

Component Analysis of Multipurpose Contact Lens Solutions To Enhance Activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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More than 125 million people wear contact lenses worldwide, and contact lens use is the single greatest risk factor for developing microbial keratitis. We tested the antibacterial activity of multipurpose contact lens solutions and their individual component preservatives against the two most common pathogens causing bacterial keratitis, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The *in vitro* antibacterial activity of five multipurpose contact lens solutions (Opti-Free GP, Boston Simplus, Boston Advance, Menicare GP, and Lobob) was assayed by the standard broth dilution method. Synergy between the preservative components found in the top performing solutions was assayed using checkerboard and time-kill assays. The ISO 14729 criteria and the standard broth dilution method were used to define an optimized contact lens solution formulation against a clinical panel of drug-susceptible and drug-resistant *P. aeruginosa* and *S. aureus* strains. Preservatives with the biguanide function group, chlorhexidine and polyaminopropylbiguanide (PAPB), had the best antistaphylococcal activity, while EDTA was the best antipseudomonal preservative. The combination of chlorhexidine and EDTA had excellent synergy against *P. aeruginosa*. A solution formulation containing chlorhexidine (30 ppm), PAPB (5 ppm), and EDTA (5,000 ppm) had three to seven times more antipseudomonal activity than anything available to consumers today. A multipurpose contact lens solution containing a combination of chlorhexidine, PAPB, and EDTA could help to reduce the incidence of microbial keratitis for contact lens users worldwide.

There are an estimated 38 million contact lens users in the United States (1) and 125 million worldwide (2). Contact lens use is the single greatest risk factor for developing microbial keratitis (3), which can cause vision loss and blindness if not diagnosed and treated promptly. The U.S. Centers for Disease Control estimates that microbial keratitis affects 5 to 10 of every 10,000 contact lens wearers (2) and accounts for ~1 million clinic visits annually in the United States (1). Bacterial infections represent ~90% of all microbial keratitis cases, with *Pseudomonas aeruginosa* being the most common pathogen, followed by *Staphylococcus aureus* (2). The remaining 10% are associated with amoebae such as *Acanthamoeba castellanii* or with fungi, including *Fusarium solani* (2).

In 2008, representatives from the American Academy of Ophthalmology, Cornea Society, American Society of Cataract and Refractive Surgery, and the Contact Lens Association of Ophthalmologists all testified before the Ophthalmic Device Panel of the U.S. Food and Drug Administration about the need to enhance the antimicrobial efficacy of contact lens solutions (4). Subsequent studies have tested the efficacy of commercially available contact lens solutions against pathogens that cause keratitis (5, 6). However, these studies tested commercial solutions as a whole and have not evaluated the efficacy of each of the component antimicrobial preservatives against *P. aeruginosa* or *S. aureus*. Furthermore, a recent publication highlights the importance of testing the activity of solutions against clinical bacterial isolates rather than against the standard laboratory ISO ATCC *P. aeruginosa* and *S. aureus* strains (7).

We hypothesized that testing the efficacy of commercially available multipurpose contact lens solutions, as well as testing their component antimicrobial preservatives alone and in differ-

ent combinations, against clinical *P. aeruginosa* and *S. aureus* isolates would allow us to develop a formulation with more-potent antibacterial activity than anything currently available to consumers today.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* strains PAO1 and PA103 were obtained from the American Type Culture Collection (ATCC) and multidrug-resistant (MDR) *P. aeruginosa* strain P4 from a tertiary care academic hospital in New York. *S. aureus* strains were methicillin-resistant *S. aureus* (MRSA) TCH 1516 (USA300) from ATCC, Sanger 252 (USA200) from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA), and methicillin-susceptible *S. aureus* UAMS1 from G. Somerville (University of Nebraska). The following fluoroquinolone-susceptible (FQs) or fluoroquinolone-resistant (FQr) clinical keratitis isolates were obtained from the collection of the Charles T. Campbell Ophthalmic Microbiology Laboratory at the University of Pittsburgh: *S. aureus* K2751 (FQs), K2738 (FQr), and K2735 (FQr) and *P. aeruginosa* K2749 (FQs), PA13 (FQr), and PA16 (FQr).

