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A traditional Ugandan *Ficus natalensis* bark cloth exhibits antimicrobial activity against Methicillin-Resistant *Staphylococcus aureus*.

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Running title: Antimicrobial activity of Ugandan bark cloth

Abstract

Aims

Surgical site, soft tissue and wound infections are some of the most prominent causes of Healthcare associated infections (HCAIs). Developing novel antimicrobial textiles and wound dressings may help alleviate the risk of developing HCAIs. We aimed to determine the antimicrobial efficacy of natural Ugandan bark cloth derived exclusively from the *Ficus natalensis* tree.

Methods and Results

Antimicrobial contact and disc diffusion assays, coupled with time-kill kinetic assays demonstrated that bark cloth inhibited the growth of a clinically relevant Methicillin-resistant *Staphylococcus aureus* (MRSA) strain and acted as a bactericidal agent causing a seven-log reduction in bacterial viability. Scanning electron microscopy was used to reveal morphological changes in the bacterial cell ultrastructure when exposed to bark cloth, which supported a proposed mechanism of antimicrobial activity.

Conclusions

The observed antimicrobial properties, combined with the physical characteristics elicited by bark cloth, suggest this product is ideally suited for wound and other skin care applications.

Significance and Impact of the study

This is the first report where a whole bark cloth product made by traditional methods has been employed as an antimicrobial fabric against MRSA. Bark cloth is a highly sustainable and renewable product and this study presents a major advance in the search for natural fabrics which could be deployed for healthcare applications.

Keywords: bark cloth; MRSA; antimicrobial; natural products; wound management; *Ficus natalensis*.

Background

Antimicrobial resistance is a major global issue (Blair *et al*. 2015). It is estimated that by 2050, mortality rates associated with antimicrobial resistant infections will exceed 10 million people per annum, superseding cancer as the leading cause of global mortality (O'Neill 2016). Healhcare associated infections (HCAIs) are a major cause of morbidity

and mortality and it is estimated that between 7% and 10% of hospitalised patients will develop such an infection type (Danasekaran *et al*. 2014). Surgical site, soft tissue and wound infections are amongst the most common types of HCAI, with Methicillin-Resistant *Staphylococcus aureus* (MRSA) being a predominant cause of infection (Haque *et al*. 2018); another notable cause of wound infections is *Pseudomonas aeruginosa*, which is a prominent organism in the case of burns and immunocompromised patients (Church *et al*. 2006; Buhl *et al*. 2015; Cardona and Wilson 2015).

One method of reducing the transmission of pathogens in healthcare environments, whilst assisting the healing process and promoting localised antisepsis, is the development of antimicrobial textiles and wound dressings (Negut *et al*. 2018). Antimicrobial wound dressings vary considerably, with many employing metals and/or synthetic compounds as the active antimicrobial agents. These may include, basic wound contact fabric, films, fabric with metal/oxide-derivative coatings (Gao and Cranston 2008; Simončič and Klemenčič 2016), graphene-oxide (Zhao *et al.* 2013) and hydrocolloid-based dressings (Capanema *et al.* 2018). Some fabrics may incorporate synthetic antimicrobials such as quaternary ammonium compounds (Kang *et al.* 2016), biguanides (Kawabata and Taylor 2004) and guanidine (Li *et al.* 2018). Whilst, antibiotic impregnated wound dressings have also been researched extensively (Simões *et al.* 2018).

As most current antimicrobial fabrics involve the use of metals and/or synthetic compounds, using antimicrobial fabrics derived from natural sources may provide clinically useful alternatives, whilst promoting a more environmentally sustainable approach. One such emerging natural product is tree bark, where previous studies have demonstrated the antimicrobial activity of products extracted from *Diplotropis ferruginea* (Cerqueira *et al.* 2011) and other tropical and subtropical tree barks (Perez *et al.* 2001). A recent study highlighted the antimicrobial activity of crude solvent extracts from the leaves, bark and fruit of *Ficus natalensis* (wild fig tree) (Awolola *et al.* 2017), part of the Moraceae tree family which are geographically distributed in southern and eastern Africa.

Throughout the present study the antimicrobial efficacy of bark from *F. natalensis* in the form of bark cloth (without an extraction process) was determined. Bark cloth is a nonwoven fibrous structure harvested from *F. natalensis* tree bark by the Baganda people of the Southern Ugandan community since at least the 13th Century. Historically, it has been used in clothing for ceremonial occasions by the Baganda community

(Nakazibwe 2005). The making of bark cloth has been passed on through many generations and is a highly skilful activity that reflects the identity of the community. UNESCO has recognised it as an 'intangible cultural heritage of humanity' (UNESCO 2008), emphasising the rationale to preserve the knowledge, skills, and the art of making bark cloth. Bark cloth harvesting occurs annually during the rainy season without damaging the tree, as the trunk is wrapped in banana leaves after harvesting to prevent dehydration (Rwawiire *et al.* 2013). The trees grow without the need for any fertilisers or pesticides and are frequently used in local agro-forestry systems to provide shade to other crops and are reputed locally to add nutrients to the soil. Figure 1 (A-D) illustrates the characteristic bark cloth making process by artisans and the Bukomansimbi Organic Tree Farmers Association (BOTFA) inspecting the bark cloth sheets, where the images were obtained during field work in 2014 and 2019 (Scott 2019).

