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# A novel comprehensive efficacy test for textiles intended for use in the healthcare setting

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ABSTRACT

Soft surfaces, including textiles are found throughout healthcare settings. Pathogens can survive for long periods of time on textiles, and can be transferred to and from the skin. Antimicrobial fabrics are used as an engineering control to prevent infection. Efficacy testing standards have limitations, including single microorganism challenges, multiple fabric plies tested, and lengthy contact times. We developed a novel method that better models in-use conditions through testing standardized mixtures of pathogens and normal skin microorganisms, artificial soils, and a 15-min contact time. Reproducible growth of all microorganisms from frozen stocks was achieved using this method. A novel rechargeable, monitorable N-halamine cotton cellulose fabric, containing 5885 ± 98 ppm of active chlorine, was evaluated with the new method using PBS, artificial sweat, and artificial sweat plus 5% serum as soil. Pathogens tested included *Acinetobacter baumannii*, *Candida albicans*, *Escherichia coli*, vancomycin-resistant *Enterococcus faecalis*, methicillin-resistant *Staphylococcus aureus,* methicillin-susceptible *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Each was tested singly and in the presence of a representative normal skin flora mixture, including: *Acinetobacter lwoffii*, *Corynebacterium striatum*, *Micrococcus luteus*, and *Staphylococcus epidermidis*. When tested singly, all microorganisms were reduced by 3.00 $\log_{10}$ or greater, regardless of artificial soil. In mixture, 4.00 $\log_{10}$ or greater reductions were achieved for all microorganisms. These results suggest that the novel testing method can be used to provide more comprehensive and realistic efficacy information for antimicrobial textiles intended for use in healthcare. Furthermore, the N-halamine fabric demonstrated efficacy against multiple pathogens, singly and in mixtures, regardless of the presence of artificial soils.

## 1. Introduction

Soft surfaces are found abundantly in the healthcare setting ranging from healthcare worker attire and wound dressings, to bed linens, privacy curtains, and upholstered furniture (Adlhart et al., 2018; Ahonen et al., 2017; Dunne et al., 2017; Koca et al., 2012; Mitchell et al., 2015). Similar to hard surfaces, soft surfaces risk becoming contaminated with pathogenic microorganisms (Burden et al., 2011; Burden et al., 2013; Du et al., 2017; Sanon and Watkins, 2012; Sattar et al., 2001; Treakle et al., 2009; Wiener-Well et al., 2011). Due to their large surface area and porous nature, soft surfaces are prone to microbial growth and biofilm development, particularly in the presence of appropriate nutrient, temperature, and moisture levels. Numerous studies have demonstrated the persistence of pathogens on hospital textiles, some of which can survive on soft surfaces for months) (Kramer et al., 2006; Lopez-Gigosos et al., 2014). Additionally, bacteria can be transferred from contaminated textiles to human skin, especially when the fabrics are moist (Sattar et al., 2001).

Because of the growing public health awareness of soft surface contamination, particularly in the healthcare setting, extensive research and development have been devoted to the generation of textiles hostile to microbial growth. Antimicrobial textiles are an effective strategy for reducing the bioburden on soft surfaces (Adlhart et al., 2018; Ahonen et al., 2017; Lopez-Gigosos et al., 2014; Morais et al., 2016; Santos et al., 2016; Sun et al., 2012; Treakle et al., 2009; Wiener-Well et al., 2011). Use of antimicrobial textiles as an engineering control in the healthcare setting has been proposed to mitigate the cross-contamination and spread of healthcare-associated infections (HAIs). Antimicrobial agents can either be incorporated into materials or grafted to fabric surface as a finishing agent after assembly of the basic fabric

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**Table 1**
Summary of major antimicrobial agents utilized in antimicrobial textiles.

| Antimicrobial agent | Mode of action | Fibers | References |
|---|---|---|---|
| Chitosan | Low molecular weight: inhibits mRNA synthesis, impacting protein synthesis<br>High molecular weight: blocks transport of essential solutes into the cell | Cotton, Polyester, Wool | Adlhart et al., 2018, Ahonen et al., 2017, Morais et al., 2016, Santos et al., 2016 |
| Metals and Metallic Salts | Generates reactive oxygen species<br>Damages cellular proteins, lipids, and DNA | Cotton, Polyester, Nylon, Wool | Adlhart et al., 2018, Ahonen et al., 2017, Dunne et al., 2017, Morais et al., 2016, Santos et al., 2016 |
| N-halamine | Interferes with critical cellular enzymes and metabolic processes | Cotton, Polyester, Nylon, Wool | Luo and Sun, 2006, Sun et al., 2012 |
| Polyhexamethylene Biguanide (PHMB) | Disrupts integrity of cell membrane | Cotton, Polyester, Nylon | Santos et al., 2016 |
| Quaternary Ammonium Compounds | Damages cell membranes, denatures proteins, and inhibits DNA synthesis | Cotton, Polyester, Nylon, Wool | Adlhart et al., 2018, Ahonen et al., 2017, Dunne et al., 2017, Morais et al., 2016, Santos et al., 2016 |
| Triclosan | Blocks lipid synthesis and disrupts cell membrane | Polyester, Nylon, Polypropylene, Cellulose Acetate, Acrylic | Morais et al., 2016 |

(Adlhart et al., 2018; Burden et al., 2013; Koca et al., 2012; Kramer et al., 2006; Mitchell et al., 2015; Sanon and Watkins, 2012; Sattar et al., 2001). To maximize benefit, the antimicrobial finishing should be effective against a broad range of microorganisms with low toxicity to the user and the environment. Additionally, the antimicrobial finishing should be durable throughout its service life, and be able to endure laundering, drying, and day-to-day use. Lastly, antimicrobial finishes should ideally not impair the textile appearance or texture (Morais et al., 2016). Numerous antimicrobial textiles are currently available, each of which differs in the mode of action of their microbial components (Table 1).

One of the most interesting antimicrobial approaches, and the textile used in this study, is the rechargeable N-halamine based fabric. The characteristics of this fabric have been previously published in detail (Luo and Sun, 2006). In brief, N-halamines are compounds containing one or more nitrogen-halogen bonds, which demonstrate a broad spectrum of biocidal activity. The antimicrobial properties of N-halamines are based on the exchange of halogen (commonly chlorine) atoms with microorganisms in the presence of water. The N-halamine-based fabric possesses several desirable characteristics suitable for application in the healthcare setting (Adlhart et al., 2018; Ahonen et al., 2017; Luo and Sun, 2006). N-halamine textiles: 1) are highly stable for a long period of time in ambient temperatures, 2) have a low dissociation constant, which makes the N-halamine much more stable and safer for human use than free chlorine or bleach, 3) are compatible with nylon, polyester, acrylics, and cotton-cellulose, 4) can be synthesized with specific concentrations (%) of N-halamine, 5) have the ability to be regenerated or recharged with a diluted bleach rinse after the halogen functional groups have been consumed, and 6) are monitorable for antimicrobial action (active chlorine components).

