Survival and detection of Bacillus cereus in the presence of Escherichia coli, Salmonella enteritidis, Staphylococcus aureus, Pseudomonas aeruginosa and Candida albicans after rechallenge in make-up removers

N. Yossa*,1, G. Arce*,1, J. Smiley*,1, M.-C. Jo Huang1, L. Yin1, R. Bell1, S. Tallent1, E. Brown1 and T. Hammack1
1Oak Ridge Institute for Science and Education, Oak Ridge, 1299 Bethel Valley Rd Oak Ridge, TN 37830, USA; 2U.S. Food and Drug Administration, Office of Regulatory Science, 5001 Campus Dr, College Park, MD 20740, USA; 3U.S. Food and Drug Administration, Office of Cosmetics and Colors, 5001 Campus Dr, College Park, MD 20740, USA and 4U.S. Food and Drug Administration, Office of Analytics and Outreach, 5001 Campus Dr, College Park, MD 20740, USA

Received 5 July 2017, Accepted 6 October 2017

Keywords: Bacillus cereus, cosmetics, mixed culture, rechallenges

Abstract

OBJECTIVE: Pathogenic contamination of cosmetics intended to be applied on or around the eye area, including make-up removers, may lead to severe eye infections. To assess the efficacy of antimicrobial preservatives in these products, we investigated the survival and detection of Bacillus cereus F 4227A spiked into make-up removers, alone and in the presence of other relevant microorganisms.

RESULTS: The population of B. cereus spiked as a pure culture increased significantly from the first to the third challenge after 30 min exposure time, going from 0.73 to 2.59 in A, from 0.80 to 2.69 in B and from 0.80 to 1.67 log CFU per mL in C (P < 0.05), Likewise, the B. cereus population from the mixed cultures had a significantly higher survival count at the third challenge: from 0.12 log MPN per mL to 2.16 log CFU per mL in A, 0.57 to 2.27 log CFU per mL in B and from undetected (LOD = 0.48 log MPN) to 0.98 log CFU per mL in C, respectively. After challenges, Staph. aureus, C. albicans and P. aeruginosa increased in B; Staph. aureus and C. albicans in C; and E. coli and Staph. aureus in D. The growth of other bacteria types was unaffected by the number of challenges, but B. cereus population was detected with the third challenge.

CONCLUSION: It is appropriate to assess the antimicrobial efficacy of preservatives using at least three challenges, especially for cosmetics that are subjected to repetitive contamination by users.

Résumé

OBJECTIF: La contamination par des bactéries pathogènes des cosmétiques destinés à être appliqués sur ou autour du contour des yeux, y compris les démaquillants, peut entraîner des infections oculaires graves. Pour évaluer l’efficacité des agents de conservation antimicrobiens dans ces produits, nous avons étudié la survie et la détection de Bacillus cereus F 4227A stimulé, dans des démaquillants, seul et en présence d’autres micro-organismes pertinents.

MÉTHODES: Quatre marques de démaquillants A, B, C et D, ont été testées trois fois (jour 0, jour 7 et jour 14) en utilisant B. cereus, en cultures pures et mixtes, à une concentration finale de 5 log CFU mL⁻¹ pour Bacillus cereus ou 6 log CFU mL⁻¹ pour les autres micro-organismes. Les échantillons inoculés ont été dilués et ensemencés en spirale après 30 min et 24 h pour chaque challenge sur des milieux sélectionnés pour la récupération des micro-organismes survivants: BACARA (B. cereus), MacConkey (E. coli), ChromID (P. aeruginosa), XLT4 (S. enteritidis), Baird Parker agar (Staph. aureus) et FDA-chlortetracycline HCL (C. albicans).

RÉSULTATS: La population de B. cereus, stimulé en culture pure, augmente significativement du premier au troisième test après 30 minutes de temps de contact, passant de 0.73 à 2.59 pour le produit A, de 0.80 à 2.69 pour le produit B et de 0.80 à 1.67 log CFU mL⁻¹ pour le produit C (P < 0.05). De même, la population de B. cereus provenant des cultures mixtes avait un taux de survie significativement plus élevé au troisième test: de 0.12 log MPN mL⁻¹ à 2.16 log UFC mL⁻¹ pour le produit A, de 0.57 à 2.27 pour le produit B, et non détecté (LOD = 0.48 log MPN) à 0.98 log CFU mL⁻¹ pour le produit C, respectivement. Après les tests, Staph. aureus, C. albicans et P. aeruginosa ont été augmentés dans B; Staph. aureus et C. albicans en C; et E. coli et Staph. aureus dans D. La croissance des autres types de bactéries n’a pas été affectée par le nombre de tests, mais la population de B. cereus a été détectée avec le troisième challenge.
Detection of *Bacillus cereus* after rechallenges

**Introduction**

Make-up removers and other cosmetic products may contain preservatives meant to prevent or control the growth of microorganisms. Despite this, cosmetics may become contaminated due to a variety of factors, including inadequate preservation, use of ingredients that favour growth of micro-organisms or consumer practices, such as the long-term repeated use of a product or the use of a contaminated cosmetic applicator (cotton pad, cotton swab, etc.). The most prevalent organisms found in contaminated products are *Pseudomonas* species, but mesophilic *Staphylococcus aureus*, *Burkholderia cepacia*, *Klebsiella pneumonia*, *Enterobacter gergoviae*, *Bacillus cereus* and yeasts and moulds have also been identified [1, 2]. Of these, *B. cereus* has been associated with clinical infections including post-traumatic endophthalmitis and eye infections, which can result in blindness [3], making it important to control this type of contamination.