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Preservatives and reagents. Mueller-Hinton broth (MHB; Spectrum Chemicals) was supplemented with CaCl_2 and MgCl_2 to make cation-adjusted MHB (Ca-MHB) (final cation concentrations, 20 to 25 mg/liter Ca^{2+} and 10 to 12.5 mg/liter Mg^{2+}). Other reagents were obtained from the following vendors: Luria Broth base (LB) from Hardy Diagnostics; Todd-Hewitt Broth base (THB) from Neogen; EDTA from Sigma; chlorhexidine gluconate (CHD) from Sigma; polyaminopropyl biguanide (PAPB) from Lotioncrafter; resazurin sodium from Sigma; and Difco D/E neutralization broth from BD.

Multipurpose contact lens solutions. Opti-Free GP (Alcon), Boston Simplus and Boston Advance (Bausch & Lomb), Menicare GP (Menicon), and Lobob (Lobob Labs) were purchased from Amazon.

Contact lenses. Senofilcon A soft silicon hydrogel lenses (Acuvue Oasys; Johnson & Johnson Vision) were purchased from Lens.com, Inc. These contact lenses were chosen because they represent a leading extended-wear silicon hydrogel lens approved by the FDA for up to 14 days of extended wear.

Determination of MICs. MIC values for contact lens solutions and their preservative components were determined using broth microdilution in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (8). Bacterial viability was determined using an optical plate reader (at an optical density at 600 nm [OD_{600}]) and resazurin indicator dye as previously described (9).

For individual contact lens solution preservatives such as CHD, PAPB, EDTA, and benzyl alcohol, concentrated stocks were purchased and then diluted in sterile water to 10 times the concentrations used in the contact lens solutions. The broth microdilution method outlined in the CLSI guidelines mentioned above was applied as follows: in the first row of wells in a 96-well plate, we added 20 μl of 5×10^6 CFU/ml of bacteria suspended in Ca-MHB, 20 μl of the 10 \times preservative, and 160 μl of Ca-MHB. Thus, the first row of wells on the 96-well plate contained 200 μl of liquid in total with 5×10^5 CFU/ml bacteria and a 1 \times concentration of the preservative. Serial dilutions were performed by transferring 100 μl from the first well into 100 μl of Ca-MHB with 5×10^5 CFU/ml bacteria in the second row of wells, mixing well, and then repeating down the rows.

MIC testing of the contact lens solutions and our optimized formulation was performed using an adaptation of the CLSI broth microdilution guidelines for antibiotics. In the first row of wells in the 96-well plate, 20 μl of 5×10^6 CFU/ml of bacteria suspended in Ca-MHB was combined with 180 μl of contact lens solution. Thus, the first well contained 10% Ca-MHB and 90% contact lens solution with 5×10^5 CFU/ml bacteria. Serial dilutions were performed by transferring 100 μl from the first well into 100 μl of Ca-MHB with 5×10^5 CFU/ml bacteria in the second row of wells, mixing well, and then repeating down the rows. Thus, going down a vertical column of wells in the 96-well plate, every well would have 5×10^5 CFU/ml bacteria, followed by a decreasing percentage (90%, 45%, 22.5%, 11.3%, 5.6%, 2.8%, 1.4%, or 0.7%) of contact lens solution and a corresponding increase in the percentage of Ca-MHB.

Traditional checkerboard and time-kill assays. Traditional checkerboard and time-kill assays were performed as previously described (10). Overnight cultures of *P. aeruginosa* (in LB) and *S. aureus* (in THB) were grown at 37°C, pelleted, washed twice, and resuspended in phosphate-buffered saline (PBS) to an OD_{600} of 0.40. Bacterial stocks were then diluted in Ca-MHB to an initial inoculum of $\sim 1 \times 10^6$ CFU/ml and contact lens solution preservatives added at the indicated concentrations. For the checkerboard assay, 96-well plates were incubated with shaking at 37°C for 20 h, the OD_{600} was monitored, resazurin was added (final concentration, 3.38 ng/ml), and color changes were assessed after 24 h of incubation at 37°C. For the time-kill assays, 96-well plates were incubated with shaking at 37°C. Aliquots (20 μl) of test solutions were taken at the indicated time points, serially diluted, and plated for CFU enumeration.

ISO 14729 assay. The ISO 14729 assay was performed as previously described (11). Briefly, 500 μl of washed and concentrated bacteria was added to 4,500 μl PBS containing CHD (30 ppm), PAPB (5 ppm), and EDTA (5,000 ppm) to reach a concentration of 1×10^6 CFU/ml, mixed,

and incubated for 1 h at room temperature. A 100- μl volume of test solution was removed, serially diluted in Dey-Engley neutralizing broth, and plated for CFU enumeration.

