosmetic matrices containing various concentrations of  
96 preservatives and we accurately predicted the number of bacterial log reductions on longer  
97 periods, which are similar to challenge-test ones.

98

## 99 **Materials and Methods**

### 100 **Chemicals and materials**

101 Cetearyl glucoside and glyceryl stearate were purchased from SEPPIC (Puteaux, France),  
102 carbomer from Gattefossé (Lyon, France), glycerin from Oleon (Ertvelde, Belgium), cetearyl  
103 isononanoate from BASF France (Lyon, France), tocopheryl acetate from DSM (Heerlen, the  
104 Netherlands), tromethamine from Azelis (Heusden, Belgium), chlorphenesin and benzyl  
105 alcohol from Thor (Compiègne, France). Eugon LT 100 supplemented broth was purchased  
106 from Indicia production (Saint Genis l'Argentière, France).

107

### 108 **Bacterial strain and culture conditions**

109 The strain used in this study is *Staphylococcus aureus* CIP 4.83 recommended by the EN ISO  
110 11930:2019 standard for cosmetic-product challenge tests. It was stored in cryovials at -80°C  
111 and resuscitated by two successive subcultures in tryptic soy broth (TSB, Biomérieux, Marcy-  
112 l'étoile, France) before each experiment. Cultures were grown at 30°C until the end of the  
113 exponential growth phase.

114

### 115 **Preparation and characterization of the emulsified model matrix**

116 The aqueous phase was first prepared with 0.25% carbomer in water and heated to 75°C before  
117 glycerin (moisturizer, 9%) was added. The oil phase is composed of 28.8% cetearyl  
118 isononanoate (emollient), 3.5% cetearyl glucoside (emulsifier), 0.2% tocopheryl acetate  
119 (antioxidant), and 2.5% glyceryl stearate (co-emulsifier). It was heated to 75-80°C before it was  
120 blended with the aqueous phase (20/80 o/w %) at 1,800 rpm using a rotor-stator homogenizer  
121 (Rayneri 33/300P, Group VMI) to obtain an emulsion. Benzyl alcohol and chlorphenesin at 7  
122 different concentrations (respectively from 1.00 to 1.85 % and from 0.30 to 0.60 %) were

123 respectively pre-mixed with glycerin or water at 40°C. Tromethamine (base, 0.15%) was finally  
124 added. The viscosity was measured using a penetrometer (PNR10, PetroMesures). A specific  
125 cone was released in 300 g of matrix and the penetration depth measured (in mm  $\pm$  0.1 mm)  
126 after 5 s. The penetrometry measured on each batch in triplicate is 33.06 mm  $\pm$  1.33 mm. The  
127 pH, measured using pH-meter (SI Analytics, Lab 870) on each batch in triplicate, is 5.76  $\pm$  0.03.  
128

## 129 **Bacterial staining and matrix inoculation**

130 Bacterial cells were harvested by centrifugation at 1,575 g for 10 min and washed twice in 150  
131 mM NaCl. The bacterial suspension was calibrated to  $1.10^{10}$  to  $1.10^{11}$  CFU.ml<sup>-1</sup> in 150 mM  
132 NaCl to observe at least 10-100 bacteria per CLSM image (290.6 x 290.6 x 1.6  $\mu\text{m}^3$ ). 300 $\mu\text{l}$  of  
133 bacterial suspension were labeled with 13  $\mu\text{l}$  calcein-AM (53.55 $\mu\text{M}$  in DMSO, Invitrogen by  
134 Thermofisher Scientific), incubated in the dark for 1h30 at 37°C and inoculated in 30 g of model  
135 cosmetic matrix which was vortexed for 30 s. The average of the bacterial concentration in the  
136 matrix is  $1.10^8$  to  $1.10^9$  bacteria/g. Calcein-AM is a viability marker that penetrates passively  
137 into a cell where it is cleaved by cytoplasmic esterases and leads to green fluorescence. Each  
138 experiment was performed respectively two or three times from independent cultures for benzyl  
139 alcohol and chlorphenesin.

140

## 141 **Enumeration of the bacterial population by drop-plate method**

142 For each enumeration, 1 g of inoculated matrix was dispersed in 9 ml of neutralization solution  
143 (Eugon LT 100 supplemented broth). After 30 minutes, the bacterial population is enumerated  
144 by serial dilution in 150 mM NaCl on tryptone soya agar (TSA, Biomérieux) using the drop-  
145 plate method [35]. Plates were incubated at 30°C for 24 to 48 h before counting. Bacterial  
146 enumeration is processed every twenty minutes for four hours after inoculation and then at least

147 once every day until seven days. Each enumeration was performed at least in duplicate.

148

## 149 **Acquisition of bacterial inactivation curves by High Content**

### 150 **Screening - Confocal Laser Scanning Microscopy (HCS-CLSM)**

151 The evolution of bacterial population was acquired upon a short time (typically 4h) for 7  
152 different concentrations in duplicate for benzyl alcohol and in triplicate for chlorphenesin. To  
153 obtain one inactivation curve, the inoculated matrix containing a specific concentration of a  
154 preservative was dropped into several wells of polystyrene 96-well microtiter plates (Greiner  
155 Bio-One, France) and CLSM acquisition was achieved in each well at a specific time to avoid  
156 photobleaching. Thanks to the HCS-CLSM, the stage was programmed to move automatically  
157 to the next well every 15 min during 4h or every hour during 13h for low concentrations.

