Factors of detection of *Bacillus cereus* strains in eye cream

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**Abstract**

*Bacillus cereus* has been associated with clinical infections and is also the cause of post-traumatic endophthalmitis as well as endogenous eye infections, which can result in blindness. Cosmetics, although preserved, can be contaminated during manufacture or use and thus cause serious health issues.

**OBJECTIVE:** We investigated the detection factors of *Bacillus* in eye cream preserved with parabens, including the non-ionic surfactants used as neutralizers such as Tween 80, a blend of Tween 60 and Span 80, Tween 20 and selective media.

**METHODS:** Eye-cream samples were first mixed with neutralizers and individually inoculated with *B. cereus* strains, *B. mycoides*, *B. subtilis* or *B. thuringiensis* at a final concentration of 5 log CFU/g. The inoculated samples with and without neutralizers were analysed after 30 min and during 84-day storage at room temperature. Presumptive colonies of *Bacillus* were enumerated on the varieties of Bacillus agar by spiral-plating techniques and most probable number (MPN) method.

**RESULTS:** The recovery counts of all *Bacillus* strains were between 4.10 and 4.58 log CFU/g in samples with Tween 80 and from 3.62 to 4.53 log CFU/g in samples with TS after 30 min. Tween 20 was the least effective neutralizer. The challenged organisms, in samples without neutralizer, *B. subtilis* ATCC 15563 and *B. cereus* 4227A were detected at 1.83 and 1.49 log CFU/g after 30 min, respectively.

**CONCLUSION:** This study showed that Tween 80 was the best neutralizer for reducing the antimicrobial effect of parabens. BACARA® and R&F plating media showed typical reaction of *Bacillus cereus* strains.

**Résumé**

*Bacillus cereus* a été associé à des infections cliniques et est également la cause de l’endophtalmie post-traumatique ainsi que des infections oculaires endogènes, ce qui peut entraîner la cécité. Des produits cosmétiques, bien que conservés, peuvent être contaminés lors de la fabrication ou de l’utilisation, et donc causer des problèmes de santé graves.

OBJECTIF: Nous avons étudié les facteurs de détection de *Bacillus* dans des crèmes pour les yeux conservées avec des parabens, y compris les tensioactifs non ioniques utilisés comme agents neutralisants tels que le Tween 80, un mélange de Tween 60 et Span 80, Tween 20 et des milieux sélectifs.

**MÉTHODES:** Des échantillons de crème pour les yeux ont d’abord été mélangés avec des neutralisants et inoculés individuellement avec des souches de *B. cereus*, *B. mycoides*, *B. subtilis*, ou *B. thuringiensis* à une concentration finale de 5 log UFC/g. Les échantillons inoculés avec et sans agents de neutralisation ont été analysés après 30 min et pendant le stockage de 84 jours à température ambiante. Les colonies de *Bacillus* ont été dénombrées sur les variétés de gelose *Bacillus* par des techniques de placage spiral et de la méthode du nombre le plus probable (MPN).

**RÉSULTATS:** Les chiffres de récupération de toutes les souches de *Bacillus* étaient entre 4,10 et 4,58 log UFC/g dans les échantillons avec de Tween 80 et 3,62 à 4,53 UFC/g dans les échantillons avec TS après 30 min. Tween 20 est le neutralisant le moins efficace. Les organismes testés dans les échantillons sans neutralisant, à savoir *B. subtilis* ATCC 15563 et *B. cereus* 4227A ont été détectés à 1,83 et 1,49 log UFC/g après 30 min, respectivement.

**CONCLUSION:** Cette étude a montré que le Tween 80 était le meilleur neutralisant pour réduire l’effet antimicrobien de parabens. Les médias Bacara® et R & F de placage ont montré la réaction typique des souches de *Bacillus cereus*.

**Introduction**

Cosmetics are defined by the Federal Food, Drug, and Cosmetic Act (FD&C Act, sec. 201(i)) as articles intended to be rubbed, poured, sprinkled, or sprayed on, introduced into or otherwise applied to the human body or any part thereof for cleansing, beautifying, promoting attractiveness or altering the appearance [1]. Cosmetics contain preservatives to maintain the integrity of the product and protect the product from growth of harmful microorganisms. However, cosmetics may become contaminated during processing from raw material or the manufacturing environment, through the loss of preservative’s effectiveness over time [2], the inadequate use of preservatives [3] and especially through the use or misuse of products by the consumers [4].

Among preservatives, parabens are the most commonly used because of their low toxicity and their effective antimicrobial activity, especially against yeasts and moulds. Parabens are esters of p-hydroxybenzoic acid. The most commonly used parabens in
cosmetics are methyl-, propyl- and butylparaben, which are used at very low levels ranging from 0.01% to 0.3% in most cosmetics [5].

Cosmetics intended for use near the eye area are of particular concern because the user could easily introduce organisms into the product with applicators or fingers potentially causing an eye infection. Significant eye infections, keratitis, perforation, endophthalmitis and even blindness have resulted from ocular infections following the use of contaminated mascara products with *Pseudomonas aeruginosa* [6], or the use of contact lenses contaminated with *Bacillus subtilis* and *Bacillus cereus* [7–9]. Recently, FDA has recalled several eye-area cosmetics contaminated with *B. cereus* [10], but there are currently no reports of eye infections linked with eye-area cosmetic products and *Bacillus cereus*.