Relatively, little research has examined the usefulness of multiple challenges for assessing preservative adequacy, in particular, identifying marginally preserved cosmetics: preservative system failure was 50% after a single challenge, 80% after two challenges and 90% after three challenges [4]. As pathogens should be inactivated completely in 24 h in multiple-use products intended for application in and around the eye, and for baby products [5], multichallenge tests could be a good indicator of the adequate product preservation, and therefore merit further exploration.

This study evaluates the preservative capacity – defined as the ability or power of a product to consistently maintain low and acceptable levels of microbes in response to microbial challenges [6] – of four brands of make-up removers in response to three spiking challenges, using pure cultures of *B. cereus* and mixtures *B. cereus* with other relevant organisms: *Staph. aureus*, *P. aeruginosa*, *E. coli*, *S. enteritidis* and *C. albicans*. Each of the four make-up removers assessed used several different preservatives, and appropriate selective media were used for the detection of each spiked microbe.

| Product A (pH 4.93) | Product B (pH 6.87) | Product C (pH 5.55) | Product D (pH 4.52) |
|---------------------|---------------------|---------------------|---------------------|
| Citrimonium bromide | Cranberry-derived sodium benzoate | Polyaminopropyl biguanide | Methyl paraben |
| Citric acid         | Sorbapple-derived potassium sorbate | Citric acid           | Phenoxethanol |
|                     | Sodium benzoate      | Sodium benzoate      |                     |
|                     | Potassium sorbate    | Potassium sorbate    |                     |

Data represent mean, n = 3. Values without (*) are direct plate counts in log CFU per mL. *Data represent counts in log MPN per mL (MPN performed on the samples that presented no viable cell counts on direct plating).*

**Materials and methods**

**Cosmetic samples**

Four brands of make-up removers (coded A, B, C and D) were randomly purchased from online sources in the United States. The preservatives found in these products are listed in Table I. The concentrations of those preservatives were not indicated on the product labels.

**Selection of the test organisms**

We selected prevalent organisms frequently associated with cosmetic contamination – two Gram-positive bacteria: *B. cereus* and *Staph. aureus*; three Gram-negative bacteria: *E. coli*, *P. aeruginosa* and *S. enteritidis*; and one yeast: *C. albicans*. Sources of these strains are as follows: *B. cereus* F 4227A, used in a previous study and resistant to parabens [7], was provided by Dr. Sandra Tallent. A wild-type K-12 strain of *E. coli* MG1655 was provided by Dr. Rachel Binet and *S. enteritidis* 5835 by Mr. Jean Taylor. Three strains, *P. aeruginosa* ATCC 27853, *Staph. aureus* ATCC 21293 and *C. albicans* ATCC 20308, were purchased from American Type Culture Collections (ATCC) (Manassas, VA). All cultures were maintained at −80°C in 20% glycerol: each strain was aseptically subcultured in nutrient broth (NB) (Difco™, Franklin Lakes, NJ) for 24 h at 30°C and stored at 4°C.

**Inoculum preparation**

Prior to our experiments, cultures were revived and grown in nutrient broth (NB) (Difco™, Sparks, MD) and incubated at 30°C or 35°C for 24 h. Fresh cultures were centrifuged (7500g, 10 min, 4°C) and washed in sterile phosphate-buffered saline (PBS) (Difco™, Sparks, MD). The cell pellets were resuspended in PBS, and the cell density of each strain was adjusted to obtain a final concentration of ~7 log CFU per mL for *Bacillus* and 8 log CFU per mL for the other organisms. The concentrations of all strains and of a mixture containing 1 mL of each strain were verified on Tryptic Soy Agar (TSA) (Difco™, Sparks, MD) by spiral plating. The four make-up remover products were inoculated with the strains as indicated in Fig. 1.

**Multiple challenge tests for preservative capacity**

The preservative capacity of each product was challenged using three (3) inoculations (Fig. 1), as follows: On day 0, we placed 100 mL of a given make-up remover into a 500-mL sterile container and artificially inoculated each sample with either 1 mL of 7 log CFU per mL of vegetative cells of *B. cereus* only or with the same amount of *B. cereus* vegetative cells in the presence of 1 mL of 8 log CFU per mL of each culture, *Staph. aureus*, *E. coli*, *S. enteritidis*, *P. aeruginosa* and *C. albicans*. These samples were re inoculated with the same amount of the same organisms on days 7 and 14. Uninoculated samples of each type of make-up remover were served as negative controls. After each inoculation, the samples were maintained at room temperature and analysed for microbial survival at two time points: 30 min and 24 h.