158 Image acquisition was performed using a Leica SP8 AOBS Confocal Laser Scanning  
159 Microscope (Leica Microsystems, France) at the MIMA2 imaging platform  
160 (<https://doi.org/10.15454/1.5572348210007727E12>). Calcein-AM is excited at 488 nm and the  
161 emitted fluorescence collected in the range 498 to 560 nm. Images size were 290.6 x 290.6 x  
162 1.6  $\mu\text{m}^3$  (512 x 512 pixels) and were acquired at 600 Hz using a 40x air objective (N.A. = 0.85)  
163 and a hybrid detector. The HCS-CLSM control software was programmed to take a mosaic of  
164 10 x 10 images per well, corresponding to a volume of  $1.3 \times 10^{-5}$  ml. The number of bacteria  
165 by mosaic was counted by binarizing each image using the MaxEntropy algorithm in an  
166 automatic macro executed in ImageJ software (National Institutes of Health, USA) [36]. The  
167 obtained number of bacteria per ml was converted per g according to the matrix density (1.15  
168 g/ml). In our experimental conditions, we consider that our threshold value is at 1 bacteria per  
169 image or 100 bacteria per mosaic, which corresponds to  $6.10^6$  bacteria/g.

170

## 171 **Primary model for bacterial inactivation on short times**



172 The log-linear model of Bigelow *et al.* [37], described in equation 1, was used to fit each CLSM  
173 inactivation curve acquired on short-times.

$$174 \log_{10}(N) = \log_{10}(N_0) - \frac{t}{Dc} \quad (1)$$

175 where  $N_0$  is the initial bacterial population,  $N$  is the bacterial population at the sampling time,  
176  $Dc$  is the decimal reduction time and  $t$  is the time (min).

177 The GinaFit freeware add-in for Microsoft Excel was used to fit each curve [39] and to obtain  
178 the  $Dc$  value to which we applied a correction factor to take into account to the correlation  
179 between CLSM enumeration and plate enumeration. Hence, we obtained a dataset of  $Dc$ , each  
180 of them corresponding to a specific concentration of one preservative.

181

## 182 **Secondary model for estimation of the $Dc$ -value according to the** 183 **concentration**

184 From obtained  $Dc$  datasets, the  $Dc$  values were fitted according to concentration using a semi-  
185 log approach, derived from Mafart *et al.* (2001) [40], and expressed in equation 2.

$$186 \log_{10}(Dc) = \log_{10}(Dc^*) - \left(\frac{C-C^*}{z_c}\right)^n \quad (2)$$

187 where  $Dc$  is the decimal reduction time for the concentration  $C$ ,  $Dc^*$  is the decimal reduction  
188 time for the reference concentration  $C^*$ ,  $z_c$  is the increase of concentration which leads to a ten-  
189 fold reduction of the decimal reduction,  $n$  is a shape parameter which can be set to 1 (model #1,  
190 linear model) or 2 (model #2, second-degree model).  $Dc^*$  and  $z_c$  were the estimated parameters.

191 The model parameters were fitted with `nls` R function according to the minimization of the  
192 residual sum of square errors (RSS). Confidence intervals of fitted parameters were assessed  
193 by bootstrap using `nlsBoot` function from `nlsMicrobio` R package [41]. The two models were  
194 compared according to the Bayesian information criterion (BIC) (equation 3). The lower the  
195 BIC, the better the model fits the dataset.

196  $BIC = p \cdot \text{Ln} \left( \frac{RSS}{p} \right) + k \cdot \text{Ln}(p)$  (3)

197 Where  $p$  is the number of experimental points and  $k$  the number of parameters of the model

198

199 **Prediction of the log-reduction of the bacterial population over a**  
200 **period of several days**

201 To predict the log reduction of the bacterial population over several days, we first predict  $D_c$   
202 with the secondary model #2 (equation 2) at some tested concentrations of preservative.

203 Afterwards, two different models were used to predict the inactivation of the bacterial  
204 population as a function of time: the log-linear model (equation 1) and IQ model (equation 4).

205 The intrinsic quenching model (Lambert et al., 2000) [38] was constructed with the hypothesis  
206 that the disinfection concentration decreases during the test period and can be described by the  
207 equation 4.

208  $\log_{10}(N) = \log_{10}(N_0) - \frac{(1-e^{-Q \cdot t})}{Q \cdot D_c}$  (4)

209 where  $N_0$  is the initial bacterial population,  $N$  is the bacterial population at the sampling time,  
210  $D_c$  is the decimal reduction time,  $t$  is the time (min) and  $Q$  is the quenching coefficient.  $Q$  was  
211 the estimated parameter.

212 The logarithm reduction of the bacterial population that should be obtained after a defined time,  
213 from 1 to 7 days was predicted. To optimize and validate the model, a dataset of log-reductions  
214 of the bacterial population was acquired by plate enumeration on the corresponding periods (1  
215 to 7 days) for 7 concentrations of each preservative, as described before. Predicted and observed  
216 log reductions were compared.