Effective testing is the key to understanding the performance of antimicrobial textiles. There are several published standards for assessing antimicrobial efficacy that include both qualitative (absorption or halo-methods) and quantitative methods (see Table 2). While the existing standards can be utilized to assess antimicrobial activity of individual textiles, they have different testing parameters and procedures. The lack of standardization and differences between each method analyzing antimicrobial activity makes meaningful comparison between the performance of different fabrics challenging (Pinho et al., 2011). The purported antimicrobial activity of one fabric determined by one method cannot readily be compared to antimicrobial activity determined by a different method. Furthermore, there exists limited data comparing the performance of distinct antimicrobial textiles in real-world scenarios.

Currently the most commonly applied standards in the U.S are the qualitative American Association of Textile Chemists and Colorists AATCC 147:2019 “Assessment of Textile Materials: Parallel Streak Method” and the quantitative AATCC 100:2011(2016e) “Antibacterial Finishes on Textile Materials: Assessment of” (AATCC, 2016; AATCC, 2019). Both standards specify testing against pure cultures of *Staphylococcus aureus* and *Klebsiella pneumoniae* as representative Gram-positive and Gram-negative pathogens. Additional microorganisms may be included at the discretion of the testing party, but are not mandated. Additionally, the standards do not define pass-fail criteria, ultimately allowing the manufacturer or testing party to determine its own antimicrobial success. The AATCC 147 method is easily reproducible and relatively easy to perform, and provides qualitative data on the fabric's bacteriostatic capabilities. The AATCC 100 is a quantitative method, but it does not specify the final concentration of bacteria to be tested (the protocol states adjust to ‘appropriate dilution’). In addition, the test swatches utilized must be able to absorb 1 mL of liquid, therefore more than one fabric swatch (comparable to plies of fabric) are commonly utilized since a single swatch usually cannot absorb the full volume, even if the proposed use of the textile would be single ply.

Standardized in-lab qualitative and quantitative tests are an essential first step in evaluating the antimicrobial activity of a fabric. However, for textiles intended for use in healthcare, there are several aspects of real-world application that are not reflected in these protocols. Antimicrobial textiles can be marketed and utilized in the healthcare setting without accompanying data validating the performance of the textile under actual use conditions. We hypothesized that a novel, more comprehensive, efficacy test for antimicrobial healthcare textiles could be used to evaluate a textile's performance in the presence of: 1) pure cultures of important HAI-causing microbes and normal skin microorganisms, 2) reproducible mixtures of skin microorganisms with pathogens, and 3) artificial soils, including artificial sweat and 5% serum. We also hypothesized that the N-halamine fabric would effectively reduce microorganism contamination under the conditions tested. Our protocol builds on the existing methods to provide a broader and more realistic characterization of the antimicrobial efficacy of fabrics intended for use in healthcare.

## 2. Materials and methods

### 2.1. Bacterial strains and materials

All bacterial strains were purchased from the American Type Culture Collection (ATCC, Manassas VA). Lyophilized samples were rehydrated and cultured onto appropriate media and subcultured at least 3 times before use. Pathogens included: *Acinetobacter baumannii* (ATCC 19606), *Candida albicans* (ATCC 24433), *Escherichia coli* (ATCC 29214), vancomycin-resistant *Enterococcus faecalis* (ATCC 51575), methicillin-resistant *Staphylococcus aureus* (ATCC 43300), methicillin-susceptible *Staphylococcus aureus* (ATCC 6538), and *Pseudomonas aeruginosa* (ATCC 10145). Representative normal skin flora included: *Acinetobacter lwoffii* (ATCC 15309), *Corynebacterium striatum* (ATCC

**Table 2**
Summary of existing antimicrobial textile testing standards.

| Reference | Principle | Summary |
|---|---|---|
| AATCC 147 | Qualitative | Agar plates are inoculated with 5 parallel streaks of *K. pneumoniae* or *S. aureus*. Textile test swatch placed in direct contact with surface of agar and incubated for 24 h. Activity assessed by zone of inhibition over 5 streaks or absence of growth directly behind fabric swatch. |
| AATCC 100 | Quantitative | Multiple textile test and control swatches are inoculated with 1 mL overnight growth of *K. pneumoniae* or *S. aureus* in nutrient broth and incubated at 37 °C for 24 h. Comparing the viable cell count from control to test swatch assesses activity. |
| JIS L 1902 Halo-Method | Qualitative | Molten nutrient agar is inoculated with a suspension of *K. pneumoniae* or *S. aureus*, added to sterile petri plates, and allowed to solidify. Fabric is placed in direct contact with surface of agar and incubated overnight at 37 °C for 24–48 h. Activity is assessed by zone of inhibition around fabric swatches. |
| JIS L1902, 2015 | Quantitative | Control and test swatches are incubated with *K. pneumoniae* or *S. aureus* suspended in heavily diluted nutrient medium. Samples are incubated at 37 °C for specific contact time. Antimicrobial activity is assessed by comparing the viable cell count from control to the test swatch. No neutralizer is applied. Pass – fail criteria provided. |
| ISO 20645, 2004 | Qualitative | Molten nutrient agar is inoculated with a suspension of *E. coli, K. pneumoniae,* or *S. aureus*, added to a sterile petri plates, and allowed to solidify. Four test fabric swatches are placed in direct contact with a separate sterile nutrient agar plate. Fabric samples are overlaid with inoculated petri plate. Plates are incubated for 18–24 h. Activity is assessed with zone of inhibition in overlaying petri plate. |
| ISO 20743, 2013 | Quantitative | Three test and control swatches are inoculated with pure *K. pneumoniae* or *S. aureus* suspended heavily diluted nutrient medium. Swatches are incubated at 37 °C for 18–24 h. Antimicrobial activity is assessed by comparing the viable cell count from control to the test swatch. No neutralizer is applied. Pass – fail criteria provided. |
| ASTM E2149 – 13a, 2013 | Quantitative | *K. pneumoniae* in nutrient broth is diluted in sterile buffer to standardize starting inoculum, and added to sterile jars. Control and test swatches are added to jars and agitated for 1 h. Antimicrobial activity is assessed by comparing the viable cell count before and after contact time. |

BAA1293), *Micrococcus luteus* (ATCC 49732), and *Staphylococcus epidermidis* (ATCC 14900).