*B. cereus* and other *Bacillus* species can produce toxins that cause gastrointestinal diseases and several clinical infections [11, 12]. A study conducted by Callegari et al. [13] on rabbits show that 100 CFU per mL of *B. cereus* was capable of causing eye inflammation within 3 h and severe iritis and abscesses thereafter. In addition to causing eye infections, all species of *Bacillus* are also undesirable in cosmetics and are regarded as indicator of filth because their spores originate in soil [14].

Non-ionic surfactants contain no specific charge and are used most often in cosmetics as emulsifiers, conditioning ingredients and solubilizing agents. Examples of non-ionic surfactants are polyoxyethylene and sorbitan esters. They are widely used in cosmetics and are considered innocuous [15]. Lalitha and Rao [16] in a fourteen-day study, added Tween 80 to the facial cream to neutralize and verify the effectiveness of different preservatives that comprised methyl- and propylparaben. Likewise, Terleckyj and Axler [17] mixed the biocide with the neutralizer before addition of the challenge organisms to determine the neutralizing capacity of the neutralizer.

This study investigated different factors that could contribute to the recovery and detection of seven strains of *Bacillus*, *B cereus* F 4227A, *B. cereus* F 60006, *B. cereus* ATCC 14579, *B. cereus* X, *B. mycoides* ATCC 6462, *B. subtilis* ATCC 15563 and *B. thuringiensis* ATCC 35866 inoculated in eye cream preserved with methyl- and propylparaben. We evaluated the effectiveness of the non-ionic surfactants, neutralizers, Tween 80, Tween 20 and a mixture of Tween 60 and Span 80 on parabens for the detection of the inoculated *Bacillus* strains in eye cream. Prior to that, the effect of the inhibitory activity of parabens at different concentrations was investigated in different broths, both with and without neutralizers.

The performance of two dilution broths and three chromogenic plating media, BACARA<sup>®</sup>, Brilliance™ *Bacillus cereus* agar and R&F *Bacillus cereus* plating media, plus a standard selective plating medium, Mannitol-egg-yolk-polymyxin B agar, was evaluated for the recovery and differentiation of *Bacillus* strains in eye cream. This is the first study reporting the recovery and enumeration of *Bacillus* species in eye cream.

**Materials and methods**

**Cosmetic samples**

A single brand of eye cream containing methyl- and propyl-parabens as traditional preservatives and also natural ingredients was purchased from retail outlets and kept in their original containers at room temperature until use. No expiration date was found on the packaging.

**Preparation of bacterial strains**

Seven strains of *Bacillus* were used, in which two were from previously characterized isolates of food-borne outbreaks from U. S. Food and Drug Administration culture collection (F) (*B. cereus* F 4227A and *B. cereus* F 60006). Five strains were purchased from American Type Culture Collection (ATCC) (Manassas, VA) (*B. cereus* ATCC 14579, *B. cereus* X, *B. mycoides* ATCC 6264, *B. subtilis* ATCC 15563 and *B. thuringiensis* ATCC 35866). All cultures were maintained at −80°C in 20% glycerol, and each strain was aseptically subcultured in nutrient broth (NB) (Difco™, Franklin Lakes, NJ) for 24 h at 30°C and stored at 4°C for use. Prior to the experiment, cultures were grown in NB and incubated at 30°C for 18 h.

Fresh cultures were centrifuged (7500 °C for 10 min, 4°C) and washed in sterile phosphate-buffered saline (PBS) (Difco™, Franklin Lakes, NJ). The cell pellets were resuspended in PBS, and the cell density of individual strains was adjusted to obtain final concentration ~7.0 log. The phase contrast microscope showed the cells in their vegetative state. The populations of individual strains were verified on Tryptic soy agar (TSA) (Difco™, Franklin Lakes, NJ) by the spiral plater.

**Determination of the inhibitory activity of methyl- and propylparabens in broths in the presence of the neutralizers**

The test *Bacillus* strains, at a concentration of ~6 log CFU per mL, were individually diluted (1:9) in the sterile Tryptic soy broth (TSB) (Difco™, Franklin Lakes, NJ) and in the modified Leethen broth (MLB) (Difco™, Franklin Lakes, NJ) containing methylparaben (MP) (Supelco, Sigma-Aldrich Co. LLC, St. Louis, MO) and propylparaben (PP) (Fluka, Sigma-Aldrich Co. LLC, St. Louis, MO) at concentrations of 0%, 0.01%, 0.05%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.8% and 1% in the presence or the absence of the neutralizers and, also, in a combination of 0.2% MP and 0.1% PP. The MIC was defined as the lowest concentration inhibiting visible growth of test organism [19], and also, the MBC was the lowest concentration at which no growth was observed after subculturing [20]. The sporidal concentrations were not evaluated.