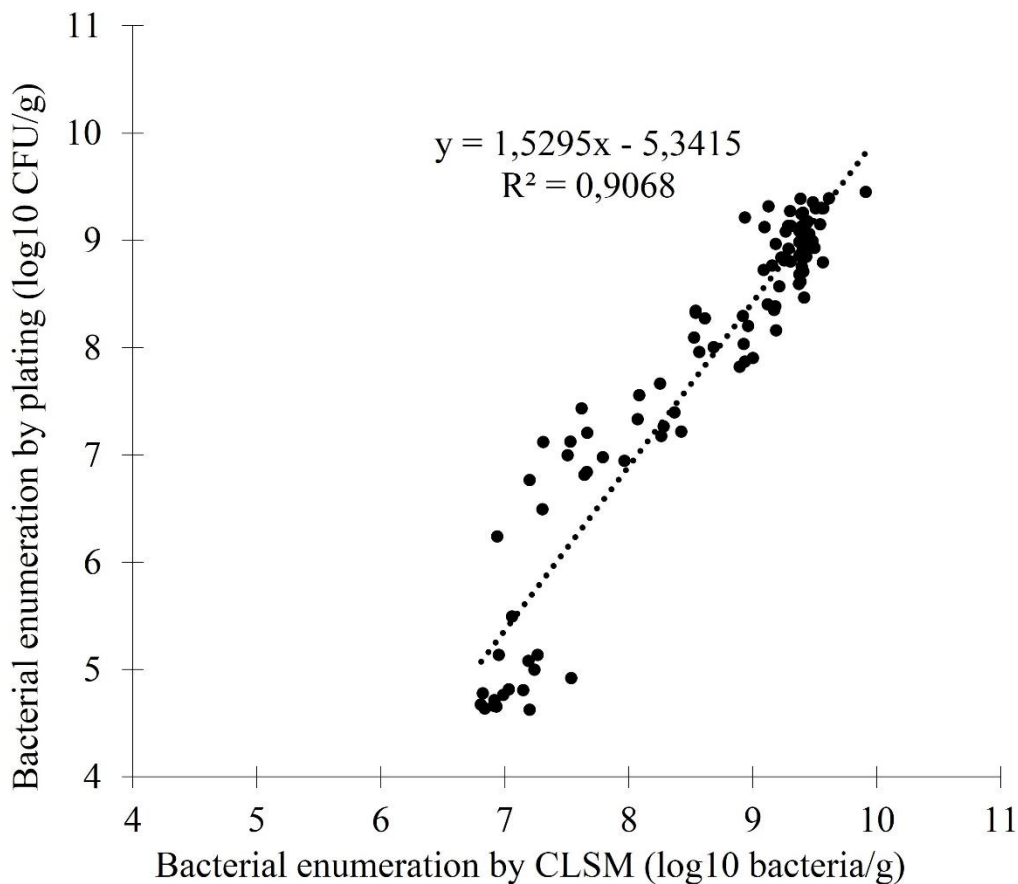
217 The model parameters were fitted with nls R function according to the minimization of the  
218 residual sum of square errors (RSS). Confidence intervals of fitted parameters were assessed  
219 by bootstrap using nlsBoot function from nlsMicrobio R package [41].

220

## 221 **Results**

### 222 **Correlation between enumeration by CLSM and plate counting**

223 Model cosmetic matrices were formulated with different concentrations of chlorphenesin or  
224 benzyl alcohol. Bacterial enumeration of *S. aureus* was achieved at several contact times  
225 (between 10 min and 4h) by both plate counting (log CFU/g) and CLSM enumeration (log  
226 bacteria/g). Fig 1 gives the relationship between both techniques. The relationship between both  
227 techniques is linear ( $y = 1.530x - 5.342$ ;  $R^2 = 0.907$ ) for a level of population over the detection  
228 threshold of the technique ( $6.10^6$  bacteria/g).