Evaluation of the effect of the C30/P5/E5000 formulation on contact lens biofilms. Biofilm studies employed a published technique (12). Briefly, senofilcon A lenses were washed with PBS and then placed in 12-well tissue culture plates with 4 ml of bacterial cell suspensions; overnight cultures were washed twice with PBS and diluted in PBS to reach an absorbance value of 0.1 at 660 nm. Lenses were incubated at 37°C for 120 min to allow adhesion of bacteria to the lens surface (adherence phase). Lenses were then transferred to new 12-well plates containing 4 ml of fresh PBS. Each lens was then placed in an Eppendorf tube filled with 2 ml of 1% THB or 1% (wt/vol) LB for *S. aureus* or *P. aeruginosa*, respectively, and was rotated at 37°C for 24 h (biofilm formation phase). Each lens was then washed in fresh PBS for 5 s to simulate the rinsing step and placed in 4 ml of CHD at 30 ppm, PAPB at 5 ppm, and EDTA at 5,000 ppm (C30/P5/E5000) or 4 ml of a PBS control and incubated at room temperature for 4 h. Lenses were washed again in fresh PBS for 5 s and transferred to a 1.5 ml Eppendorf tube containing 1 ml of PBS and 1-mm-diameter silicon beads. In order to break up the biofilm on the contact lenses, the tubes were rigorously shaken at 6,000 rpm for 1 min twice, with 1 min of cooling down on ice between agitations. The bacterial suspensions were serially diluted in Dey-Engley neutralizing broth, and serial dilutions were plated on THB and LB agar plates for *S. aureus* and *P. aeruginosa*, respectively, to evaluate viability.

RESULTS

Activity of five commercial contact lens solutions against MRSA and *P. aeruginosa*. We tested five multipurpose contact lens solutions from major manufacturers in the United States: Boston Simplus, Boston Advance, Opti-Free, Menicare GP, and Lobob. The antibacterial preservatives found in each solution are listed in Fig. 1A. The MIC of each solution against methicillin-resistant *S. aureus* (MRSA) TCH 1516 and *P. aeruginosa* PAO1 was determined by CLSI broth microdilution methodology (8). Boston Simplus had the most potent antistaphylococcal activity, with a MIC of 1.5% (Fig. 1B), while Menicare GP had the most potent antipseudomonal activity, with a MIC of 23% (Fig. 1C). All multipurpose solutions tested were less effective against *P. aeruginosa* than against MRSA. The same results were observed when we tested the multipurpose contact lens solutions against three *S. aureus* and three *P. aeruginosa* clinical keratitis isolates (see Fig. S1 in the supplemental material).

Preservatives with a biguanide functional group have the highest anti-MRSA activity, while EDTA has the highest antipseudomonal activity. We sought to determine which preservative(s) found in each top performing solution yielded the antibacterial effects observed. Boston Simplus, with the highest anti-MRSA activity, utilizes the biguanide-containing preservatives CHD and PAPB. CHD and PAPB were equally active against MRSA, with MICs of 2.5 ppm and 2.5 ppm, and were less active against *P. aeruginosa*, with MICs of 15 ppm and 20 ppm, respectively. No synergy of CHD and PAPB in combination was observed for either MRSA or *P. aeruginosa* (Fig. 1D). Menicare GP, the most active solution against *P. aeruginosa*, utilizes EDTA and benzyl alcohol as preservatives. The MICs of EDTA were 2,500 ppm against *P. aeruginosa* and 300 ppm against MRSA. The MICs of benzyl alcohol were 5,000 ppm against *P. aeruginosa* and 10,000 ppm against MRSA. Synergy of EDTA and benzyl alcohol was observed against *P. aeruginosa* but not against MRSA (Fig. 1E).