**Sample preparation**

In this first study, the eye-cream samples were mixed with the neutralizers before the addition of the challenge organisms to determine the neutralizing efficacy of the neutralizers and to help in the detection of the *Bacillus* population. In the BAM method for cosmetics [18], Tween 80 is added to the cream at a concentration of 1:1 for microbial analysis. Twenty grams of cream was placed into a 50-mL polystyrene sterile conical tube, and 20 mL of Tween 20 (Fisher; Thermo Scientific Inc, Pittsburgh, PA), 20 mL of 50% of Tween 80 (Fisher; Thermo Scientific Inc, Pittsburgh, PA), or 20 mL of PBS, a blend of 10 mL of 20% Tween 60 (Fisher; Thermo Scientific Inc, Pittsburgh, PA) and 10 mL of Span 80 (Sigma-Aldrich Co. LLC, St Louis, MO), was added to it and vortex-mixed. Sterile glass beads were also included to the mixture to facilitate homogenization and to obtain a good emulsion.

**Sample inoculation**

Two hundred µL of fresh *Bacillus* culture (~7 log CFU per mL cells) was individually added to the 20-g sample with or without neutralizer to obtain ~5 log CFU per g cream. The samples were mixed

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thoroughly and kept at room temperature. After 30 min, 1 mL of
the sample was aseptically placed in the 15-mL conical tube diluted
and spiral-plated on the selected plating media with the easySpiral®
(Interscience, Rockland, MA). The samples were stored at room
temperature and analysed after 3, 7, 14, 28, 56 and 84 days.
Uninoculated cream samples were plated on MLA and used as a
negative control.

Challenge test
The product was challenged with seven strains of Bacillus accord-
ing to the slightly modified method of Personal Care Products
Council (PCPC) [21]. One millilitre of the inoculated samples was
placed in a 15-mL tube, and 9 mL of the sterile diluting MLB was
added and vortexed; 100 µL was spiral-plated onto modified Leth-
een agar (MLA) (Difco™, Franklin Lakes, NJ). The analysis
was performed on MLA at the same time intervals as above.

Microbial evaluation of cream samples neutralized with T80, T20
and TS
On each sampling date, 1 mL of inoculated sample was placed into
a 15-mL sterile conical tube and 9 mL of sterile MLB or Tryptone-
Azolectin-Tween (TAT) (Difco™, Franklin Lakes, NJ) was added to
each tube and vortex-mixed. Appropriate diluted suspensions were
spiral-plated onto Bacillus cereus rapid agar (BACARA™) (bioMer-
ieux, Durham, NC), Brilliance™ Bacillus cereus agar (BBC) (Thermo
Fisher Scientific Inc. Asheville, NC), Mannitol-egg-yolk-polymyxin B
agar (MYP) (Difco™, Franklin Lakes, NJ) and R&F™ Bacillus cereus
agar (RF) (R&F Laboratories, Downers Grove, IL). The presumptive-
positive Bacillus colonies were enumerated according to the charac-
teristics described in Table I. The uninoculated samples were also
analysed and used as controls.

In addition to direct plating, most probable number (MPN) was
derived from the use of eight wells and six dilutions in 96-deep-well
plates (Fisher) for enumeration of Bacillus in samples that showed
no growth on the surface-plating and the recovery of the cells
affected by the preservatives. Wells were filled with MLB, and
200 µL of inoculated samples was serially diluted to the sixth dilu-
tion inside the wells. Wells were incubated at 30°C for 24 h. Samples
were spot-plated on the same four plating media used for
direct plating. Colonies with characteristics described in Table I
were reported as positive and scored for MPN calculation. Direct
plating was performed to quantify the viable cells present in the
samples, and MPN method was performed to insure that there were
no surviving cells in the samples.

Bacillus was biochemically confirmed with a Vitek 2 (bioMerieux,
Durham, NC) using a BCL card (bioMerieux, Durham, NC). Bacte-
rial suspensions were prepared in 3 mL of saline and adjusted to
MacFarland standard of 1.8–2.2 using the Vitek 2 DensiChek (bio-
Merieux, Durham, NC). BCL cards were automatically filled in the
VITEK vacuum chamber, sealed, incubated and read automatically.
Identification results were reported as correct identification to a sin-
gle species, slashline when the generated biopattern was insuffi-
cient to discriminate between more than one species, low
discrimination or unidentified.

Statistical analysis
The experiment was repeated in triplicate for each time period.
Recovery of Bacillus populations obtained at each sampling period
from the cream samples was converted to log CFU per g. The data
were analysed by repeated measures ANOVA using SAS 9.4 mixed
procedure (SAS, Cary, NC). Differences were accepted as statistical
different at P < 0.05.