All bacteriological media, chemicals, and disposable laboratory supplies were purchase from ThermoFisher Scientific (Watham MA) unless otherwise noted.

#### 2.1.1. Preparation of individual microorganism testing inoculum for single microorganism challenge

Two well-isolated colonies from the stock plate were aseptically transferred to a sterile 1 mL BHI broth and incubated overnight at 37 °C with agitation. The overnight suspension was aseptically transferred to sterile 1.5 mL microcentrifuge tubes and centrifuged at 6000 RPM for 10 min. The supernatant was removed using aseptic technique with care not to disturb the microbial pellet. The pellet was resuspended with selected soils – PBS, artificial sweat, or 5% serum in artificial sweat. The suspension was standardized spectrophotometrically (600 nm) to about $7.5 \times 10^7$ CFU/mL.

### 2.2. Preparation of microorganism mixtures for use in mixture challenge

The goal of the mixture was to grow representative countable colonies of each individual component of the mixture. In order to achieve inter-trial reproducibility, mixtures were prepared and frozen in large batches. This procedure has 4 main steps: 1) freezing individual microorganisms at a consistent absorbance, 2) identifying the concentration of microorganism in frozen aliquot, 3) mixing specific quantities of each to obtain the desired concentration, and 4) preparing frozen aliquots of the final mixture.

Step 1: For each microorganism to be incorporated into the mixture, 5 well-isolated colonies from the stock plate were aseptically transferred to sterile 5 mL BHI broths in duplicate. BHI broths were incubated at 37 °C with agitation to about mid-exponential phase, as determined by a standard growth curve procedure. Individual microorganisms were adjusted spectrophotometrically to 0.582 absorbance at 600 nm. The adjusted microorganism was mixed in a 1:1 ratio with 40% sterile glycerol (final 20% glycerol) in a sterile 15 mL tube and vortexed for 2 min. One mL of the microorganism-glycerol preparation was aliquoted to sterile low-temperature cryovials and frozen at −80 °C. A purity plate was set up with remaining microorganism-glycerol preparation.

Step 2: A single cryovial of each microorganism was thawed at room temperature and subsequently vortexed for 1 min to homogenously resuspend microorganisms. The vial was serial diluted in PBS to $1{:}10^{10}$. One hundred μL of each dilution was plated in duplicate to sterile BHI plates using the spread plate method. Plates were incubated at 37 °C overnight. Each dilution was evaluated for growth, and CFU per mL for the frozen stock was calculated.

Step 3: In order to make 10 mL of mixed microorganisms with a concentration of $7.5 \times 10^7$C FU/mL, the volume of each individual microorganism to be mixed was calculated using the formula (C1)(V1) = (C2)(V2). A sample table utilized to calculate microorganism mixtures is shown in Table 3. The media into which each microorganism was mixed was a 1:1 mixture of PBS in 40% glycerol (effective 20% glycerol) at the pre-determined final mixture volume.

Step 4: Once each microorganism was added to the PBS - glycerol solution, the tube was vortexed for 2 min. One mL aliquots were prepared and frozen at −80 °C.

### 2.3. Preparation of artificial soils

The artificial soils utilized in this protocol were: sterile phosphate buffered saline (PBS, the control soil), sterile artificial sweat, and sterile 5% serum in artificial sweat. All solutions were stored at 4 °C.

An artificial sweat solution was prepared according to the European Standard EN 1800:2011 using sodium chloride, sodium hydroxide, urea, and lactic acid and pH adjusted to 6.5. To generate the serum in artificial sweat solution, sterile pooled human serum was added to an artificial sweat solution to a final concentration of 5%.

### 2.4. N-halamine fabric synthesis

The synthesis method for the N-halamine fabric has been previously published (Luo and Sun, 2006). In brief, free radicals were generated on

**Table 3**
Example of a calculation table utilized to generate mixtures of microorganisms.

| | C1 | V1 | C2 | V2 |
|---|---|---|---|---|
| Microorganism | Stock titer (in 1 mL) | Volume required of each stock (mL) | Desired final concentration (CFU/mL) | Desired final volume (mL) |
| *A. lwoffii* | $2.90 \times 10^{10}$ | $1.29 \times 10^{-2}$ | $1.50 \times 10^7$ | 25 |
| *C. striatum* | $5.95 \times 10^8$ | $6.30 \times 10^{-1}$ | $1.50 \times 10^7$ | 25 |
| *M. luteus* | $1.50 \times 10^9$ | $2.50 \times 10^{-1}$ | $1.50 \times 10^7$ | 25 |
| *S. epidermidis* | $7.70 \times 10^9$ | $4.87 \times 10^{-2}$ | $1.50 \times 10^7$ | 25 |
| *C. albicans* | $4.70 \times 10^8$ | $7.98 \times 10^{-1}$ | $1.50 \times 10^7$ | 25 |

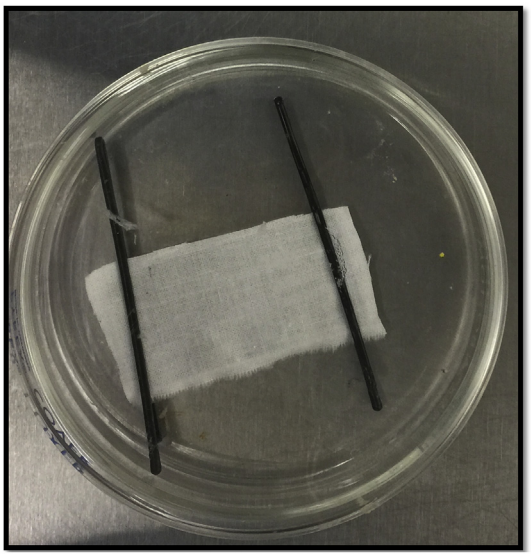

**Fig. 1.** Fabric assembly for testing. Fabric swatch is wrapped around a 6.0 × 3.5 cm stainless steel coupon and fastened with bobby pins. The entire assembly is within a sterile glass petri dish.

the cotton cellulose backbone molecules in order to graft methacrylamide (MMA) onto the cotton cellulose fabric. Once MMA monomers are polymerized onto the fabric, a diluted bleach solution (pH 7) is used to convert some of the amide groups to N-halamines. Fabric was washed 8–10 times in distilled water and allowed to dry in ambient air. The available chlorine content (% chlorine) of the fabric was determined by iodometric titration. The N-halamine fabric prepared for this study contained 5885 ± 98 ppm of active chlorine.