**Detection and microbial evaluation**

The FDA BAM cosmetic method for liquid products was used for direct plating enumeration [8]. Briefly, 1 mL from each of the inoculated samples was diluted into 9 mL MLB. Serial dilutions
were performed and plated onto appropriate agars for each organism using the easySpiral® spiral-plating system (Inter-
science, Rockland, MA). Bacillus cereus populations were plated onto two FDA BAM reference plates, B. cereus rapid agar
BACARA® (bioMerieux, Durham, NC) and mannitol-egg yolk-poly
myxin plates (MYP) (Difco™, Sparks, MD); Staph. aureus popu
lations were plated onto Baird Parker plates (Difco™, Sparks,
MD); E. coli onto MacConkey (Difco™, Sparks, MD); P. aeruginos
a onto ChromID plates (bioMerieux, Durham, NC); S. en-
teritidis onto XLT4 plates (Difco™, Sparks, MD); and C. albicans
onto PDA + chlorotetracycline HCL (Difco™, Sparks, MD). The BACARA
and MYP plates were incubated at 30 ± 2°C for 24 h. The plates for other bacteria were incubated at 35°C for 24 h, except
for the plates of C. albicans, which were incubated at 25 ± 2°C
for at least 5 days. Unincubated samples were used as negative
controls. Direct plating was performed to quantify the number of
viable cells present in the samples, and the most probable number
(MPN) method was performed to determine whether there were
surviving cells of Bacillus in the samples.

Colonies of B. cereus were manually enumerated, whereas the
other microbes were enumerated using the Automatic HD colony
counter Scan® 1200 (Interscience, Rockland, MA).

For plates on which no growth was seen after direct plating,
MPN technique was used to enumerate B. cereus, using a six
dilution scheme in 96-deep-well plates (Fisher, Thermo Scientific
Inc., Pittsburgh, PA). Inoculated samples were 10-fold diluted in
the wells with MLB. These plates were incubated at 30°C for
24 h, and then samples were spot-plated on BACARA and MYP.
Colonies with Bacillus characteristics were reported as positive
and enumerated by the MPN calculator.

Statistical analysis
We repeated the experiment in triplicate. The concentration of the
cells was first expressed as CFU per mL and then converted to log
CFU per mL values to determine the average and standard error.
These data were analysed by repeated measures ANOVA using the
SAS 9.4 mixed procedure (SAS, Cary, NC). Differences of P < 0.05
were accepted as statistically significant.

Results
The counts of B. cereus obtained from plating on BACARA were
not significantly different from those obtained from the MYP plates.
Detection of *Bacillus cereus* after rechallenges

N. Yossa et al.

Enumeration of a pure culture of *B. cereus* in make-up removers

Pure cultures of vegetative *Bacillus* cells, at a final concentration of 5 log CFU per mL, were repeatedly challenged in Products A, B, C and D at days 0, 7 and 14 and analysed after 30-min and 24-h exposure time. Results of enumerations are displayed in Fig. 2.

Product A *Bacillus* cells were enumerated at 0.55, 0.57 and 2.59 log CFU per mL after 30 min of exposure time at days 0, 7 and 14 (equivalent to the first, second and third challenges), respectively. After 30-min exposure time, the reduction was approximately 4 log CFU per mL after the 1st and 2nd challenges, and down to 2 log CFU per mL after the 3rd challenge. After 24-h exposure time of the 1st challenge, the remaining population was significantly reduced to a concentration of 0.18 log MPN per mL, while at day 8 (i.e. 24 h after the second challenge), the surviving cells were 0.57 log CFU per mL, and at day 15 (i.e. 24 h after the third challenge), the cells were 1.73 log CFU per mL.

Product B *B. cereus* enumeration was 0.60, 0.67 and 2.69 log CFU per mL after 30 min at day 0, 7 and day 14, respectively. A decrease of approximately 4 log CFU per mL in the number of cells was observed after the 1st and 2nd challenges, and of 2 log CFU per mL after the 3rd challenge. The population at days 1 and 8 remained the same, having a density of 0.57 log CFU per mL, and was 2.32 log CFU per mL at day 15.

Product C The detection of the cells was 0.8, 0.57 and 1.66 log CFU per mL 30 min after each challenge, and 1.13, under the limit of detection (DL 0.08 log MPN per mL) and 1.12 log CFU per mL after days 1, 8 and 15, respectively. The *B. cereus* population decreased approximately 4 log CFU per mL after 30 min of the 1st and 2nd challenges, and 3 log after the 3rd challenge.

Table II Survival and growth of test strains after three repetitive challenges in Product A