Results
Minimal bactericidal concentration of methyl- (MP) and
propylparabens (PP)
Initially, an attempt was made to determine the MIC as well as
the MBC. However, some media interfered with the neutralizers
and the parabens and formed a turbid solution rendering the
reading of the MIC plates impossible. The same observation was
reported by Mizuba and Sheikh [26]. Therefore, we proceeded
with the MBC. As controls, all the organisms grew in the broths
containing the neutralizers, but not the preservatives. The MBC
of MP and PP of all the strains was 0.3% and 0.05% in TSB,
respectively. In MLB, the MBC of MP and PP was 0.4% and
0.2%, respectively. The bactericidal concentration of the parabens
increased with the addition of T80, T20 and TS in MLB
(Table II). Likewise, additional T80, T60 and T20 in MLB reduced
the antimicrobial effect of the parabens and allowed the growth
of the population of Bacillus in the combination of 0.2% MP and
0.1% PP (Table III).

Table 1 Principles and features of isolation and differentiation of Bacillus cereus of selective media

| Media       | Antibiotic       | Activity                   | Colony                  | Inhibition         | References |
|-------------|------------------|----------------------------|-------------------------|--------------------|------------|
| BACARA™     | -                | Phospholipase              | Pink/orangey halo       | Gram-/+, Non- B. cereus | [22]       |
|             |                  | Lecithinase                |                         |                    |            |
| BBC¹        | Polymyxin B      | ß-glucosidase              | Blue/green              | Gram-/+, Non- B. cereus | [23]       |
|             | Trime thoprim    |                            |                         |                    |            |
|             | Polymyxin B      | Lecithinase                | Pink halo               | Gram-               | [24]       |
| MYP²        |                  | No mannitol fermentation   | Turquoise               | Gram-/+, Non- B. cereus | [25]       |
|             | Polymyxin B      | Phospholipase              |                         |                    |            |
| RF³         | Cefazidine       |                            |                         |                    |            |
|             | Polymyxin B      |                            |                         |                    |            |

*Proprietary formula; ¹Brilliance™ Bacillus cereus agar; ²Mannitol-egg-yolk-polymyxin B agar; ³R&F Bacillus cereus agar.

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Identification of selected Bacillus strains on selective agar

The appearance of the media listed in Table I was used to identify and enumerate the isolates on the plating media. All the strains grew on all plates (Fig. 1), as would be expected based on the characteristics described in Table I.

Challenge test

The uninoculated cream (negative control) did not show any growth of organisms on MLA, so the product did not appear to be contaminated with any microorganisms. The result of the challenge test is summarized in Fig. 2. The preservatives seemed to be effective on all the strains, as all bacterial populations were reduced by more than 3 log CFU per g after 30 min and continued gradually decreasing below the detection limit (0.04 log MPN per g) after 54 days, except B. cereus 4227A. B. cereus 4227A was the most resistant with a population of 1.49 log CFU per g after 30 min, 1.12 log MPN per g after 28 days and 0.51 log MPN per g after 84 days.

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Table II Minimum bactericidal concentration of parabens (mg mL⁻¹) with or without neutralizers

| Bacillus* strains | Methylparaben (MP) % | Propylparaben (PP) % |
|------------------|---------------------|---------------------|
|                  | TSB | MLB | MLB + T20 | MLB + T80 | MLB + TS | TSB | MLB | MLB + T20 | MLB + T80 | MLB + TS |
| F 4227A          | 0.3 | 0.4 | 0.8      | 0.8      | 0.4      | 0.05 | 0.2 | 0.5      | 0.8      | 0.4      |
| F 60006          | 0.3 | 0.4 | 0.8      | 0.8      | 0.4      | 0.05 | 0.2 | 0.5      | 0.8      | 0.4      |
| ATCC 14579       | 0.3 | 0.4 | 0.8      | 0.8      | 0.5      | 0.05 | 0.2 | 0.5      | 0.8      | 0.5      |
| B. cereus X      | 0.3 | 0.4 | 0.8      | 0.8      | 0.5      | 0.05 | 0.2 | 0.5      | 0.8      | 0.5      |
| ATCC 6462        | 0.3 | 0.4 | 0.8      | 1        | 0.5      | 0.05 | 0.2 | 0.5      | 0.8      | 0.5      |
| ATCC 15563       | 0.3 | 0.4 | 0.8      | 0.8      | 0.5      | 0.05 | 0.2 | 0.5      | 0.8      | 0.5      |
| ATCC 35866       | 0.3 | 0.4 | 0.8      | 0.8      | 0.5      | 0.05 | 0.2 | 0.5      | 0.8      | 0.5      |

*B. cereus F 4227A, B. cereus F 60006, B. mycoides ATCC 6462, B. cereus ATCC 14579, B. subtilis ATCC 15563, B. thuringiensis ATCC 35866. About 6 log CFU per mL of each cell was inoculated into the broths containing paraben with and without neutralizers; the controls contained no paraben to determine the MBC.

Broths: TSB, Tryptic soy broth; MLB, modified Letheen broth; Neutralizers: T80, Tween 80; T20, Tween 20, TS, Tween 60 + Span 80.