### 2.5. Test procedure

Rectangular swatches of control (untreated) and test (N-halamine) fabrics were cut to about (6.0 × 3.5 cm) ± 0.5 cm. One-ply fabric swatches were wrapped around non-absorbent 6.0 × 3.5 cm stainless steel coupons (Metal Samples Co, Munford, AL) and secured using two metal bobby pins (purchased at a local retailer). Assembled carriers were placed in glass petri plates (Fig. 1). Before use, assembled control fabric swatches and carriers were autoclaved.

A single test procedure includes a total of 4 test swatches: positive control (non-antimicrobial fabric with bacteria), negative control (antimicrobial fabric with no bacteria), and 2 tests (antimicrobial fabric with bacteria). The positive control and 2 test swatches were inoculated with 10 μL of the standardized bacterial suspension in selected soil (PBS, artificial sweat, or 5% serum in artificial sweat) to the center of the fabric within the glass petri plate and incubated at room temperature for 15 min (15-min contact time). The negative control was not inoculated with the microbial suspension.

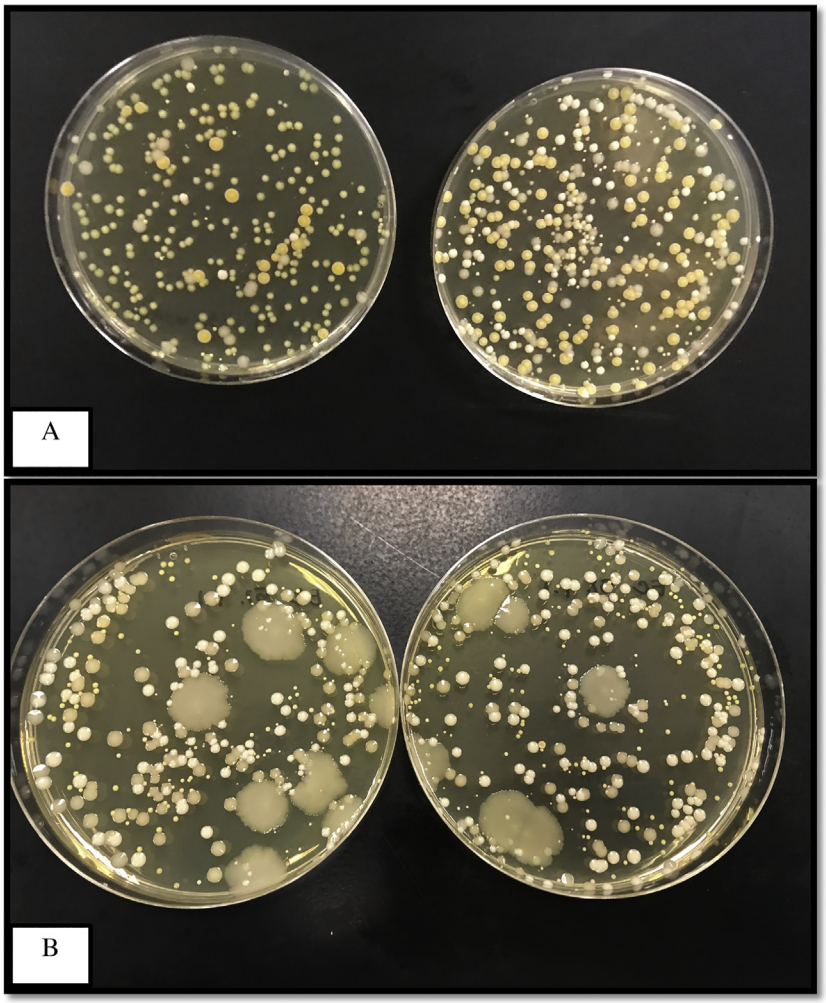


**Fig. 2.** Mixture plate replicates after one freeze-thaw cycle: A) Normal flora and MRSA, B) Normal flora and *E. coli*.

The antimicrobial action of the fabric was stopped after 15 min by utilizing DE Neutralizing broth. All swatches were transferred using sterile forceps to individual 50 mL conical tubes containing 15 mL of neutralizing broth. The tubes were incubated at room temperature for 10 min with constant agitation on a wrist action shaker.

The neutralizing broth for each fabric swatch was serially diluted in sterile 1× PBS, and 100 μL of each dilution was plated in duplicate to BHI agar. Following overnight incubation at 37 °C, plates were evaluated visually for growth and colonies counted. All trials were performed in duplicate. Average percent and $\log_{10}$ reductions in comparison to the positive control were calculated. *t*-tests were performed in JMP® (SAS® Software) to determine statistical significance between non-antimicrobial cotton cellulose and N-halamine fabric performance. Antimicrobial textiles that achieved ≥3.00 $\log_{10}$ reduction (99.9%) in all soils were considered appropriate for use in the healthcare setting (EPA, 2012).

## 3. Results

### 3.1. Microorganism growth and reproducibility in the test procedure

The testing protocol was successful in producing both adequate colony growth of all single and mixtures of microorganisms and the soils had no impact on the growth of the microorganisms. For single microorganism trials, the procedure consistently generated growth of every microorganism attempted. Each trial was performed in duplicate and quantity of growth was consistent between associated pairs of plates. For trials that involved mixtures of microorganisms, the procedure consistently generated growth of each representative colony type in about equivalent concentrations. The maximum number of microorganisms attempted with this procedure was five. Each mixture trial was also performed in duplicate, and representative colonies from each microorganism in the mixture were comparable in quantity between associated pairs of plates. Fig. 2 shows plate replicates with A) normal flora and MRSA and B) normal flora and *E. coli*. Colony morphologies were easily distinguishable and were confirmed utilizing a Gram-stain.

Frozen aliquot preparation generated the desired volume of broth containing about equivalent CFU/mL of each representative microorganism. Freezing aliquots of mixtures, i.e., "frozen stocks," of microorganisms as outlined in the procedure successfully allowed for thawing and utilization of the microorganism mixtures in future trials. Microorganism growth between subsequent freeze-thaw cycles was consistent between trials, and reliably generated plates with countable colonies of representative microorganisms. Fig. 3 displays plate images for all mixtures.

### 3.2. Single microorganism reductions

Overall, there was a statistically significant reduction in all microorganisms on the N-halamine fabric after 15 min of contact in the presence of all soils ($P < .05$) (Table 4). All microorganisms were reduced by 3.00 $\log_{10}$ or greater, and the combined average reduction for all microorganisms was 4.91 $\log_{10}$ (99.9581%). When separated by soil type, the overall average $\log_{10}$ reductions were 5.10 for PBS, 4.86 for artificial sweat, and 4.78 for 5% serum (Table 5). No statistical significance was found between soil types ($P > .05$).