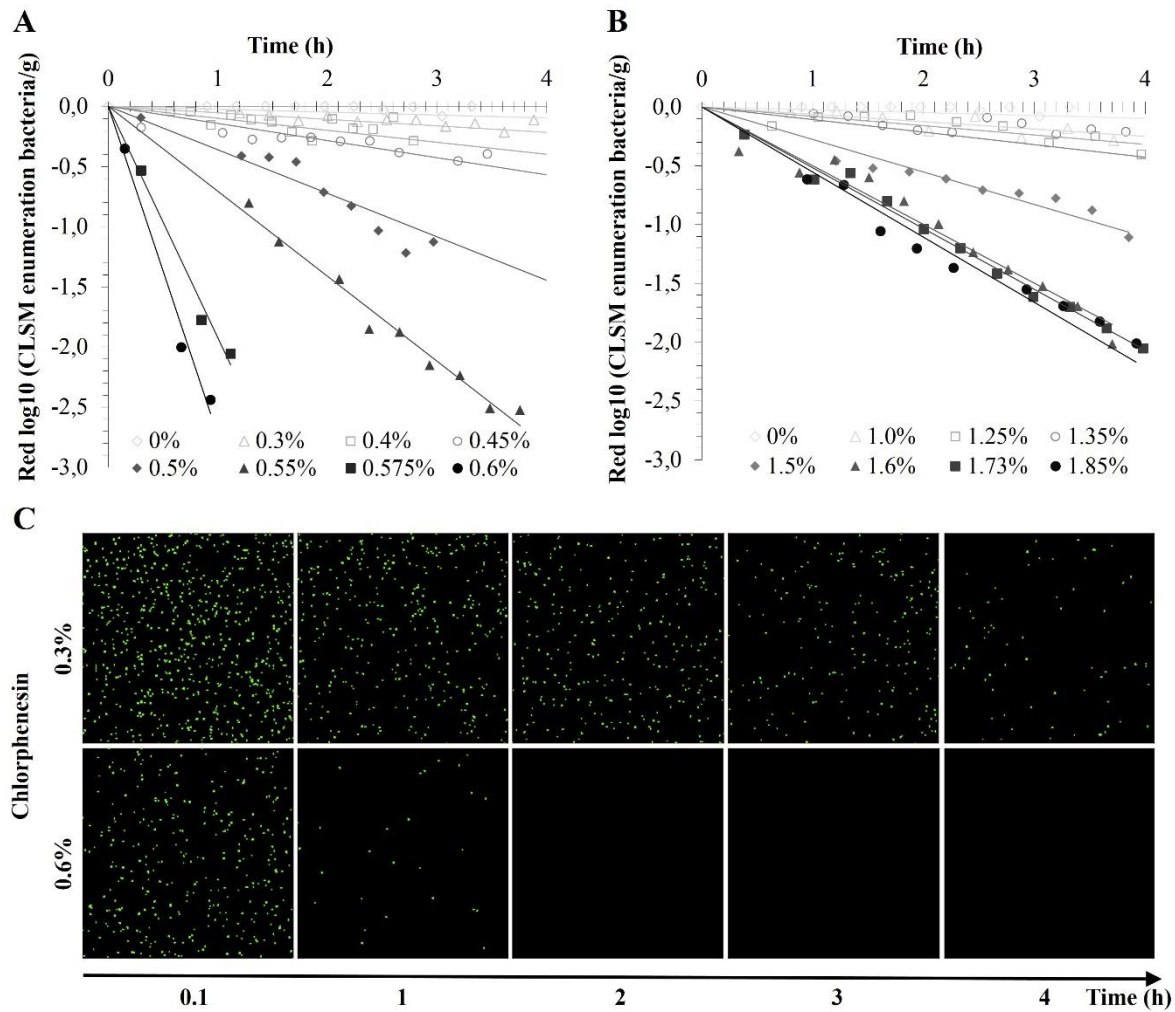
229

230 **Fig 1. Correlation between bacterial enumeration by plating (log<sub>10</sub> CFU/g) and bacterial**  
231 **enumeration by CLSM imaging (log<sub>10</sub> bacteria/g).**

232

## 233 **Inactivation of *S. aureus* according to the concentration of the** 234 **preservative**

235 Fig 2 shows the kinetics of bacterial reduction obtained by CLSM during four hours for seven  
236 different concentrations of chlorphenesin (Fig 2A) or benzyl alcohol (Fig 2B). According to the  
237 correlation between enumeration by CLSM and plate counting (Fig 1), we only took into  
238 account data in the range of population above  $6 \cdot 10^6$  (maximum  $2.5 \log_{10}$  reductions). The higher  
239 the concentration of preservative the higher the slope of inactivation and the lower the Dc. For  
240 chlorphenesin, 0.3% is the smallest concentration for which Dc is measurable ( $17.89 \text{ h} \pm 1.12$ )  
241 on a CLSM kinetics (maximum 17h). For the range between 0.40 and 0.50%, Dc varies  
242 between  $10.05 \text{ h} \pm 0.44$  and  $3.55 \text{ h} \pm 1.04$ . For the range between 0.55 to 0.60%, Dc varies  
243 between  $1.48 \text{ h} \pm 0.10$  and  $0.45 \text{ h} \pm 0.07$ . For benzyl alcohol, Dc for the smallest concentration  
244 1% is  $28.09 \text{ h} \pm 7.50$ . From 1.5% Dc increasingly decreases to reach  $1.07 \text{ h} \pm 0.05$  at 1.85 %.  
245 To obtain similar log reductions of *S. aureus*, the concentrations of benzyl alcohol should be  
246 higher than those of chlorphenesin. For example, we obtained one log reduction in  $0.45 \text{ h} \pm$   
247  $0.07$  with 0.6% chlorphenesin whereas  $1.07 \text{ h} \pm 0.05$  is necessary with 1.85% benzyl alcohol.  
248 Fig 2C illustrates the loss of fluorescence of *S. aureus* in a model matrix with 0.3% and 0.6%  
249 chlorphenesin over time. With 0.3% chlorphenesin, the number of fluorescent bacteria  
250 decreases very slowly over time. At 4 h, the slight decrease of fluorescent bacteria number  
251 corresponds to a bacterial reduction of about 0.2 log bacteria/g (Fig 2A). In contrast, with 0.6%  
252 chlorphenesin, the fluorescent bacteria number decreased rapidly in 1h which corresponds to a  
253 reduction of  $5 \cdot 10^2$  bacteria/g (Fig 2A). After 2h, no bacteria were visible anymore.