Table III Effect of the neutralizers on a combination of .2% of methylparaben and .1% of propylparaben

| Bacillus strains | Combination (0.2% MP + 0.1% PP)* |
|------------------|----------------------------------|
|                  | TSB | MLB | MLB + T20 | MLB + T80 | MLB + TS |
| F 4227A          | –   | –   | +         | +         | +        |
| F 60006          | –   | –   | +         | +         | +        |
| ATCC 14579       | –   | –   | +         | +         | +        |
| B. cereus X      | –   | –   | +         | +         | +        |
| ATCC 6462        | –   | –   | +         | +         | +        |
| ATCC 15563       | –   | –   | +         | +         | +        |
| ATCC 35866       | –   | –   | +         | +         | +        |

*About 6 log CFU per mL of each organism was inoculated into the broths containing the combination of 0.2% of methylparaben (MP) and 0.1% of propylparaben (PP) with and without neutralizers and was kept for 24 h to 48 h at 30°C.

MLB, modified Letheen broth; TSB, Tryptic soy broth; T20, Tween 20; T80, Tween 20; TS, blend of Tween 60 and Span 80; –, no growth; +, growth.

Identification of selected Bacillus strains on selective agar

The appearance of the media listed in Table I was used to identify and enumerate the isolates on the plating media. All the strains grew on all plates (Fig. 1), as would be expected based on the characteristics described in Table I.

Challenge test

The uninoculated cream (negative control) did not show any growth of organisms on MLA, so the product did not appear to be contaminated with any microorganisms. The result of the challenge test is summarized in Fig. 2. The preservatives seemed to be effective on all the strains, as all bacterial populations were reduced by more than 3 log CFU per g after 30 min and continued gradually decreasing below the detection limit (0.04 log MPN per g) after 54 days, except B. cereus 4227A. B. cereus 4227A was the most resistant with a population of 1.49 log CFU per g after 30 min, 1.12 log MPN per g after 28 days and 0.51 log MPN per g after 84 days.

Figure 1 Comparison of selected Bacillus strains on selective media. 1 – B. cereus F 4227A, 2 – B. cereus F 60006, 3 – B. cereus X, 4 – B. mycoides ATCC 6462, 5 – B. cereus ATCC 14579, 6 – B. subtilis ATCC 15563, B. thuringiensis ATCC 35866. One millilitre of 24-h actively growing culture of the selected strains was individually spotted on BACARA®, BBC, MYP and RF and incubated for 24 h at 30°C. BBC, Brilliance Bacillus cereus agar; MYP, Mannitol-egg-yolk-polymyxin B agar; RF, R&F Bacillus cereus agar.
Comparison of Bacillus population in plating and dilution media

Recovery of Bacillus strains spiked in eye creams neutralized with Tween 20, Tween 80 and Tween 60+ Span 80 at various time intervals showed no significant differences ($P > 0.05$) among the plating media using the same neutralizer. Also, all main effects such as neutralizers, strains, plating media and time were tested, and no difference ($P > 0.05$) was seen on the bacterial growth diluted with the broths MLB and TAT. However, we saw different time course patterns among neutralizers and also different growth curves among the strains (Fig. 3).

Recovery of Bacillus population in eye cream neutralized with T80, T20 and TS at various times

Results of the recovery of Bacillus are represented in Table IV.
Table IV Recovery of Bacillus strains in eye-cream samples containing parabens with and without neutralizers after 30 min, 3, 7, 14, 21, 28, 54 and 84 days