CHD and EDTA are synergistic against *P. aeruginosa*. Using checkerboard assays to test combinations of component pre-

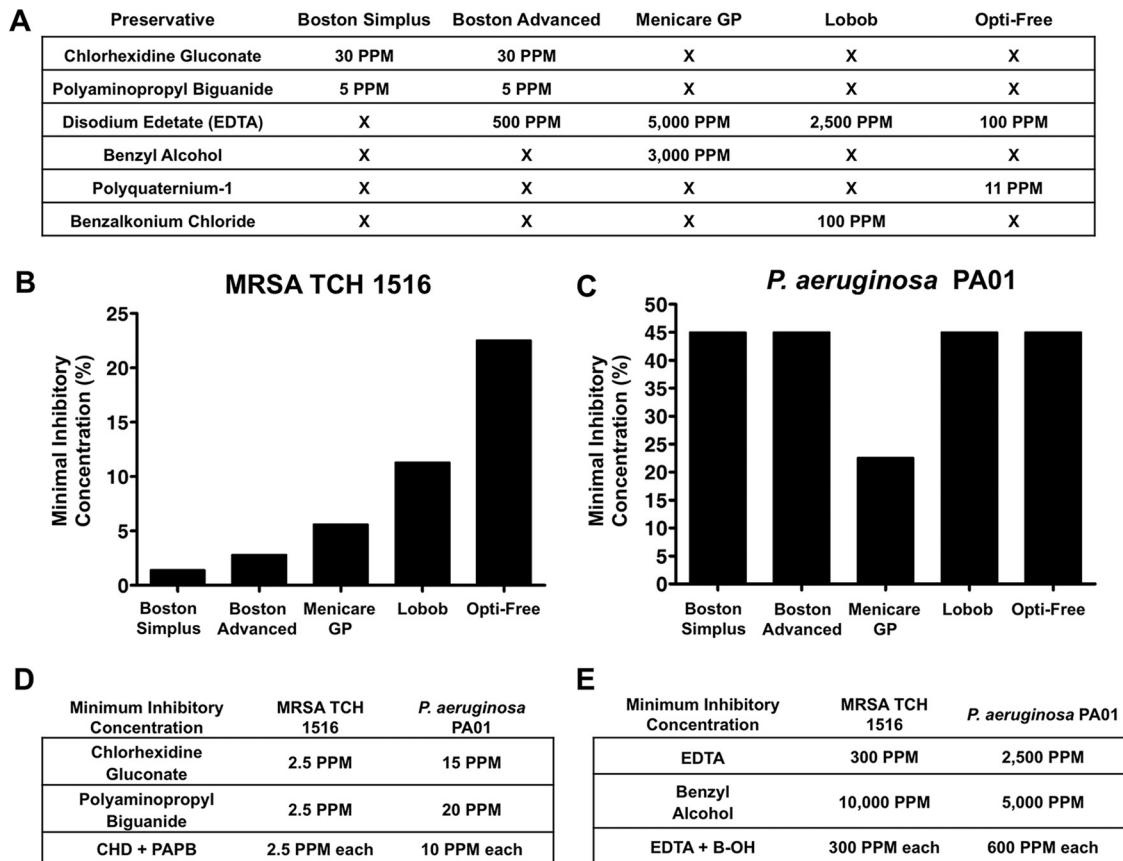


FIG 1 Antibacterial efficacy of multipurpose contact lens solutions and their individual preservative components against *P. aeruginosa* and *S. aureus*. (A) The preservative concentrations of each of the contact lens solutions tested. (B and C) Contact lens solutions were serially diluted in cation-adjusted Mueller-Hinton broth, and MICs for MRSA TCH 1516 (B) and *P. aeruginosa* PA01 (C) were determined by CLSI broth microdilution methodology. (D and E) The MICs of individual preservatives determined by CLSI broth microdilution methodology. All data are representative of results of 3 independent experiments. B-OH, benzyl alcohol.

servatives found in Boston Simplus and Menicare GP, we discovered that the most potent synergistic combination against *P. aeruginosa* was CHD plus EDTA (Fig. 2A). Used together, a solution of 4 ppm CHD (~1/4 MIC) plus 300 ppm EDTA (~1/8 MIC) was sufficient to eradicate *P. aeruginosa*, with a corresponding fractional inhibitory concentration index of 0.39. The bactericidal activity of this combination was extremely rapid, with a $>4 \log_{10}$ reduction in *P. aeruginosa* numbers in 2 h in quantitative killing assays (Fig. 2B). Synergy of CHD plus EDTA was also observed against three *P. aeruginosa* clinical keratitis isolates, with fraction inhibitory concentration index values of less than 0.13 as calculated by checkerboard assays (see Fig. S2 in the supplemental material).