254

255 **Fig 2. *S. aureus* inactivation kinetics obtained by HCS-CLSM in cosmetic model matrices**  
 256 **with several concentrations of chlorphenesin (A) and benzyl alcohol (B). Example of the**  
 257 **loss of bacterial fluorescence assessed by HCS-CLSM over time for two concentrations of**  
 258 **chlorphenesin (C).**

259

260 **Estimation of Dc value according to the preservative concentration**

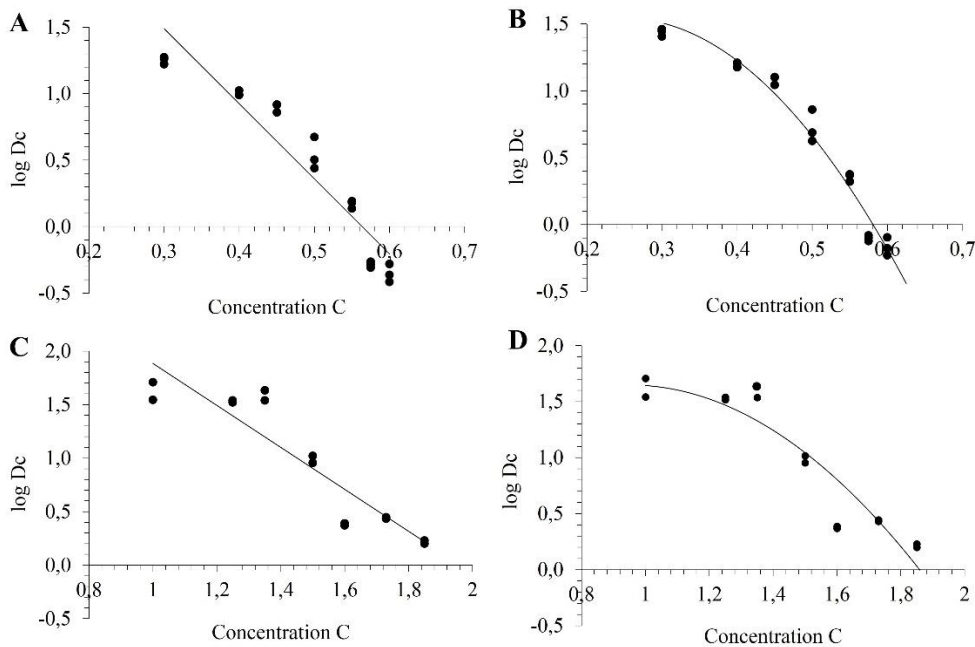
261 Semi-log models were used to fit datasets of Dc values upon the preservative concentration.  
 262 The shape parameter  $n$  was set at 1 in model #1 (linear-model, Fig 3 A and C) and set at 2 in  
 263 model#2 (second-degree model, Fig 3 B and D). Model parameters of the two models, Dc\* and  
 264  $z_c$ , are given in Table 1 together with the RSS and BIC for both preservatives. Second-degree

265 model allows the lowest BIC for both preservatives, meaning that the shape parameter is  
 266 significant. Accordingly to the BIC, model #1 does not fit well and was not used for the  
 267 following prediction.

268

269 **Table 1. Estimated parameters (and their 95% CI intervals) and performance criteria of**  
 270 **both secondary models.**

	Chlorphenesin		Benzyl alcohol	
	<i>model#1</i>	<i>model#2</i>	<i>model#1</i>	<i>model#2</i>
<b>n</b>	1	2	1	2
<b>Number of data</b>	21	21	14	14
<b><i>C*</i></b>	0.25	0.25	0.95	0.95
<b>log (<i>Dc*</i>)</b>	1.96 [1.76-2.16]	1.54 [1.47 – 1.62]	1.98 [1.70 – 2.22]	1.65 [1.44 – 1.78]
<b><i>z<sub>c</sub></i></b>	0.18 [0.15-0.21]	0.27 [0.26 – 0.28]	0.51 [0.41 – 0.65]	0.71 [0.64 – 0.79]
<b><i>RSS</i></b>	0.76	0.20	0.72	0.60
<b><i>BIC</i></b>	-63.49	-91.48	-36.22	-38.86



271

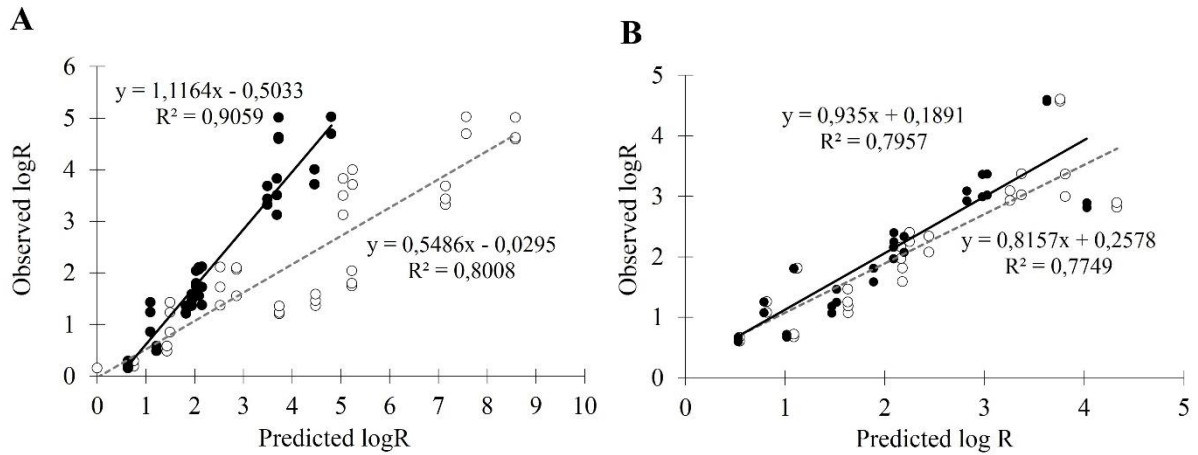
272 **Fig 3. Relation between the Dc value and the concentration of chlorphenesin (A, B) and**  
 273 **benzyl alcohol (C, D) by fitting of model#1 (A and C) and model#2 (B and D).**