| Broths | Strains | 30 min | 3 day | 7 day | 14 day | 21 day | 28 day | 56 day | 84 day |
|--------|---------|--------|-------|-------|--------|--------|--------|--------|--------|
| MLB    | F 4227A | 1.49 ± 0.34 | 1.19 ± 0.44 | 1.21 ± 0.40 | 0.64 ± 0.29 | 0.81 ± 0.23 | 1.12 ± 0.27 | 0.55 ± 0.23 | 0.51 ± 0.18 |
| MLB    | F 60006 | 1.65 ± 0.34 | 0.44 ± 0.44 | 1.19 ± 0.40 | 0.29 ± 0.29 | 0.16 ± 0.22 | 0.15 ± 0.27 | 0.14 ± 0.22 | 0.15 ± 0.18 |
| MLB    | ATTC 14579 | 0.25 ± 0.34 | 0.36 ± 0.44 | 0.35 ± 0.40 | NG | 0.13 ± 0.29 | NG | NG | NG |
| MLB    | B. cereus X | 0.13 ± 0.34 | NG | 0.42 ± 0.40 | 0.13 ± 0.29 | NG | NG | NG | NG |
| MLB    | ATTC 6462 | 1.79 ± 0.34 | NG | NG | NG | NG | NG | NG | NG |
| MLB    | ATTC 15563 | 1.83 ± 0.34 | NG | NG | NG | NG | NG | NG | NG |
| MLB    | ATTC 35866 | 0.39 ± 0.34 | NG | 0.33 ± 0.44 | NG | NG | NG | NG | NG |
| MLB + T80 | F 4227A | 4.66 ± 0.12 | 1.64 ± 0.15 | 0.84 ± 0.14 | 0.54 ± 0.10 | 0.46 ± 0.08 | 0.58 ± 0.1* | 0.58 ± 0.09* | 0.39 ± 0.06 |
| MLB + T80 | F 4227A | 4.19 ± 0.12 | 3.48 ± 0.15 | 2.18 ± 0.14 | 1.74 ± 0.10 | 1.64 ± 0.08 | 1.9 ± 0.1* | 1.88 ± 0.09* | 1.56 ± 0.06 |
| MLB + T80 | ATTC 14579 | 0.74 ± 0.12 | 2.00 ± 0.15 | 0.71 ± 0.14 | 0.54 ± 0.10 | 0.46 ± 0.08 | 0.58 ± 0.1* | 0.58 ± 0.09* | 0.39 ± 0.06 |
| MLB + T80 | ATTC 6462 | 1.12 ± 0.12 | 4.18 ± 0.15 | 3.34 ± 0.14 | NG | NG | NG | NG | NG |
| MLB + T80 | ATTC 15563 | 4.10 ± 0.24 | 2.75 ± 0.31 | 0.91 ± 0.14 | NG | NG | NG | NG | NG |
| MLB + T80 | ATTC 35866 | 4.58 ± 0.12 | 3.67 ± 0.15 | 0.08 ± 0.14 | NG | NG | NG | NG | NG |
| MLB + T20 | F 4227A | 2.66 ± 0.12 | 0.58 ± 0.15 | 0.59 ± 0.14 | 0.49 ± 0.10 | 0.78 ± 0.08 | 0.68 ± 0.09 | NG | NG |
| MLB + T20 | F 4227A | 4.19 ± 0.12 | 3.48 ± 0.15 | 2.18 ± 0.14 | 1.74 ± 0.10 | 1.64 ± 0.08 | 1.9 ± 0.1* | 1.88 ± 0.09* | 1.56 ± 0.06 |
| MLB + T20 | ATTC 14579 | 4.31 ± 0.12 | 2.90 ± 0.15 | 2.67 ± 0.14 | 0.80 ± 0.10 | 0.10 ± 0.08 | NG | NG | NG |
| MLB + T20 | ATTC 6462 | 4.62 ± 0.12 | 4.18 ± 0.15 | 3.34 ± 0.14 | NG | NG | NG | NG | NG |
| MLB + T20 | ATTC 15563 | 4.10 ± 0.24 | 2.75 ± 0.31 | 0.91 ± 0.14 | NG | NG | NG | NG | NG |
| MLB + T20 | ATTC 35866 | 4.58 ± 0.12 | 3.67 ± 0.15 | 0.08 ± 0.14 | NG | NG | NG | NG | NG |
| MLB + TS | F 4227A | 4.04 ± 0.12 | 0.46 ± 0.15 | 0.49 ± 0.14 | 0.82 ± 0.10 | 0.63 ± 0.08* | 0.63 ± 0.08* | 0.17 ± 0.08* | NG |
| MLB + TS | F 4227A | 3.73 ± 0.12 | 0.20 ± 0.15 | 0.24 ± 0.14 | 0.04 ± 0.10 | 0.08 ± 0.08* | 0.20 ± 0.09* | 0.01 ± 0.08* | NG |
| MLB + TS | ATTC 14579 | 3.91 ± 0.12 | NG | NG | NG | NG | NG | NG | NG |
| MLB + TS | ATTC 6462 | 4.53 ± 0.12 | NG | NG | NG | NG | NG | NG | NG |
| MLB + TS | ATTC 15563 | 3.62 ± 0.24 | 0.23 ± 0.31 | 0.13 ± 0.14 | NG | 0.0 ± 0.1* | NG | NG | NG |
| MLB + TS | ATTC 35866 | 4.63 ± 0.12 | 0.11 ± 0.15 | 0.03 ± 0.14 | NG | 0.1 ± 0.1* | NG | NG | NG |

Data represent mean ± SEM, n = 3; NG = no growth.
Values without (*) are direct plate counts in log CFU per g.
*Data represent counts in log MPN per g (MPN performed on the samples that presented no viable cell counts on direct plating).
MLB, modified Letheen broth; TSB, Tryptic soy broth; T20, Tween 20; T80, Tween 80; TS, blend of Tween 60 and Span 80.

Day 0
There was no growth of organisms on un inoculated (negative control) cream samples plated on MLA. The growth of Bacillus in samples containing T20 ranged from 3.28 to 2.03 log CFU per g with B. subtilis having the highest concentration and B. cereus X the lowest. In samples neutralized with TS, the growth of Bacillus varied between 4.53 and 3.62 log CFU per g with B. mycoides being the highest and B. subtilis the lowest. The population in the sample neutralized with T80 was not significantly different (P > 0.05) ranging from 4.58 to 4.10 log CFU per g of B. thuringiensis and subtilis, respectively.

Day 3
No significant differences (P > 0.05) were seen among the strains in the samples without neutralizer and in the samples containing the neutralizers T20 and TS, with the exception of B. cereus 4227A. Significant differences were observed among the strains in cream samples containing T80.

B. cereus 4227A populations in the samples without neutralizer were 1.19 log MPN per g. The highest count in samples with T20 was seen with B. subtilis at 0.70 log MPN per g followed by B. cereus 4227A at 0.58 log MPN per g; B. cereus and B. thuringiensis were under the detection limit. B. cereus 4227A in samples with TS was 0.46 log MPN per g; B. mycoides and B. cereus 14579 were under the detection limit. The bacterial populations in sample containing T80 ranged between 4.18 and 1.64 log CFU per g of B. mycoides and B. cereus 4227A, respectively.