| Bacteria       | Challenge | Day    | CFU per mL or MPN* per mL | P value |
|----------------|-----------|--------|---------------------------|---------|
| *B. cereus* F 4227A | 1st       | 30 min | 0.12                      | 0.821   |
|                |           | 24 h   | 0.00                      | 1       |
|                | 2nd       | 7      | 0.36                      | 0.497   |
|                |           | 8      | 0.24                      | 0.651   |
|                |           | 3rd    | 14                         | 2.16    |
|                |           |        | 15                         | 1.23    |
|                |           |        |                             | 0.0206  |
| *Staph. aureus* ATCC 21293 | 1st      | 30 min | 4.52                      | -0.0001 |
|                |           | 24 h   | 0.77                      | 0.1492  |
|                | 2nd       | 7      | 4.77                      | -0.0001 |
|                |           | 8      | 1.43                      | 0.0072  |
|                |           | 3rd    | 14                         | 5.05    |
|                |           |        | 15                         | 0       |
| *E. coli* MG1655 | 1st       | 30 min | 0                         | 1       |
|                |           | 24 h   | 0.67                      | 0.2095  |
|                | 2nd       | 7      | 0                         | 1       |
|                |           | 8      | 0                         | 1       |
|                |           | 3rd    | 14                         | 0       |
|                |           |        | 15                         | 0       |
| *S. enteritidis* 5835 | 1st      | 30 min | 4.65                      | -0.0001 |
|                |           | 24 h   | 3.05                      | -0.0001 |
|                | 2nd       | 7      | 3.58                      | -0.0001 |
|                |           | 8      | 3.81                      | -0.0001 |
|                | 3rd       | 14     | 5.75                      | -0.0001 |
|                |           | 15     | 4.34                      | -0.0001 |
| *P. aeruginosa* ATCC 27855 | 1st      | 30 min | 3.75                      | -0.0001 |
|                |           | 24 h   | 0                         | 1       |
|                | 2nd       | 7      | 3.58                      | -0.0001 |
|                |           | 8      | 0                         | 1       |
|                | 3rd       | 14     | 3.35                      | -0.0001 |
| *C. albicans* ATCC 20308 | 1st      | 30 min | 3.88                      | -0.0001 |
|                |           | 24 h   | 2.09                      | -0.0001 |
|                | 2nd       | 7      | 3.71                      | -0.0001 |
|                |           | 8      | 2.17                      | -0.0001 |
|                | 3rd       | 14     | 3.84                      | 0.0333  |

Data represent mean, n = 3. Values without (*) are direct plate counts in log CFU per mL.

*Data represent counts in log MPN per mL (MPN performed on the samples that presented no viable cell counts on direct plating).

Table III Survival and growth of test strains after three repetitive challenges in Product B

| Bacteria       | Challenge | Day    | CFU per mL or MPN* per mL | P value |
|----------------|-----------|--------|---------------------------|---------|
| *B. cereus* F 4227A | 1st       | 30 min | 0.57                      | 0.3014  |
|                |           | 24 h   | 0.03*                     | 0.9612  |
| *Staph. aureus* ATCC 21293 | 1st      | 30 min | 4.13                      | -0.0001 |
|                |           | 24 h   | 1.7                       | 0.0026  |
| *E. coli* MG1655 | 1st       | 30 min | 5.34                      | -0.0001 |
|                |           | 24 h   | 0.77                      | 0.1492  |
|                | 2nd       | 7      | 8                         | 3.04    |
|                |           | 3rd    | 14                         | 14.16   |
|                |           |        | 15                         | 2.58    |
| *S. enteritidis* 5835 | 1st      | 30 min | 5.56                      | -0.0001 |
|                |           | 24 h   | 1.72                      | 0.0019  |
|                | 2nd       | 7      | 5.73                      | -0.0001 |
|                |           | 3rd    | 14                         | 5.75    |
|                |           |        | 15                         | 1.45    |
| *P. aeruginosa* ATCC 27855 | 1st      | 30 min | 4.13                      | -0.0001 |
|                |           | 24 h   | 0.00                      | 1       |
|                | 2nd       | 7      | 4.48                      | -0.0001 |
|                |           | 8      | 0.00                      | 1       |
|                | 3rd       | 14     | 4.92                      | -0.0001 |
| *C. albicans* ATCC 20308 | 1st      | 30 min | 3.97                      | -0.0001 |
|                |           | 24 h   | 0                         | 1       |
|                | 2nd       | 7      | 4.28                      | -0.0001 |
|                |           | 8      | 1.65                      | 0.0028  |
|                | 3rd       | 14     | 3.89                      | -0.0001 |

Data represent mean, n = 3. Values without (*) are direct plate counts in log CFU per mL.

*Data represent counts in log MPN per mL (MPN performed on the samples that presented no viable cell counts on direct plating).
Table IV Survival and growth of test strains after three repetitive challenges in Product C

| Bacteria       | Challenge | Day | CFU      | P value |
|----------------|-----------|-----|----------|---------|
| B. cereus      | 1st       | 30 min | 0.00 | 1       |
| F 4227A        | 2nd       | 24 h   | 0.00 | 1       |
|                | 3rd       | 7      | 0.00 | 1       |
|                | 8         | 0.98   | 0.075 | 0.1031 |
| Staph. aureus  | 1st       | 30 min | 0.57 | 0.3039 |
| ATCC 21293     | 2nd       | 24 h   | 0.00 | 1       |
|                | 3rd       | 7      | 0.77 | 0.1646 |
|                | 8         | 0.67   | 0.2267 | 0.0002 |
| B. cereus      | ATCC 20308| 24 h | 0.00 | 1       |
|                | 2nd       | 7      | 0.00 | 1       |
|                | 3rd       | 14     | 1.70 | 0.0002 |
| E. coli MG1655 | 1st       | 30 min | 0.00 | 1       |
|                | 2nd       | 24 h   | 0.00 | 1       |
|                | 3rd       | 7      | 0.00 | 1       |
|                | 8         | 0.00   | 1     | 0.0000 |
| S. enteritidis | 1st       | 30 min | 2.26 | <0.0001|
| 5835           | 2nd       | 24 h   | 0     | 1       |
|                | 7         | 0      | 1     | 0.0000 |
|                | 8         | 1      | 0     | 0.0000 |
|                | 14        | 1.11   | 0.0452 | 0.0000 |
|                | 15        | 0      | 1     | 0.0000 |
| P. aeruginosa  | 1st       | 30 min | 1.23 | 0.0258 |
| ATCC 27855     | 2nd       | 24 h   | 0     | 1       |
|                | 7         | 0.67   | 0.2267 | 0.0001 |
|                | 8         | 0.00   | 1     | 0.0000 |
|                | 14        | 1.43   | 0.0097 | 0.0000 |
|                | 15        | 0.00   | 1     | 0.0000 |
| C. albicans    | 1st       | 30 min | 1.49 | 0.007  |
| ATCC 20308     | 2nd       | 24 h   | 0.67 | 0.2267 |
|                | 7         | 2.12   | 0.0001 | 0.0000 |
|                | 8         | 1.9    | 0.0006 | 0.0000 |
|                | 14        | 0.67   | 0.2267 | 0.0000 |