### 3.3. Mixed microorganism reductions

Mixtures containing the 4 representative normal flora microorganisms, with and without an additional pathogen, were tested against the N-halamine fabric with a 15-min contact time. Overall, there was a statistically significant reduction in all microorganisms in the mixtures on the N-halamine fabric in the presence of all soils ($P < .05$) (Table 6). All microorganisms were reduced by 4.00 $\log_{10}$ or greater, and the overall average $\log_{10}$ reduction for all mixtures was 4.86. When separated by soil type, the overall average $\log_{10}$ reductions were 4.86 for PBS, 5.03 for artificial sweat, and 4.86 for 5% serum (Table 7). No statistical significance was found between soil types ($P > .05$). Fig. 4 shows scanning electron micrograph images of untreated control and N-halamine fabric with the normal flora mix plus *C. albicans*.

## 4. Discussion

Despite improved hygiene practices and dynamic infection prevention programs, the transmission of microorganisms in the healthcare setting remains of great concern. It is well documented that soft surfaces in the healthcare setting, particularly fabric, are contaminated with pathogens, and therefore play a role in the chain of infection, including the transfer of microbes from fabric to human skin (Boutin et al., 2014; Koca et al., 2012; Lopez-Gigosos et al., 2014; Nordstrom et al., 2012; Scott et al., 2015). Incorporating antimicrobial soft surfaces in the healthcare setting can serve as an engineering control to reduce the spread of HAIs. While there are several published standards for antimicrobial efficacy testing, none account for the dynamic environment in the healthcare setting. In this study we present a more comprehensive testing method to evaluate textile performance against a number of important HAI causing microbes, including bacteria and fungi, as well as interfering soils, including sweat and serum. We also present the performance of a novel rechargeable, monitorable N-halamine fabric using this protocol (Luo and Sun, 2006).

While there are several published standards that evaluate the efficacy of antimicrobial textiles, those standards do not reflect exposures to microorganisms in a realistic setting, evaluating the activity against single microorganisms only in near perfect conditions, and supplying the test microorganisms with nutrients in a high-level of moisture for up to 24 h of contact with the antimicrobial agent. Ideally, antimicrobial textiles should exert effects rapidly, over brief periods of time, and should be tested in conditions that mimic in-use conditions as much as possible. While testing the fabric against individual microorganisms is valuable, it is important to also challenge the fabric against a combination of microbes as textiles are in close contact with skin, which hosts a myriad of microbes. Furthermore, the addition of common soils that may decrease the activity or deactivate the antimicrobial property all together should be incorporated into testing algorithms.

A unique characteristic of this method is that it specifically evaluates healthcare-specific antimicrobial textiles in simulated in-use conditions. Many antimicrobial fabrics are intended for use in clothing such as scrubs and lab coats, as well as other soft surfaces such as bed linens, privacy curtains, and upholstered furniture. These fabrics may have direct contact with human skin, pathogens, as well as exposure with organic soils such as blood and other body fluids. The current methods used to test the efficacy of antimicrobial materials do not fully address the potential impact of organic soils, nor do they take into account the human skin environment, including sweat and normal skin microorganisms. The fabrics currently worn by healthcare workers are in close contact with their own skin as well as that of their patients. It is unknown how a diverse microbial environment impacts the performance of antimicrobial soft surfaces. No studies exist that evaluate the performance of antimicrobial fabrics in the presence of these factors. This more extensive testing provides a more rigorous performance evaluation, enabling a better understanding of antimicrobial efficacy by including commonly encountered pathogens in the presence of normal flora and soils with potential to alter the performance of the fabric.

We also report a reproducible method for mixing microorganisms at relatively equal concentrations. By freezing mixtures in large batches, testing parties will minimize inter-trial variability. While the method as presented focuses on the skin and healthcare environment, it is designed to be flexible. Depending on where the antimicrobial textile will be implemented, the microorganism types and concentration can easily be altered, as well as adjusting soils and contact time.