A formulation of CHD, PAPB, and EDTA showed excellent antibacterial activity against MRSA and *P. aeruginosa*. The combination of CHD and PAPB in Boston Simplus had strong activity against MRSA (Fig. 1B), but the EDTA concentration in this product is too low for synergy against *P. aeruginosa*. Such synergy was achieved by combining the EDTA concentration of Menicare GP with the CHD and PAPB concentrations of Boston Simplus. A formulation of CHD at 30 ppm, PAPB at 5 ppm, and EDTA at 5,000 ppm (C30/P5/E5000) satisfies the international criteria for contact lens solution efficacy against bacterial pathogens described in ISO 14729. In just 1 h, the concentrations of

MRSA and *P. aeruginosa* were reduced by $>4 \log_{10}$ (Fig. 2C), which was far less than the manufacturer's recommended disinfection time for either Boston Simplus (4 h) or Menicare GP (6 h). A $>4 \log_{10}$ reduction in CFU per milliliter was also observed after just 1 h against all six clinical keratitis isolates (see Fig. S3 in the supplemental material). The C30/P5/E5000 formulation was also extremely effective against a panel of clinical *S. aureus* and *P. aeruginosa* isolates, including MRSA and multidrug-resistant *P. aeruginosa*, as well as against our six clinical keratitis strains. The favorable MIC of C30/P5/E5000 was 3% to 6% against all strains tested (Fig. 2D).

The C30/P5/E5000 formulation is able to eradicate *S. aureus* and *P. aeruginosa* biofilms that have formed on contact lens surfaces. Among all isolates or our clinical keratitis isolates, *S. aureus* K2738 and *P. aeruginosa* K2749 were the most mucoid and robust biofilm producers. We used a previously published protocol (12) to grow mature *S. aureus* K2738 and *P. aeruginosa* K2749 biofilms on a popular brand of silicon hydrogel lenses. Treatment of these biofilm-coated contact lenses with C30/P5/E5000 for 4 h at room temperature, the minimum recommended disinfection time for most multipurpose contact lens solutions, resulted in a $>4 \log_{10}$ reduction in the numbers of viable *S. aureus* and *P. aeruginosa* (Fig. 3).