Product D The cell count for B. cereus was 2.31, 2.02 and 2.09 log CFU per mL after 30 min of the 1st, 2nd and 3rd challenge tests, respectively. Twenty-four hours later, the cells had decreased, not by a statistically significant amount (P ≥ 0.05), showing 1.86, 1.9 and 1.76 log CFU per mL at days 1, 8 and 15, respectively. After 30-min exposure time, cell population was reduced by approximately 3 log CFU per mL after each challenge.

Enumeration of the mixed culture in make-up removers

Mixed cultures of B. cereus vegetative cells (5 log CFU per mL) and Staph. aureus, E. coli, S. enteritidis, P. aeruginosa and C. albicans (6 log CFU per mL) were challenged in Products A, B, C and D at day 0, day 7 and day 14. After 30-min and 24-h exposure time, the samples were analysed. The results are displayed in Tables III-V.

Product A (Table II)
The population of B. cereus dropped below the limit of detection for the spiral-plating process after the first two challenges after 30 min, and remained practically undetectable, even after 24 h. Upon 3rd challenge, the population decreased to 2.16 log CFU per mL after 30 min and continued to drop to 1.23 log CFU per mL. S. enteritidis and C. albicans survived after the 24 h of each challenge with a count of 3.05, 3.81 and 4.34 log CFU per mL for S. enteritidis, and 2.09, 2.17 and 1.13 log CFU per mL for C. albicans, respectively. In contrast, populations of Staph. aureus, E. coli and P. aeruginosa were under the detection limit (0.08 log MPN per mL) after 24 h of the 3rd challenge (day 15). It appears that the preservatives in Product A maintain effective after three challenges for Staph. aureus, E. coli and P. aeruginosa, but not for B. cereus, S. enteritidis and C. albicans.

Table V Survival and growth of test strains after three repetitive challenges in Product D

| Bacteria       | Challenge | Day | CFU per mL or MPN* per mL | P value |
|----------------|-----------|-----|---------------------------|---------|
| B. cereus      | 1st       | 30 min | 1.39 | 0.0119 |
| F 4227A        | 2nd       | 24 h   | 1.19* | 0.0312 |
|                | 3rd       | 14     | 0.67  | <0.0001 |
| Staph. aureus  | 1st       | 30 min | 4.43  | <0.0001 |
| ATCC 21293     | 2nd       | 24 h   | 2.75  | <0.0001 |
|                | 3rd       | 7      | 4.62  | <0.0001 |
| E. coli MG1655 | 1st       | 30 min | 3.37  | <0.0001 |
|                | 2nd       | 24 h   | 0.83  | 0.1941 |
|                | 3rd       | 8      | 2.03  | 0.0003 |
| S. enteritidis | 1st       | 30 min | 3.64  | <0.0001 |
| 5835           | 2nd       | 24 h   | 1     | 0.0000 |
|                | 3rd       | 14     | 3.98  | <0.0001 |
| P. aeruginosa  | 1st       | 30 min | 1.39  | 0.0119 |
| ATCC 27855     | 2nd       | 24 h   | 1     | 0.0000 |
|                | 3rd       | 14     | 3.98  | <0.0001 |
| C. albicans    | 1st       | 30 min | 2.53  | <0.0001 |
| ATCC 20308     | 2nd       | 24 h   | 1     | 0.0000 |
|                | 3rd       | 14     | 2.23  | <0.0001 |

Data represent mean, n = 3. Values without (*) are direct plate counts in log CFU per mL.
*Data represent counts in log MPN per mL (MPN performed on the samples that presented no viable cell counts on direct plating).

© 2017 U.S. Food and Drug Administration. International Journal of Cosmetic Science published by John Wiley & Sons Ltd on behalf of Society of Cosmetic Scientists and the Société Française de Cosmétologie

International Journal of Cosmetic Science, 40, 67–74
At day 8 (after the 2nd challenge) and from 4.61 to 3.67 log CFU per mL, respectively. The population at days 14 and 15 was 0.98 and 1.03 log CFU per mL, respectively. The only organism that survived at day 15, 24 h after the 3rd challenge, was B. cereus, which remained constant after each challenge. In addition to the antimicrobial capacity of those preservatives in Product D, the length of exposure time in the product and the method used for growing the cells were factors affecting the microbial growth, increase the microbial density, and enhance or increase the microbial population.