A cut off of ≥3.0 $\log_{10}$ (99.9%) was used as the pass-fail criteria for

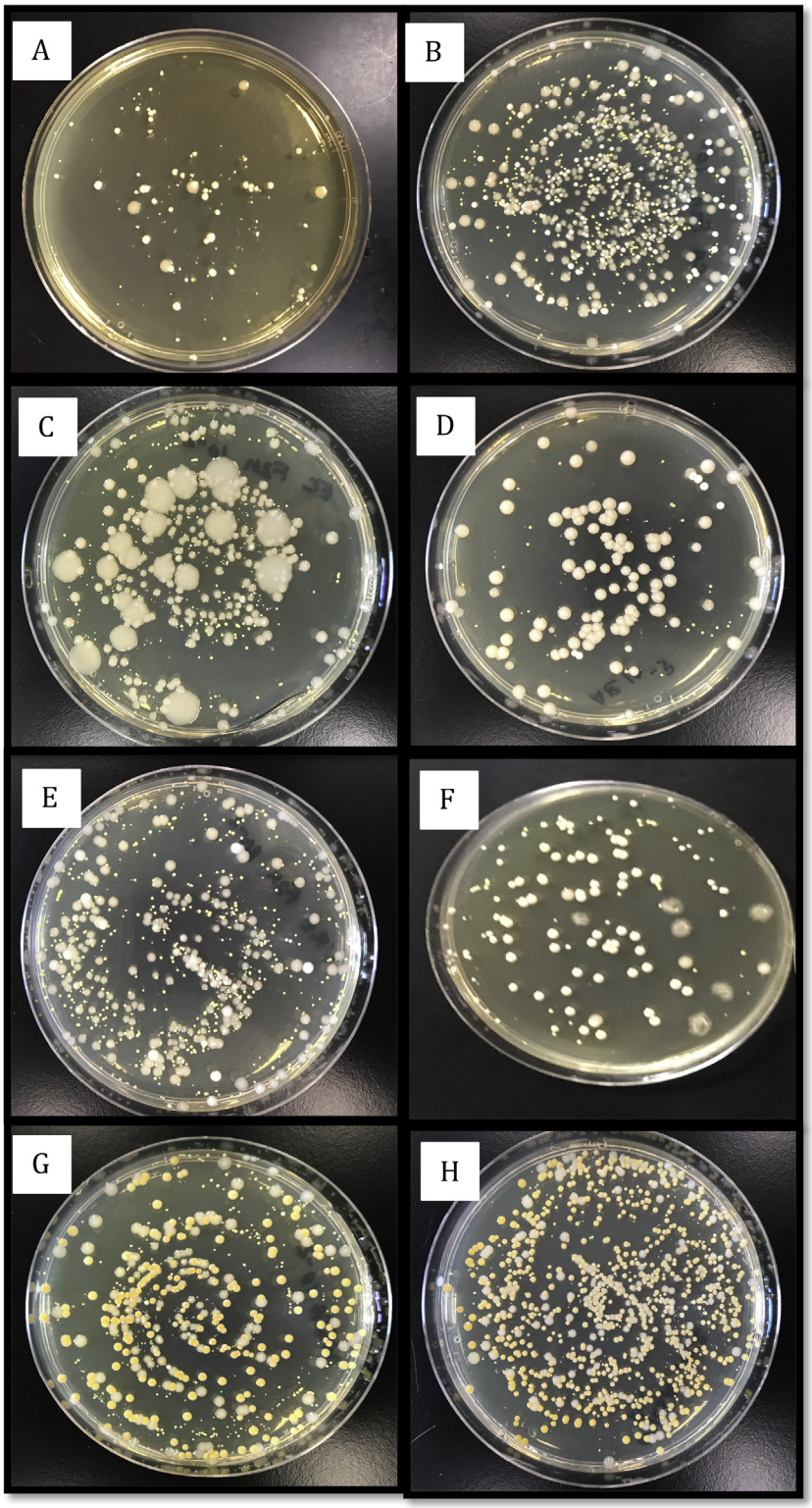


**Fig. 3.** Plate images of single pathogens with normal flora mixtures (A. *lwoffii, C. striatum, M. luteus, S. epidermidis*). Pathogens: A: No pathogen, B: *C. albicans*, C: *E. coli*, D: *A. baumannii*, E: VRE, F: *P. aeruginosa*, G: MSSA, H: MRSA.

the soft surfaces utilized in this study. In the absence of guidelines for soft surface $\log_{10}$ and percent reductions, the cut off of $\geq 3.0$ $\log_{10}$ (99.9%) was chosen based on the existing EPA product performances guidelines for evaluating disinfection of hard surfaces embedded with antimicrobials (EPA, 2012).

To date, the N-halamine cotton cellulose fabric is the first antimicrobial fabric fully tested with the proposed more comprehensive antimicrobial efficacy test method. It has been reported that "clean" service textiles can be contaminated within hours after donning in healthcare settings (Burden et al., 2011; Mitchell et al., 2015; Ohl et al., 2012), increasing the risk of occupational exposure. We therefore chose 15 min as the contact time to challenge the fabric. Overall, the N-

**Table 4**
Overall average $log_{10}$ and percent reductions for individual microorganisms after a 15 min contact time with the N-halamine fabric. All averages include 4 replicates for each artificial soil (PBS, artificial sweat, and 5% serum in artificial sweat).

| Microorganism | $Log_{10}$ reduction (SD) | Percent reduction (SD) |
|---|---|---|
| *Acinetobacter baumannii* | 5.37 (0.06) | 100.0000 (0.0000) |
| *Acinetobacter lwoffii* | 4.87 (0.97) | 100.0000 (0.0000) |
| *Corynebacterium striatum* | 3.64 (0.25) | 100.0000 (0.0000) |
| *Candida albicans* | 4.84 (0.53) | 99.9993 (0.0011) |
| *Enterococcus faecalis* (VRE) | 6.76 (0.65) | 100.0000 (0.0000) |
| *Escherichia coli* | 5.17 (0.28) | 99.9968 (0.0054) |
| *Micrococcus luteus* | 4.29 (0.45) | 100.0000 (0.0000) |
| *Pseudomonas aeruginosa* | 4.41 (0.76) | 100.0000 (0.0000) |
| *Staphylococcus aureus* (MSSA) | 3.90 (0.65) | 99.6479 (0.4318) |
| *Staphylococcus aureus* (MRSA) | 6.38 (0.03) | 100.0000 (0.0000) |
| *Staphylococcus epidermidis* | 4.36 (0.75) | 99.8952 (0.0895) |

**Table 5**
Average $log_{10}$ reduction and percent reduction for individual microorganisms by matrix ($n$ = 4 for each microorganism/soil combination) and statistical significance of soil.

| Microorganism | Soil | $Log_{10}$ reduction | Percent reduction | *P*-Value |
|---|---|---|---|---|
| *A. lwoffii* | PBS | 4.19 | 100.0000 | 0.0005 |
| | Art. Sweat | 3.98 | 100.0000 | 0.0032 |
| | 5% Serum | 6.48 | 100.0000 | 0.0001 |
| *C. striatum* | PBS | 3.64 | 100.0000 | 0.0089 |
| | Art. Sweat | 3.84 | 100.0000 | 0.0344 |
| | 5% Serum | 3.46 | 100.0000 | 0.0147 |
| *M. luteus* | PBS | 4.52 | 100.0000 | 0.0006 |
| | Art. Sweat | 4.55 | 100.0000 | 0.0004 |
| | 5% Serum | 3.80 | 100.0000 | 0.0086 |
| *S. epidermidis* | PBS | 4.83 | 99.9877 | 0.0020 |
| | Art. Sweat | 4.91 | 99.9917 | 0.0087 |
| | 5% Serum | 3.34 | 99.7061 | 0.0378 |
| *A. baumannii* | PBS | 5.81 | 100.0000 | 0.0212 |
| | Art. Sweat | 5.19 | 100.0000 | 0.0043 |
| | 5% Serum | 5.11 | 100.0000 | 0.0002 |
| *C. albicans* | PBS | 5.61 | 99.9980 | 0.0038 |
| | Art. Sweat | 4.46 | 100.0000 | 0.0003 |
| | 5% Serum | 4.47 | 100.0000 | 0.0001 |
| *E. faecalis* (VRE) | PBS | 6.46 | 100.0000 | 0.0063 |
| | Art. Sweat | 7.53 | 100.0000 | 0.0001 |
| | 5% Serum | 6.29 | 100.0000 | 0.0002 |
| *E. coli* | PBS | 5.18 | 100.0000 | 0.0046 |
| | Art. Sweat | 5.58 | 100.0000 | 0.0020 |
| | 5% Serum | 4.76 | 99.9903 | 0.0232 |
| *P. aeruginosa* | PBS | 5.29 | 100.0000 | 0.0044 |
| | Art. Sweat | 3.91 | 100.0000 | 0.0015 |
| | 5% Serum | 4.04 | 100.0000 | 0.0002 |
| *S. aureus* (MSSA) | PBS | 4.33 | 100.0000 | 0.0042 |
| | Art. Sweat | 3.05 | 98.9534 | 0.0019 |
| | 5% Serum | 4.33 | 99.9903 | 0.0042 |
| *S. aureus* (MRSA) | PBS | 6.27 | 100.0000 | 0.0286 |
| | Art. Sweat | 6.43 | 100.0000 | 0.0090 |
| | 5% Serum | 6.45 | 100.0000 | 0.0001 |

halamine fabric was highly effective at reducing the number of microorganisms within 15 min, with an average $log_{10}$ reduction of 4.93 (99.9757% reduction) of microbial load for all trials, including individual microorganisms and microorganism mixtures, in all 3 soils. The antimicrobial fabric did not show variability between soils, indicating that sweat and proteinaceous serum do not impede the action of the N-halamine at the concentrations tested.