274

## 275 **Prediction of bacterial log reduction on long periods**

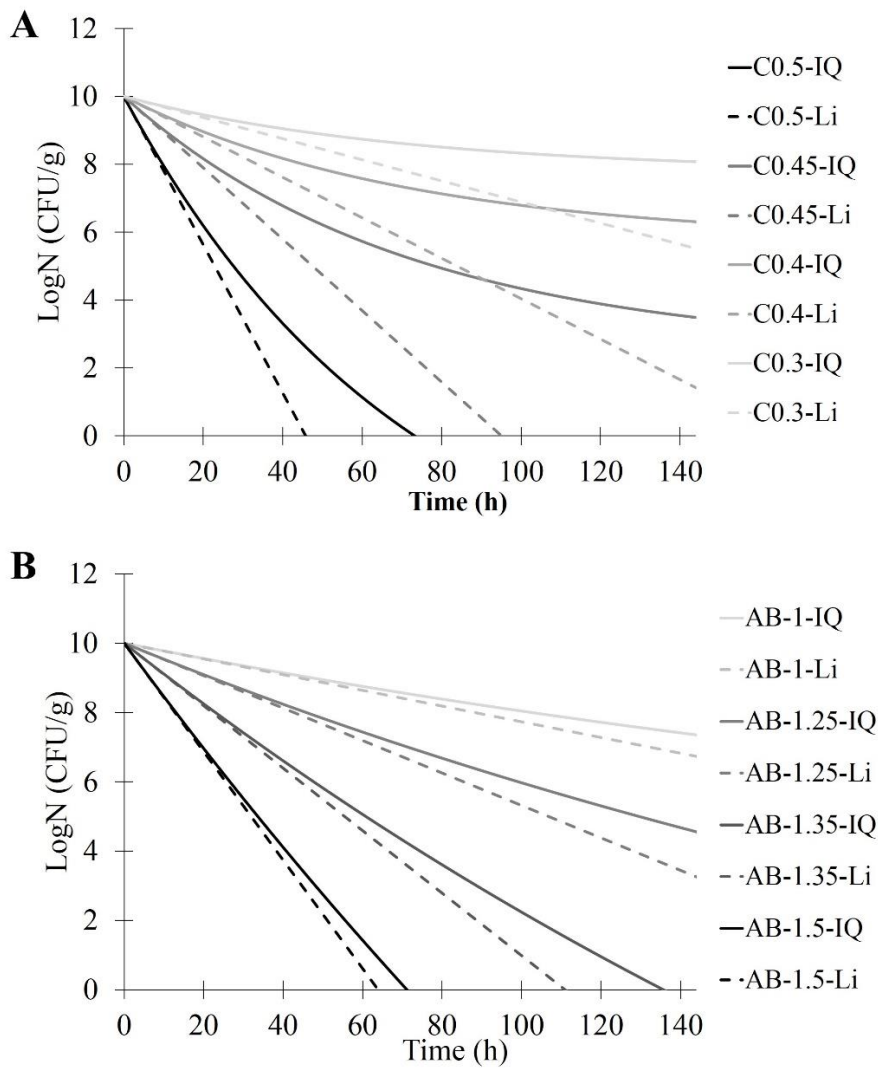
276 Dc values at specific concentrations were first estimated from model #2. The logarithm  
277 reduction of the bacterial population was then calculated for specific times (from 1 to 7 days)  
278 using the Bigelow linear-model (equation 1) or the IQ model (equation 4). Fig 4 presents the  
279 relationships between predicted and experimental bacterial reductions. For both preservatives,  
280 the best combination is obtained when using IQ model for log-reduction estimation. For  
281 chlorphenesin (Fig 4A), the IQ model prediction for log-reduction datasets is far better than the  
282 linear model. The Q coefficient could be optimized at 0.0141 (CI 95% 0.0124 – 0.0156) and  
283 the slope of regression curve is 1.12 ( $R^2=0.906$ ). Linear model is less relevant with a slope  
284 around 0.55 and lower  $R^2$ . For benzyl alcohol (Fig 4 B), predictions with both models are less  
285 different than for chlorphenesin. The Q coefficient is optimized at 0.0043 (CI 95% 0.0022 –  
286 0.0076) and the slope of the regression curve with the IQ model is 0.93 ( $R^2=0.796$ ). For both  
287 preservatives, one could note that the prediction is relevant only for maximum 5 log-reductions  
288 because of the initial level of contamination and the experimental protocol used to obtain the  
289 observed datasets. Fig 5 shows the prediction of the evolution of bacterial enumerations over  
290 seven days for 4 tested concentrations of chlorphenesin (Fig 5A) and benzyl alcohol (Fig 5B)  
291 with Bigelow linear-model (dotted lines) or IQ model (plain lines). These curves could be  
292 generated for any concentrations in the range of 0.3 to 0.6 % of chlorphenesin and 1 to 1.9 %  
293 for alcohol benzyl from model #2 and IQ model with respective Q to 0.014 for chlorphenesin  
294 and 0.003 for benzyl alcohol.