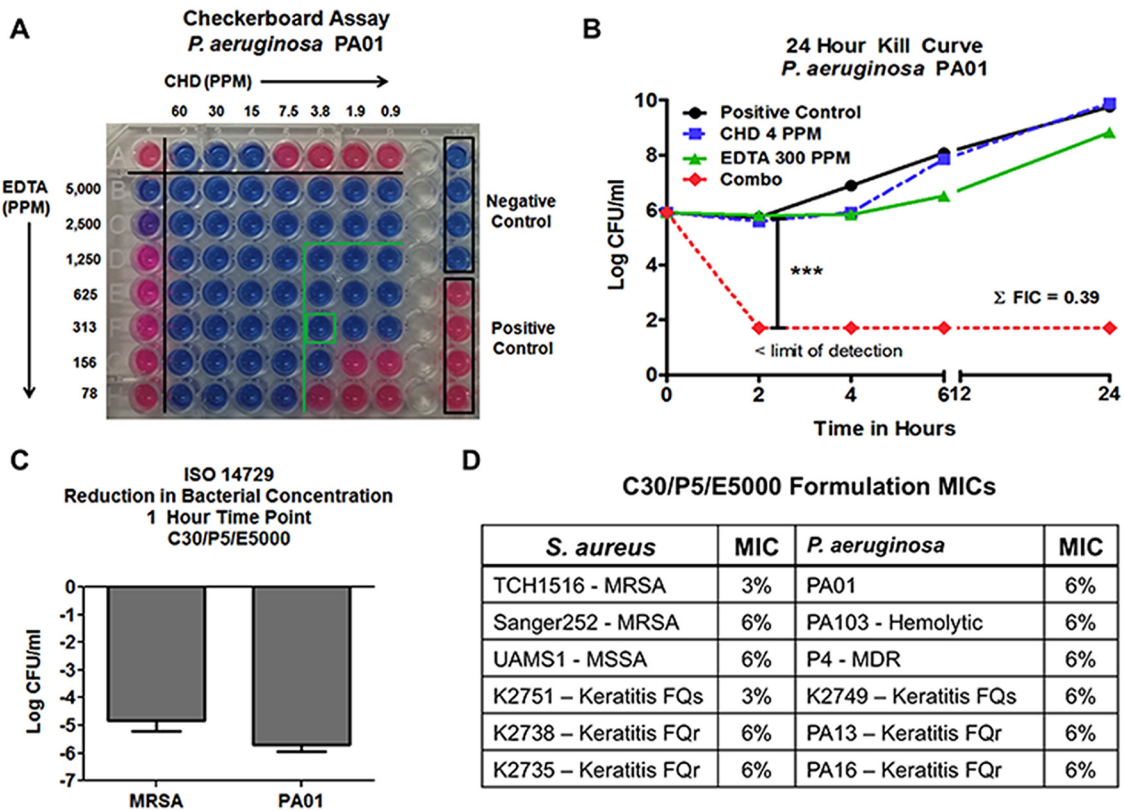


FIG 2 Synergy of CHD plus EDTA against *P. aeruginosa* and optimized activity of CHD plus PAPB plus EDTA against both *P. aeruginosa* and *S. aureus*. (A) Checkerboard assay testing the combination of CHD and EDTA against *P. aeruginosa*. A resazurin probe was used to assess bacterial viability, which is indicated as follows: blue, no viable bacteria; red, viable bacteria. Blue wells bounded by green bars in the bottom right quadrant have fractional inhibitory concentration (FIC) values of <0.5. The green box demarks approximate concentrations used in the time-kill curve analysis whose results are shown in panel B. (B) Time-kill curve. Data plotted are means \pm standard errors of the means (SEM) and represent the averages of results of triplicates from 3 independent experiments. ***, $P < 0.001$ (two-way analysis of variance [ANOVA]). (C) Reduction in concentrations of bacteria after 1 h of incubation in a phosphate-buffered saline solution containing CHD (30 ppm), PAPB (5 ppm), and EDTA (5,000 ppm). ISO 14729 testing guidelines were followed. Data plotted are means \pm SEM and represent the averages of results of 3 independent experiments. (D) MICs of the C30/P5/E5000 formulation against a panel of clinical *P. aeruginosa* and *S. aureus* isolates, including multidrug-resistant strains. MSSA, methicillin-susceptible *S. aureus*.

DISCUSSION

With millions of daily users, contact lens-related microbial keratitis continues to be a significant health problem. Contact lenses interfere with several innate immune defense mechanisms of the

eye (13). Furthermore, most contact lens users are noncompliant with proper lens cleaning and care procedures (14), with significant percentages reporting reuse of old contact lens solution or topping off their existing solution each night. In this setting, a contact lens solution with rapid killing activity against the major keratitis-causing pathogens, even when diluted significantly, could reduce the incidence of keratitis. By harnessing the synergy of CHD and EDTA against *P. aeruginosa*, the C30/P5/E5000 formulation has 3 to 7 times more antipseudomonal activity than any of the commonly used multipurpose contact lens solution available today. C30/P5/E5000 is also equivalent to the best solutions tested against *S. aureus*. Finally, C30/P5/E5000 demonstrates excellent activity against both planktonic and biofilm-associated keratitis isolates of *P. aeruginosa* and *S. aureus*. Since CHD and PAPB are effective against acanthamoebae (15) and fungal eye pathogens (16) and EDTA is effective against *P. aeruginosa* biofilms and *S. aureus* biofilms (17–19), a C30/P5/E5000 formulation could provide a one-step solution to reducing contact lens-related keratitis of all causes.

Silicon hydrogen contact lens, 24 h bacterial biofilms Treatment with C30/P5/E5000 for 4 h

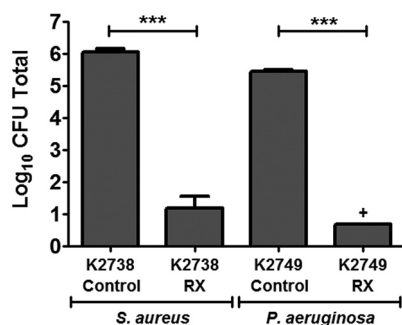


FIG 3 Treatment of *S. aureus* and *P. aeruginosa* biofilms formed on silicon hydrogel contact lenses with the C30/P5/E5000 formulation. Data plotted are means \pm SEM, with 6 contact lenses per group. ***, $P < 0.001$ (two-tailed Student's *t* test). +, below the limit of detection.

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