Day 7
There were no significant differences (P > 0.05) among the strains in the samples without neutralizer and in the samples containing the neutralizers T20 and TS, with the exception of B. cereus 4227A and 6006 in T20, and B. cereus 4227A in no neutralizer and in TS samples. Significant differences (P < 0.05) were observed among the strains in cream samples with T80, but not with B. thuringiensis.

B. cereus 4227 A was at a level of 1.21 log MPN per g in the samples without neutralizer, and in T20 samples, B. cereus 4227A and 6006 were at levels of 0.59 and 0.50 log MPN per g, respectively. B. cereus 14579, B. subtilis and B. mycoides were undetected (detection limit = 0.04 log CFU per g). In samples neutralized with

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TS, *B. cereus* 4227A was 0.49 log MPN per g, and *B. cereus* 14579 and *B. mycoides* remained under the detection limit. The bacterial population in sample containing T80 ranged between 3.3 log MPN per g and 0.08 log MPN per g of *B. mycoides* and *B. thuringiensis*, respectively.

**Day 14**

There were no significant differences \( (P > 0.05) \) among the strains in the samples without neutralizer and in the samples containing the neutralizers T20 and TS, with the exception of *B. cereus* 4227A. Significant differences \( (P < 0.05) \) were observed among the strains in cream samples with T80, with the exception of *B. cereus* X, *B. mycoides*, *B. subtilis* and *B. thuringiensis*.

*B. cereus* 4227A in sample without neutralizer decreased to 0.64 log MPN per g, and *B. cereus* 14579, *B. mycoides*, *B. subtilis* and *B. thuringiensis* were under detection limit. Samples containing T20 had a survival rate of 0.49 log MPN per g for *B. cereus* 4227A, and the rest of strains were under detection limit. Likewise, samples with TS showed the survival rates of *B. cereus* 4227A and 6006 at 0.81 and 0.04 log MPN per g, respectively, whereas the remaining strains were undetected (limit: 0.04 log MPN per g). *B. cereus* 4227A, 6006 and 14579 decreased to levels of 0.54, 1.74 and 0.80 log MPN per g, respectively.

**Day 21**

No significant differences \( (P > 0.05) \) were observed among the strains in any of the samples except the strain of *B. cereus* 4227A in samples without neutralizer and in samples incorporated with T20 and TS with concentrations of 0.81, 0.78 and 0.63 log MPN per g, respectively. Also, the levels of strains of *B. cereus* 4227A and 6006 from samples with T80 with 0.46 and 1.63 log MPN per g \( (P < 0.05) \) were significantly different from one another.

**Day 28**

No significant differences \( (P > 0.05) \) were observed among the strains in all samples except the strain of *B. cereus* 4227A in samples without neutralizer and in samples incorporated with T20 and TS with concentrations of 1.12, 0.68 and 0.61 log MPN per g, respectively. Also, the levels of strains of *B. cereus* 4227A and 6006 from samples with T80 with 0.61 and 1.88 log MPN per g \( (P < 0.05) \) were significantly different.

**Day 56**

On day 56, levels of *B. cereus* 4227A were significantly higher \( (P < 0.05) \) in samples without neutralizer and also in the samples containing TS with population levels of 0.54 and 0.17 log MPN per g, respectively. *B. cereus* 4227A and 6006 were at levels of 0.62 and 1.78 log MPN per g, respectively, in the samples containing T80; the rest of bacterial population was below the level of detection (0.04 log MPN per g).

**Day 84**

At day 84, *B. cereus* 4227A was found at a significantly high level \( (P < 0.05) \) among the *Bacillus* populations in the samples without neutralizer with a population density of 0.51 log MPN per g. Other populations were under the limit of detection (0.04 log MPN per g). *B. cereus* 4227A and 6006 population decreased slightly at 0.39 and 1.56 log MPN per g, respectively, in the eye-cream samples mixed with T80.

**Effect of neutralizers added to the product**

The neutralizers were added to the product to inactivate the preservatives and to prevent microbial inhibition. T20 had the least influence on the parabens followed by T60. T80 reduced the antimicrobial efficacy of the parabens to a greater extent. However, the efficacy of TS was comparable to T80 at the first time point \( (P > 0.05) \).

The neutralizing ability of the three agents varied with each test organism and at each time interval, except day 0 for T80 samples, where there was no difference \( (P > 0.05) \) among the strains. In T80 samples, *B. cereus* 6006 (1.56 log MPN per g) and 4227A (0.39 log MPN per g) survived for 84 days whereas in TS samples, *B. cereus* 4227A (0.17 log MPN per g) survived for 56 days and *B. cereus* 6006 (0.2 log MPN per g) survived for 28 days. The most resistant strain in T20 samples was *B. cereus* 4227A (0.66 log MPN per g), surviving for 28 days.