295



296

297 **Fig 4. Correlation between the observed bacterial log-reductions and the predicted ones**  
 298 **using Bigelow linear-model (white dots) or IQ model (black dots) for chlorphenesin (A)**  
 299 **and benzyl alcohol (B).**



300



301 **Fig 5. Illustration of the possible prediction of the evolution of the bacterial population**  
302 **over seven days for four concentrations of chlorphenesin (A) or benzyl alcohol (B) with**  
303 **Bigelow linear-model (dotted lines) or IQ model (plain lines).**

304

## 305 **Discussion**

306 Challenge-tests are necessary to assess the efficiency of preservation in cosmetic products.  
307 Nevertheless, the procedure of the challenge-test is time-consuming due to the numerous  
308 enumerations by plate-counting necessary and to the results that are available only 48h after the  
309 last assessment point (day 28). By consequence, the challenge-test method lacks reactivity and  
310 flexibility for optimizing the preservation of a formula. In this study, we propose a new  
311 alternative method allowing the prediction of the log reduction of a bacterial population in long-  
312 term preservation by acquiring data on short-time periods. This method relies on the acquisition  
313 of CLSM kinetics of bacterial inactivation in the presence of several concentrations of  
314 preservatives during short times: acquisition during 4h is generally enough to evaluate  $D_c$  but  
315 13h could be necessary for very low concentrations. Bacteria are first stained with a viability  
316 fluorescent marker, calcein-AM. This marker is widely used to assess bacterial viability by  
317 CLSM or by flow cytometry [31, 42]. Its precursor diffuses passively into the cytoplasm, where  
318 it is cleaved by intracellular esterases into green-fluorescent calcein [43]. This non-permeant  
319 fluorescent dye is released out of the cell when the membrane is permeabilized (dead cell).  
320 We have first shown that the enumeration obtained by CSLM and dedicated image analysis can  
321 be correlated to bacterial plate-counting during the action (10 min to 4h) of the preservative  
322 (chlorphenesin or benzyl alcohol at specific concentrations). Our detection threshold by CLSM  
323 imaging is  $6.10^6$  bacteria/g which is lower than the one obtained by CLSM by Auty *et al.* [29]

324 (1.10<sup>8</sup> bacteria/ml). This is probably due to the observed surface which was enlarged to a mosaic  
325 of 100 CLSM images. Here, bacterial CLSM enumeration is always higher (between 0.5 and 1  
326 log) than the enumeration by plate counting. Auty *et al.* [29] also compared enumeration by  
327 CLSM and plate counting before they assess the viability of human probiotic strains in dairy  
328 products. They used Live/Dead BacLight marker and also underlined an overestimation of the  
329 CLSM enumeration of about 1 log. They suggested that this might be due to the bacterial  
330 clumping on plates. Indeed, the accuracy of enumeration by plate counting is usually estimated  
331 in the range of 0.3 to 0.7 log [23, 44]. However, we can notice that the difference between both  
332 techniques increases when enumeration decreases. Lower enumerations correspond to bacterial  
333 populations that remain alive after action of the preservative. Among this persistent population,  
334 a high fraction of bacteria is under stress which could explain why this fraction could not have  
335 the ability to recover and grow on agar plates while it is still stained by the viability marker by  
336 CLSM [24].

337 We used CLSM enumeration technique to follow the action of two preservatives at different  
338 concentrations in model cosmetic matrices. The bacterial inactivation kinetics was assessed by  
339 acquiring the calcein-AM loss of fluorescence over a few hours. These acquisitions were only  
340 possible thanks to the HCS-module of the CLSM. The automated high content screening (HCS)  
341 system is an emerging software solution that allows a CLSM to acquire automatically high  
342 content images for analysis of numerous samples, thanks to an automatically xyz-positioning  
343 in multiple wells as a function of time [45]. Automatic movements from well to well allow to  
344 acquire images for the same sample over time while avoiding photobleaching by enlightening  
345 each well only once. Moreover, we can also investigate several preservative concentrations over  
346 the same time lapse.

347 The CLSM method used during this study is very well suited to evaluate the efficiency of  
348 preservatives that cause membrane permeabilization. Chlorphenesin is a phenol ether with a

349 chlorine atom and it belongs to the class of organo-halogen organic compounds. Phenols disrupt  
350 the cytoplasmic membrane and induce leakage of potassium ions of the cytosol. Their  
351 halogenation is known to improve their antibacterial activity [46]. Benzyl alcohol is an organic  
352 aromatic alcohol. Alcohols are known to damage cell membranes and denature bacterial  
353 proteins that are essential to the cell metabolism which leads to the cell lysis [46].

354 Chlorphenesin seems to be more effective than benzyl alcohol against *S. aureus* in the model  
355 cosmetic matrix. We observed that obtaining the same logarithm reduction needs lower  
356 concentrations of chlorphenesin than benzyl alcohol. According to the literature, the partition  
357 coefficient (logP) can be a parameter influencing bacterial inactivation [47]. The higher the  
358 logP the higher the antibacterial activity. Chlorphenesin could have a better ability than benzyl  
359 alcohol to intercalate into the bacterial membrane of *S. aureus* because its logP is higher (1.713)  
360 than the one of benzyl alcohol (1.100) [48, 49].