The microorganisms tested comprised a broad spectrum of microorganisms, including Gram-positive bacteria, Gram-negative bacteria, and yeast. Furthermore, the fabric was able to kill multiple microorganism populations simultaneously, which is most representative of real-world application. When testing the fabric against individual microorganisms, the N-halamine was able to reduce or completely

**Table 6**
Overall average $log_{10}$ and percent reductions for normal flora plus pathogen mixtures after a 15 min contact time with the N-halamine fabric. All averages include 4 replicates for each artificial soil: PBS, artificial sweat, and 5% serum in artificial sweat.

| Microorganisms | $Log_{10}$ reduction (SD) | Percent reduction (SD) |
|---|---|---|
| Normal flora mixture | 5.11 (0.57) | 100.0000 (0.0000) |
| Normal Flora + *A. baumannii* | 5.03 (0.39) | 100.0000 (0.0000) |
| Normal Flora + *C. albicans* | 4.91 (0.23) | 100.0000 (0.0000) |
| Normal Flora + *E. coli* | 4.89 (0.08) | 100.0000 (0.0000) |
| Normal Flora + *E. faecalis* (VRE) | 4.68 (0.29) | 100.0000 (0.0000) |
| Normal Flora + *S. aureus* (MSSA) | 4.92 (0.18) | 100.0000 (0.0000) |
| Normal Flora + *S. aureus* (MRSA) | 5.03 (0.32) | 100.0000 (0.0000) |
| Normal Flora + *P. aeruginosa* | 5.08 (0.05) | 100.0000 (0.0000) |

**Table 7**
Average $log_{10}$ and percent reduction for normal flora plus pathogen mixtures by matrix and statistical significance of soil (n = 4 for each microorganism/soil mixture).

| Microorganisms | Soil | $Log_{10}$ reduction | Percent reduction | P-Value |
|---|---|---|---|---|
| Normal flora mixture | PBS | 5.36 | 100.0000 | 0.0001 |
| | Art. Sweat | 5.51 | 100.0000 | 0.0001 |
| | 5% Serum | 4.46 | 100.0000 | 0.0032 |
| Normal Flora + *A. baumannii* | PBS | 4.68 | 100.0000 | 0.0012 |
| | Art. Sweat | 5.25 | 100.0000 | 0.0003 |
| | 5% Serum | 5.15 | 100.0000 | 0.0015 |
| Normal Flora + *C. albicans* | PBS | 4.67 | 100.0000 | 0.0085 |
| | Art. Sweat | 4.99 | 100.0000 | 0.0014 |
| | 5% Serum | 5.07 | 100.0000 | 0.0004 |
| Normal Flora + *E. coli* | PBS | 4.91 | 100.0000 | 0.0029 |
| | Art. Sweat | 4.96 | 100.0000 | 0.0009 |
| | 5% Serum | 4.80 | 100.0000 | 0.0023 |
| Normal Flora + VRE | PBS | 4.94 | 100.0000 | 0.0013 |
| | Art. Sweat | 4.38 | 100.0000 | 0.0008 |
| | 5% Serum | 4.74 | 100.0000 | 0.0015 |
| Normal Flora + *P. aeruginosa* | PBS | 4.76 | 100.0000 | 0.0048 |
| | Art. Sweat | 5.19 | 100.0000 | 0.0018 |
| | 5% Serum | 4.29 | 100.0000 | 0.0014 |
| Normal Flora + MSSA | PBS | 4.73 | 100.0000 | 0.0007 |
| | Art. Sweat | 5.08 | 100.0000 | 0.0013 |
| | 5% Serum | 4.96 | 100.0000 | 0.0023 |
| Normal Flora + MRSA | PBS | 4.81 | 100.0000 | 0.0055 |
| | Art. Sweat | 4.88 | 100.0000 | 0.0006 |
| | 5% Serum | 5.40 | 100.0000 | 0.0066 |

eliminate major HAI-causing microorganisms, including antibiotic resistant and encapsulated strains. When testing against single microorganisms, the fabric performed best against Gram-negative microorganisms, *A. lwoffii, A. baumannii, E. coli,* and *P. aeruginosa.* The average $log_{10}$ reduction for Gram-negative microorganisms was 4.96 (> 99.99% reduction). Among all microorganisms tested, Gram-positives, namely *Staphylococcus* spp., were the most challenging to eliminate. This is consistent with previously published data regarding the differences between Gram-positive and –negative bacteria in their susceptibility to antimicrobial agents and disinfectants (Santos et al., 2016). Generally, the Gram-positive bacteria have a thicker cell wall, which has been suggested to provide an enhanced level of protection against the penetration of agents.

When tested against mixed microorganism, a 100% reduction was achieved with all mixes. The improved performance may be due to microorganism concentrations: all trials, mixed and single, were performed with about 5.00 $log_{10}$ of bacteria applied to the fabric. However, in the mixtures, each bacterial species was at a lower concentration, therefore improving the ability of the fabric to kill each individual species. In healthcare applications, mixed bacterial populations are highly likely.