**Comparison of surviving B. cereus cells spiked as a pure and mixed cultures in Products A, B, C and D**

Differences resulting from the surviving population of B. cereus spiked as a pure culture into make-up removers, were counted directly from the spiral-plate technique with no need of enrichment, except from the samples of day 1 and day 8 for Products A and C, respectively, which showed no growth on the original unenriched plates. However, the detection of B. cereus inoculated into a mixed culture was usually obtained with enrichment.

**Discussion**

Four brands of make-up removers were challenged with selected micro-organisms three times: on days 0, 7 and 14, using the same concentration of cultures for each challenge. Results showed that several factors affected B. cereus detection: the types of micro-organisms included in each mixed culture, the number of challenges, the length of exposure time in the product and the method used for growing the cells. Significant numbers of vegetative cells of B. cereus, spiked as pure cultures into Products A, B and C, survived beyond the initial 30 min of the 3rd challenge and 24-h time points. This survival could be due to a gradual degradation of preservative effectiveness in those three products or that the antimicrobial capacity of those preservatives had been exhausted after killing the inoculated cells during the first two challenges. Notably, cells of B. cereus that were inoculated into Product D, which was preserved with phe-noxyethanol and methylparaben, showed no survival differences during the subsequent challenges. This suggests that among the four products tested in this study, the preservative in Product D was the least effective. However, the preservatives of all the products were gradually losing their efficacy over the course of the repeated challenges, except the two preservatives in Product D, which remained constant after each challenge. In addition to the preservatives, the ingredients in the product formulation may influence the microbial growth, enhance or increase the microbial population.

**Figure 3** B. cereus counts in log CFU per mL as pure culture or mixed cultures in four make-up removers A, B, C and D during three challenges. The stars ‘*’ represent results in log MPN per mL.
activity of a preservative [9]. Products B and D were mostly made from botanical extracts, which are ingredients that could serve as a microbial nutrient in a product formulation, and Products A and C contained caprylic glycol, propylene glycol and EDTA, respectively, which are ingredients that could increase or enhance the microbial activity of a preservative.

As shown in Fig. 3, the surviving population of *B. cereus* that had been spiked in as a mixed culture with other microbes was lower in comparison with the survival of *B. cereus* that had been spiked as pure culture (Fig. 3). Nonetheless, *B. cereus* cells from the mixed cultures were still detectable without enrichment after the 3rd challenge in all products, even though enrichment had been required at least once during the 1st and 2nd challenges. One possibility is that the *Bacillus* cells were being suppressed by those other bacteria or may have been injured to the extent that they could not be detected by spiral plating but could resume growth after the enrichment procedure. It is also possible that the preservatives may have been strong enough to kill the spiked bacteria during the first two challenges, but were insufficient after that point.

The preservative used in Product A, cetrimonium bromide, is one of the quaternary ammonium compounds (QACs), a group of amphoteric surfactants used to control bacteria growth in clinical and industrial environments [10], as well as in cosmetics. Solutions of QACs can be either bactericidal or bacteriostatic, according to the concentration used and the duration of exposure. The antimicrobial action of QACs depends on the n-alkyl length and involves the perturbation of the cytoplasmic and outer membrane lipid bilayers through the association of positively charged quaternary nitrogen with the polar head groups of acidic phospholipids [11]. In our study, the three time-challenge tests demonstrated the survival of *B. cereus*, *S. enteritidis* and *C. albicans* populations. Other organisms were either significantly reduced or undetectable after 24-h exposure. These differences in the recovery and survival could be due to certain organisms being more susceptible than others to the effects of cetrimonium bromide. Lundr et al. [12] reported QAC tolerance by *P. aeruginosa* or *Staph. aureus*. Tabata et al. [13] demonstrated that QAC tolerance was related to the physiology of the organism, specifically the expression of efflux pumps [14, 15]. We did not observe that tolerance in this study, as both *P. aeruginosa* and *Staph. aureus* were susceptible to cetrimonium bromide, although *S. enteritidis* and *B. cereus* did tolerate the preservative. Studies have shown that at higher concentrations, QACs are lethal to vegetative bacteria, yeasts, moulds, algae and lipopolipid viruses, but not to bacterial spores, mycobacteria or hydrophilic viruses [16, 17]. Nonetheless, Hoogerheide [18] demonstrated that although spores are not destroyed, small amounts of cetyltrimethyl-ammonium bromide (C16TAB) will prevent them from germinating or will destroy them when germination occurs. In this study, the efficacy of cetrimonium bromide might have been reduced during the subsequent challenges because of the microbial density.

Product B was preserved with sodium benzoate and potassium sorbate; both are commonly used in the food industry [19], pharmaceutical preparations and cosmetic products. These have been found to be effective against a wide spectrum of organisms, including aerobic bacteria, yeasts and moulds [20]. The inhibitory effect of these two preservatives varies with the inoculum level, the organisms, the length of shelf life, the temperature, the pH and the preservation concentration. Multiple investigations have shown that sodium benzoate and potassium sorbate are equally effective against bacteria, yeasts and moulds. Interestingly, Stanoevic et al. [21] demonstrated that sodium benzoate and potassium sorbate had an additive effect on *Aspergillus flavus* and *E. coli* but not on *B. subtilis*, *B. mycoides*, *Staph. aureus*, *P. aeruginosa* and *C. albicans*. In our study, the survival of *C. albicans*, *B. cereus* and *P. aeruginosa* populations increased with each subsequent challenge. Although no population changes were observed among *E. coli*, *S. enteritidis* and *Staph. aureus* during the subsequent challenges in products preserved with sodium benzoate and potassium sorbate, those three microbes were resistant to the preservatives. Our data demonstrated that the rates of inactivation for those organisms were independent of the subsequent challenges. Once the preservatives became overloaded by the spiked strains, their capacity to inactivate the test organisms may have been exceeded and the inhibition process slowed down.