361 In this study, we were able to predict the number of log reduction at any time for one  
362 preservative at any concentration in a specific range from inactivation datasets obtained over  
363 short-term times. We fitted the datasets with two models describing the effect of the  
364 concentration on the log reduction time. These models derived from Mafart models [40] can  
365 take several forms by setting the shape parameter at 1 (linear model#1) or 2 (second-degree  
366 model#2). Mafart *et al.* (2001) compared these two first semi-log models for describing the  
367 effect of pH on the heat resistance of spores (reduction time  $D_T$ ) and showed that second-degree  
368 model presents a better safety than the linear one. From our side, we used the BIC calculation  
369 to choose the most relevant model while adjusting the minimum number of parameters. BICs  
370 of models#2 are better than model#1 for both chlorphenesin and benzyl alcohol (Table 1). This  
371 indicates that the preservative concentration and the contact time do not have a similar impact  
372 on the reduction time. As noticed by Mafart *et al.* (2001) for the effect of pH on the resistance  
373 of spores, we can hypothesize that the relationship between  $D_c$  and the preservative

374 concentration is more complex than that of the effect of temperature on heat resistance. Hence,  
375 the linear model was discarded from the following prediction.

376 The next step was to predict, from the Dc values calculated with model#2, the log reduction of  
377 the bacterial population on longer times (up to seven days) using two primary models, the  
378 Bigelow linear model and the IQ model. The Q coefficient is the characteristic parameter of the  
379 IQ model which indicates the level of quenching of the preservative in the matrix (Lambert et  
380 al 2000). Below 0.005, which appears to be the case of benzyl alcohol, the level of quenching  
381 is very low and the inactivation curves are quite similar to linear log-survivor curves. On the  
382 contrary, the Q coefficient for chlorphenesin is 0.014 which indicates a quenching of the  
383 preservative in the matrix. The level of quenching increases over time as it is demonstrated by  
384 the comparison between the predictions of linear-model and IQ models (Fig 5). As it is not  
385 similar for both preservatives, we can hypothesize that it is influenced by the interactions  
386 between the antimicrobial and the matrix. As the model cosmetic matrix used here is an  
387 emulsion, we can hypothesize that chlorphenesin which has a higher log P (1.713) than benzyl  
388 alcohol (1.100) could progressively partition into the hydrophobic droplets, thus losing its  
389 preservative efficiency. Pernin et al. (2019) studied the antimicrobial activity of two natural  
390 phenolic compounds, ferulic acid and eugenol, against *Listeria monocytogenes* in a model oil-  
391 in-water emulsion. They showed that eugenol, which has the highest logP, loses its antibacterial  
392 efficacy in emulsified systems, in contrast of ferulic acid. The authors suggest that once in the  
393 emulsion, the more hydrophobic antimicrobial agent would preferentially partition in the lipid  
394 droplets and thus the remaining concentration in the aqueous phase would not be able to inhibit  
395 microorganisms [50]. Polarity, antimicrobial charge, and environmental conditions such as  
396 temperature, ionic strength, and pH can also play a major role in the effectiveness of an  
397 antimicrobial [51]. Electrostatic and hydrophobic interactions between antimicrobials and the  
398 matrix constituents, such as lipids, proteins and charged polysaccharides, could interfere with

399 the antimicrobial activity [51]. For example, the addition of bovine meat proteins decreases the  
400 antimicrobial activity of phenolic compounds [52, 53]. Some gelling agents, such as  
401 hydroxypropylmethylcellulose, may be associated with the loss of effectiveness of  
402 preservatives [54]. Emulsifiers could also participate in the reduction of antimicrobial activity  
403 by sequestering antimicrobial molecules in micelles [50, 55, 56].

404 Nevertheless, from the estimations of  $D_c$  with model#2 and then of the log-survivors from IQ  
405 model, we propose here a method of prediction of the efficiency of two preservatives. The log-  
406 reduction of *S. aureus* population could be estimated at any concentration and after any time in  
407 a period of a few days for both tested preservatives.

408 This prediction is matrix- and preservative- dependent. The Q parameter is a characteristic of  
409 the interactions between them. This method should be challenged for many other couples of  
410 preservatives and matrices before it can be used for industrial prediction purposes. Moreover,  
411 some other microorganisms should be tested besides *S. aureus*, i.e. environmental strains  
412 isolated from contaminated cosmetic products. Calcein-AM is relevant for many bacteria  
413 including some Gram negative ones such as *Salmonella* [57]. However, it doesn't work for  
414 some species including *Escherichia coli* [58]. Indeed, some strains, such as *Pseudomonas*  
415 *aeruginosa*, have efflux pumps that release the fluorescence outside the alive bacteria and  
416 prevent cell visualization [59]. To limit these pump interferences, it was suggested to add  
417 sodium azide in the staining solution [59], as used for the observation of biofilms [32].  
418 Unfortunately, we cannot add this molecule in cosmetic matrices because it could modify the  
419 structure and composition of the formula. Hence, other impermeant fluorescent dyes should be  
420 evaluated.

421

## 422 **Conclusions**

423 In this paper, we propose a rapid HCS-CLSM method associated with modeling to predict the

424 preservative efficacy in a cosmetic matrix. This method could provide a quick evaluation of  
425 preservative efficiency and save a lot of time by replacing many microbiological analyses. It  
426 could be beneficially used for screening preservatives or for optimizing the formulation of a  
427 cosmetic product. Nevertheless this model has to be challenged in the future and adapted for  
428 several bacterial species, preservatives and matrices.

429

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436

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