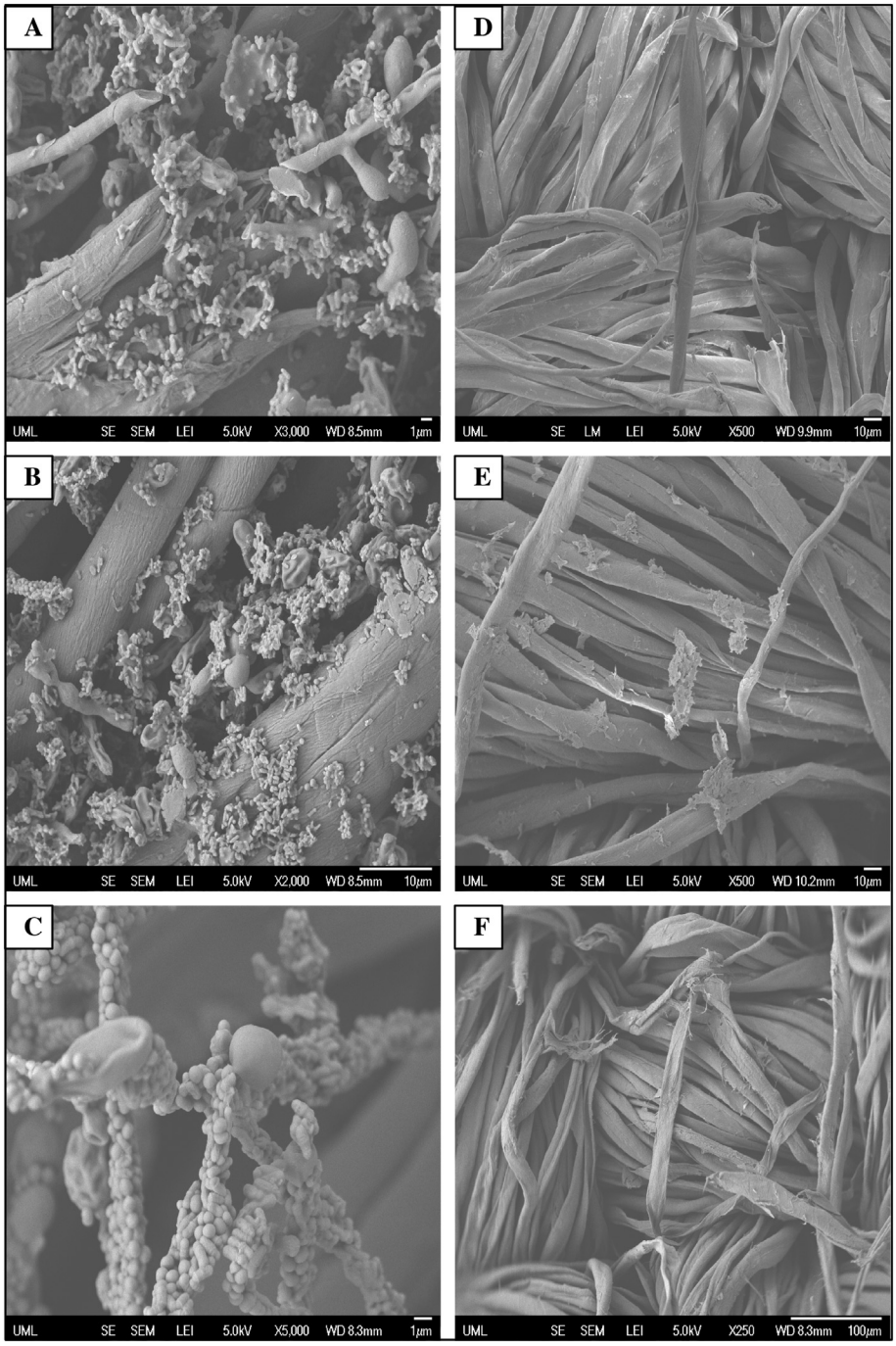


**Fig. 4.** SEM Images of untreated cotton cellulose fabric (A, B, C) vs. N-halamine treated fabric after exposure to normal flora mixture + *C. albicans* (D, E, F).

It is important to note that the N-halamine fabric significantly reduced microorganisms classified as normal skin flora, both individual strains and in mixtures. Therefore, there are limitations surrounding implementation of this fabric into everyday-use soft surfaces that will be in close contact with the skin, as this may negatively impact the dynamic and critical skin microbiome. Previously published data regarding long-term topical antimicrobial treatment showed shifts in residential flora, and ultimately reduced healthy skin flora critical for preventing the colonization of opportunistic or pathogenic microbes (SanMiguel et al., 2017). There are also occasions when normal skin flora, *S. epidermidis* in particular, can cause serious nosocomial infections, particularly in individuals with indwelling devices (Gomes et al., 2014; SanMiguel et al., 2017). Such circumstances include: immunocompromised, chronic indwelling catheters or chest tubes, other percutaneous catheters, mechanical ventilation, chronic wounds, and burns. Therefore, in these circumstances it might be advantageous to reduce the number of normal flora microorganisms to prevent serious infections. A possible means of doing so would be incorporating N-halamine based textiles into bandages, tapes and other coverings that occlude the sites of percutaneous infection or utilizing N-halamine fabric for bed linens and wound bandages. Further study is required in order to determine how the N-halamine fabric would alter skin microbiome in otherwise healthy individuals.

These data suggest that this technology has the potential to greatly reduce the bioburden of microbial populations on textiles quickly, and therefore is a strong candidate for implementation in the healthcare setting. It could also be valuable to incorporate N-halamine antimicrobial soft surfaces into high touch areas within high-risk patient

accommodations, such as operating rooms and precaution rooms. N-halamine based textiles can be incorporated into privacy curtains, bed linens, as well as precaution gowns.

There are several unique advantages to utilizing N-halamine based textiles over others currently commercially available. N-halamine based antimicrobial fabrics possess many appealing characteristics, especially for healthcare soft surfaces. N-halamine fabrics can be grafted to several types of textiles, including cotton cellulose and polyester. The concentration of chlorine can be controlled based on the MAA graft, remaining activity can be monitored, and it can be recharged once the chlorine is consumed, as previously reported (Luo and Sun, 2006). Most importantly for healthcare textiles, N-halamine is a quick acting antimicrobial with a broad spectrum of bactericidal activity.

There are limitations to this study. The lack of a recognized minimum standard for antimicrobial fabric performance is problematic for all efficacy protocols. In addition, the ideal bacterial and soil concentrations are unknown. Future studies should address the level of contamination and microorganism concentrations found in healthcare settings, as well as performance of other types of antimicrobial textiles. Additional microbes that should be tested may include more resistant bacteria such as vancomycin-intermediate and vancomycin-resistant *S. aureus* (VISA and VRSA), carbapenem-resistant Enterobacteriaceae (CRE), and *Candida auris*, an emerging multi-drug resistant yeast. We also suggest the inclusion of spore-forming microorganisms and their corresponding spores, such as *Clostridioides difficile*. Additional interfering soils may help better characterize antimicrobial fabrics, particularly those intended for use in the healthcare setting. Suggested soils include whole blood, sebum, urine, and feces. The N-halamine fabric should be tested at different chlorine concentrations to determine the most effective level, and samples should be tested following multiple laundering and recharge cycles to determine the lifespan of the fabric.

This novel, quantitative method provides a more realistic challenge for textiles intended for use in the healthcare setting by utilizing HAI-causing microbes as challenge microorganisms, as well as mixtures of microorganisms. Additionally, this method includes potentially inhibiting factors, such as sweat and serum, to aid in qualifying the real-world effectiveness of antimicrobial textiles. Based on the overall performance of the fabric with the novel efficacy method, the rechargeable, monitorable N-halamine based fabric is a strong candidate for implementation into healthcare soft surfaces.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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