Product C also contained polyaminopropyl biguanide and citric acid in addition to potassium sorbate and sodium benzoate. Polyaminopropyl biguanides, which are polymeric biguanides consist of polycationic linear polymers with a hydrophobic backbone and multiple cationic groupings separated by methylene chains [22], are used widely to control microbes in pharmacological formulations and cosmetics [23]. Their antibacterial action is believed to result from rapid binding of multiple positively charged domains to the negatively charged cell membranes of both Gram-negative and Gram-positive bacteria, which bridges acidic phospholipids through the hydrophobic methylene groups. Over time, this leads to membrane dysfunction and total loss of the permeability barrier, resulting in increasing fluidity, loss of integration and cellular destruction [24, 25]. The preservative combination used in Product C was highly effective at inactivating selected organisms during the three challenges, especially *E. coli* which was undetectable from day 0. The other organisms decreased at each challenge with time exposure except *B. cereus*, which was detected after the third challenge.

The final product tested, Product D, was preserved with both methylparaben and phenoxyethanol. Methylparaben is the methyl ester of p-hydroxybenzoic acid and is often used for antibacterial and fungicidal preservation of food, pharmaceuticals and cosmetics [26, 27]. Its mode of action is the disruption of membrane potential; this disruption interferes with both membrane transport and energy generation. Cells exposed to parabens leak their intracellular contents but show no overt changes in cell structure and can recover when exposed to preservative-free media. On the other hand, phenoxyethanol is glycol ether, which disrupts cell membranes by solubilizing lipids and may also denature important proteins [28]. Phenoxyethanol at 0.5% to 2.0% is often used in combination with parabens because it can kill bacteria and stabilize formulations [29]. However, in our study, levels of *B. cereus*, *S. aureus* and *E. coli* cells were high 24 h after the third challenge, while the populations of *C. albicans* and *P. aeruginosa* remained undetected 24 h after each challenge. Product D was the least preserved product.

Our study demonstrates the importance of challenging a cosmetic product with micro-organisms at least three times to properly assess preservative capacity. Although early studies, such as Brahan [30], claimed that multiple challenges did not provide much more information than single challenge trials, our data show that by the third challenge, the preservatives of three products were no longer able to suppress the growth of populations of *B. cereus*, spiked either solely or as a mixture with other organisms. This multichallenge procedure also provides a better model of how products become contaminated by consumers, who typically use make-up removers multiple times. This use pattern presents potential risks associated with the loss of the preservative efficacy subsequent to recontamination. The robustness of a given preservative might be
best assessed by repeated microbial challenges at high inoculation levels. Another important finding of our study is that the inactivation rate for the most sensitive organisms is not the same for all the organisms in the mixtures. For example, populations of S. aureus may remain steady across challenges, but other microbes, such as P. aeruginosa in Product B, may increase significantly.

**Conclusion**

Our repeated challenge study evaluated the capacity of preservatives to continually suppress the growth and overall load of spiked organisms in four different brands of make-up removers. We detected vegetative cells of B. cereus from pure and mixed cultures, without enrichment, in all four brands of make-up removers after the 3rd challenge. These results demonstrate that three challenges are a useful technique for assessing preservative efficacy and may help identify which organisms are most likely to be problematic for specific cosmetic formulations.

**Acknowledgements**

Office of Cosmetics and Colors at Center of Food Safety and Applied Nutrition, FDA has funded this study. The authors thank Lili Fox Vélez for scientific writing support.