**Discussion**

In this study, the efficacy of the neutralizers, the dilution broths and the plating media was investigated for the detection of seven *Bacillus* strains inoculated in eye creams preserved with parabens. The study was carried on through day 84 because most cosmetic products do not have an expiration date and consumers utilize them until they are finished. The seven strains of *Bacillus* challenged in eye creams preserved with methyl- and propylparabens decreased over time, and drastically in the samples that were not neutralized. They were all undetected after 56 days except of *B. cereus* F 4227A, *B. mycoides* and *B. subtilis* were the most sensitive strains and remained undetected from the 3rd day.

Span and Tween are mild non-ionic surfactants; their use is not only limited as emulsifiers, solubilizers, wetting agents in a number of home-care applications, but also as inactivating agents of a wide range of biocides in cosmetics. In most cosmetics, parabens are used at levels ranging from 0.01% to 0.3%, and because of their synergistic effects, methyl- and propylparabens are used in a combination of 0.2% methylparaben and 0.1% propylparaben. In the EU’s Cosmetics Directive, the maximal concentration of paraben that can be used is 0.4% individually or 0.8% as a mixture of esters [27]. For this reason, we investigated the MBC of methyl- and propylparaben individually in the range between 0.01% and 1% in the broths first with and without neutralizers and the effect of the combination of 0.2% and 0.1% of methyl- and propylparaben, respectively, on the cells of *Bacillus*. The intention was to have a glimpse on the influence of the neutralizers on the parabens in a simple solution before the usage of a complex milieu such as the cream.

Methyl- and propylparaben used individually were bactericidal at low concentrations in TS B. The concentration of methyl- and propylparabens that inactivated the growth of the viable cells of *B. cereus* ATCC 6462 was 0.3% and 0.05%, respectively. Aalto et al. [28] found that 0.2% methyl- and 0.0125% propylparabens were the minimum inhibitory concentration need to inactivate *B. mycoides* ATCC 6462. The bactericidal concentration in MLB increased with 0.1% for methylparaben and by 4% for propylparaben, and additional neutralizers added to MLB augmented the bactericidal concentrations of both paraben with Tween 80 having
a greater influence on the antimicrobial effect of the preservatives. This is in agreement with other studies that stipulate that higher concentrations of parabens, compared to plain medium, are required in the presence of T80 to inhibit the growth of microorganisms [29, 30]. In the same study, Pisano and Kostenbauder demonstrated that the principal effect of Tween was to bind the parabens and there was no direct influence of the Tween on the growth of the organism. On the other hand, the combined parabens were bactericidal for all the organisms in TSB and MLB, but not when the neutralizers were added to ML B. These results showed that by adding the neutralizers in the cream for microbial analysis, the neutralizers could negate the effect of parabens in cosmetic creams for better detection of microorganisms. Studies have shown that low concentrations of non-ionic surfactants enhanced the effect of the biocide, whereas higher concentrations led to various degree of inactivation [31]. Neutralizers are known to neutralize these preservatives by the formation of a complex between the macromolecule and the parabens; Blaug and Ahsan [29] reported that the binding capacity increased with the molecular weight of the parabens, and also by the solubilization of the preservatives in the surfactant’s micelles [32, 33]. The use of neutralizers before the inoculation of the cells into cream samples reduced the inhibitory effect of the preservatives considerably with Tween 80 yielding a greater influence on the antimicrobial effect of the preservatives. Detection of higher cell concentrations and its neutralizing activity lasting longer than the ones of Tween 60+ Span 80 and Tween 20. Tween 60+ Span 80 in return yielded a high cell concentration just after 30 min, and the cells recovered in Tween 20 were reduced by ≤2 logs compared to the other neutralizers. For example, B. cereus X population was 4.3 in T80 samples. 4.19 in TS samples and 2.03 log CFU per g in T20 samples. The same population in the broth (MLB) where no additional neutralizer was added was 0.13 log MPN per g. This result showed that the neutralizers allowed the detection of B. cereus population after 30 min, which will be helpful for the testing of organisms in cosmetic products during microbial analysis. In general, the populations of B. cereus gradually decreased with the time and were not detected even after a pre-enrichment and remained under the detection limit (0.04 log MPN per g) except those of B. cereus F 4227A and F 60006 in T80 samples that survived for 84 days. This could be due to the resistance ability of some strains over the others, and also the pre-enriched samples that presented no cell growths were not heat shock for further investigations.

**BACARA**°, MYP and R&F agars presented a typical growth reaction with orange and pink colonies surrounded by a white halo for BACARA° and MYP, respectively, and homogeneous blue-turquoise colonies surrounded by a blue halo for R&F. We conclude that T80 was the best neutralizer. The chromogenic plating media BACARA° and R&F allowed good isolation and identification of B. cereus. In the light of this study, we will evaluate these plating media for the detection of B. cereus in heterogeneous milieu in different cosmetic products.

In conclusion, T80 and TS reduce the antimicrobial efficacy of the parabens and can be used in addition to the neutralizing broths (MLB or TAT) for the growth of B. cereus; BACARA° and MYP can be utilized for the detection of Bacillus cereus in eye creams. However, in the future, these agars will need to be tested with different cream formulations.

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