**References**

1. Neza, E. and Centini, M. Microbiologically contaminated and over-preserved cosmetic products according Rapex 2008–2014. Cosmetics 3, 3 (2015).
2. Wong, S., Street, D., Delgado, S.I. and Klonz, K.C. Recalls of foods and cosmetics due to microbial contamination reported to the U.S. Food Drug Administration. J. Food Protect. 61, 1113–1116 (2000).
3. Bottone, E.J. Bacillus cereus, a volatile human pathogen. Clin. Microbiol. Rev. 23, 382–398 (2010).
4. Cosmetic Toiletry and Fragrance Association (CTFA). A study of the use of rechallenge in preservation in testing of cosmetics. CTFA, Cos. J., 13, 19–22 (1981).
5. Orth, D.S. Linear regression method for rapid determination of cosmetic preservative efficacy. J. Soc. Cosmet. Chem. 30, 321–313 (1979).
6. Hugbo, P.G., Onyekeleli, A.O. and Igue, I. Microbial contamination and preservative capacity of some brands of cosmetic creams. Trop. J. Pharm. Res. 2, 229–234 (2003).
7. Yoss, N., Arce, G., Huang, M-C.J., Yin, L., Brown, E. and Hammad, T. Factors of detection of Bacillus cereus strains in eye cream. Int. J. Cosmet. Sci. 39, 179–187 (2016).
8. U.S. FDA. BAM. Microbiological Methods for Cosmetics. https://www.fda.gov/food/foodscience/research/laboratorymethods/uc m073598.htm (2016).
9. English, D.J. Microbiology in cosmetics-Challenges in cosmetic manufacturing. http://www.english.com/sites/default/files/field s/English_Don_pres.pdf (2017).
10. Brannan, D.K. Cosmetic Microbiology: A Practical Handbook. CRC Press Inc, Boca Raton, Fla (1997).
11. Gilbert, P. and Al-Tawe, A.N.A. Antimicrobial activity of some alkyl-trimethyl ammonium bromides. Lett. Appl. Microbiol. 1, 101–105 (1985).
12. Langsrud, S., Sundheim, G. and Borgmann-Strahsén, R. Intrinsically and acquired resistance to quaternary ammonium compounds in food-related Pseudomonas spp. J. Appl. Microbiol. 95, 874–882 (2003).
13. Tabata, A., Naganuma, H., Maeda, T., Murakami, K., Miyake, Y. and Kourai, H. Correlation between resistance of Pseudomonas aeruginosa to quaternary ammonium compounds and expression of outer membrane protein OprF. Antimicrob. Agents Chemother. 47, 2093–2099 (2003).
14. Chuanchuen, R., Narasaki, C.T. and Schweizer, H.P. The MexJK efflux pump of Pseudomonas aeruginosa requires OprM for antibiotic efflux but not for efflux of triclosan. J. Bacteriol. 184, 5036–5044 (2002).
15. Chuanchuen, R., Beinlich, K., Hoang, T.T., Becker, A., Karkhoff-Schweizer, R.B. and Schweizer, H.P. Cross-resistance between triclosan and antibiotics in Pseudomonas aeruginosa is mediated by multidrug efflux pumps: exposure of a susceptible mutant strain to triclosan selects nfxB mutants overexpressing MexCD-Opr. J. Antimicrob. Agents Chemother. 45, 428–432 (2001).
16. Fredell, D.I. Biological properties and applications of cationic surfactants. In: Cationic Surfacants (Cross, J. and Singer, E.J., eds.), pp. 31–60. Marcel Dekker, New York (1994).
17. Merianos, J.J. Surface-active agents. In: Disinfection, Sterilization and Preservation (Block, J.C., ed.), pp. 282–306. Lipinskiott Williams and Wilkins, Philadelphia (2001).
18. Hoogerheide, J.C. The germicidal properties of certain quaternary ammonium salts with special reference to cetyltrimethyl-ammonium bromide. J. Bacteriol. 49, 277–289 (1945).
19. Sofos, J.N. and Busta, F.F. Antimicrobial activity of sorbate. J. Food Prot. 44, 614–622 (1981).
20. Jin, T., Zhang, H. and Boyd, G. Incorporation of preservatives in polyactic acid films for inactivating E. coli O157:H7 and extending microbiological shelf life of strawberry puree. J. Food Prot. 73, 812–818 (2010).
21. Stanojevic, D., Comic, L., Stefanovic, O. and Solujic-Sukludak, S.I. Antimicrobial effects of sodium benzoate, sodium nitrate and potassium sorbate and their synergistic action in vitro. Biol. J. Agri. Sci. 15, 307–311 (2009).
22. Gilbert, P. and Moore, L.E. Cationic antiseptics: diversity of action under a common epithet. J. Appl. Microbiol. 99, 703–715 (2005).
23. Rembe, J-D., Fromm-Dornieden, C., Shaefer, N., Boehm, J.K. and Stuermer, E.K. Comparing two polymeric biguanides: chemical distinction, antiseptic efficacy and cytotoxicity of polyaminopropyl biguanide and polyhexamethylene biguanide. J. Med. Microbiol. 65, 867–876 (2016).
24. Broxton, P., Woodcock, P.M. and Gilbert, P. A study of the antibacterial activity of some polyhexamethylene biguanides towards Escherichia coli ATCC 8739. J. Appl. Bacteriol. 54, 345–353 (1983).
25. Broxton, P., Woodcock, P.M., Heatley, F. and Gilbert, P. Interaction of some polyhexamethylene biguanides and membrane phospholipids in Escherichia coli. J. Appl. Bacteriol. 57, 115–124 (1984a).
26. Aalto, T.R., Firman, M.C. and Rigler, N.E. P-Hydroxybenzoic acid Esters as preservatives. Uses, Antibacterial and antifungal studies, properties and determination. J. Am. Pharmaceut. Assoc. 8, 489–491 (1953).
27. U.S. FDA. Parabens. http://www.fda.gov/cosmetics/productsingredients/ingredients/uc m128042.htm (2016).
28. Geis, P.A. Common cosmetic preservatives. In: Cosmetic Microbiology. A Practical Approach (Geis, P.A., ed.), 2nd ed. pp. 227–281. CRC Press, Boca Raton (2006).
29. Lalitha, C. and Prasada Rao, P.V.V. Antimicrobial efficacy of low level cosmetic preservatives. WJPPS 3, 1685–1696 (2014).
30. Brannan, D.K. Cosmetic preservation. J. Soc. Cosm. Chem. 46, 199–220 (1995).