Other related research on bark cloth reported the thermo-physiological properties of the bark cloth, where it was observed to have lower thermal absorptivity than cotton fibre and higher moisture absorption (Rwawiire and Tomkova 2013). In addition, bark cloth based green epoxy composites showed sufficient tensile strength suitable for automotive panels (Rwawiire *et al.* 2015). Natural Ugandan bark cloth has a stiff texture, is porous, varies in thickness (0.672 mm ± 0.05) with random fibre arrangement, but most areas show diagonal fabric grain (Fig. 2A) (Venkatraman *et al.* 2020). In the same study, surface morphological assessment using Energy Dispersive Spectroscopy (EDX) showed traces of calcium and sodium. Based on this comprehensive analysis, it was recommended as a sustainable material for developing small scale, niche fashion collections, which could preserve this endangered art of making bark cloth (Venkatraman *et al.* 2020).

In the present study, we aimed to examine the feasibility of using bark cloth as an antimicrobial fabric for clinical use within wound care management. This was achieved using a combination of antimicrobial susceptibility assays coupled with scanning electron microscopy to elucidate the nature of the observed antimicrobial effects.

Materials and methods

Bacterial strains

Methicillin-resistant *Staphylococcus aureus* strain USA300 (ST8) JE2 (Fey *et al*. 2013) and *Pseudomonas aeruginosa* strain PAO1 (Holloway 1955) were cultured using Mueller Hinton (MH) agar or broth (Oxoid, UK) and incubated under aerobic conditions at 37 °C for 24 h, with agitation (150 rpm) for broth cultures.

Bark cloth preparation

Bark cloth was made by local artisans using traditional techniques and kindly supplied by Dr K. Scott (Istituto Marangoni, London UK). Briefly, bark from the *F. natalensis* was harvested, pressed and shaped with stretching and allowed to dry naturally under sunlight. To determine the durability of bark cloth, four samples each measuring 30 x 21 cm (Fig. 3) were washed and rinsed with ECE detergent (phosphate powder with no optical brighteners used in British Standard wash tests recommended for textiles) in a container with 5 L of cold water. The fabric was submerged ten times with no wringing. The bark cloth was then rinsed in another container of water (5 L) to remove any detergent and dried at room temperature for 24 h. Prior to microbiological experimentation, the fabric was exposed to ultraviolet light for 1 h with frequent rotation.

Antimicrobial susceptibility

Contact assay

Contact assays were performed in accordance with a modified Deutsches Institut für Normung (DIN) 58940-3 agar diffusion standard method (Wiegand *et al*., 2015). Briefly, 6 mm diameter circular discs of both washed and unwashed bark cloth were added to agar plates pre-inoculated with 100 µL of *S. aureus* strain USA300 or *P. aeruginosa* strain PAO1, which were adjusted to an Optical Density of 0.5 (± 0.05) at 600 nm (OD_{600}). Aliquots of 10 µL phosphate buffered saline (PBS) were added to fabric disc samples to facilitate diffusion. Inoculated agar plates were incubated for 24 h at 37 °C under aerobic conditions and the size of bacterial growth inhibitory zones were determined using a digital calliper gauge. Filter paper discs (6 mm diameter; Whatman, Merck UK) with 10 µL PBS were included as a control. All assays were performed in triplicate with biological replicates (n = 3).

Antimicrobial extraction and disk diffusion assay

Antimicrobial susceptibility was determined using a modified EUCAST disk diffusion method (Matuschek *et al*., 2015). Briefly, unwashed and washed bark cloth samples (surface area: 1 cm^2) were exposed to 500 µL analytical grade 95% ethanol, PBS or dimethylsulfoxide (DMSO) for 24 h. Supernatants were harvested and 10 µL of each were added to 6 mm diameter circular filter paper discs (Whatman, Merck UK) on the surface of MH agar which was pre-inoculated with 100 µL of *S. aureus* strain USA300 at an OD_{600} of 0.5 (± 0.05). Plates were incubated for 24 h at 37 °C and bacterial growth inhibitory zones were measured. Solvent only controls were included for comparison and assays were performed in triplicate with biological replicates (*n* = 3).

Time-kill kinetic assays

An overnight culture of *S. aureus* strain USA300 was adjusted to an OD_{600} of 0.0025 (approximately 1x 10^7 Colony Forming Units (CFU) mL^{-1}) in MH broth and 1 cm^2 unwashed bark cloth samples were added. Cultures were incubated for 24 h at 37 °C with agitation and bacterial viability was determined at 0 h, 2 h, 4 h, 6 h and 24 h (Miles *et al.* 1938; Hickey *et al*. 2019). Control cultures excluding bark cloth were included and bark cloth was also incubated in MH broth without bacterial inoculation to confirm sterility. All assays were performed in biological triplicates (*n* = 3).

Scanning Electron Microscopy (SEM)

After 24 h incubation following the time-kill kinetic assay, bark cloth samples were fixed in 4% v/v glutaraldehyde for 24 h at 4 °C. Fixed samples were rinsed with sterile deionised water and subjected to an ethanol gradient from 10% to 30%, 50%, 70%, 90% and 100% v/v absolute ethanol (Liauw *et al*. 2019). Samples were dried by desiccation over 24 h and were sputter coated with gold (Polaron, UK) for 30 s (parameters: power 5 mA, 30 s, 800 V, vacuum 0.09 mbar, argon gas) prior to imaging using a JEOL JSM 5600LV scanning electron microscope.

Statistical analysis

One or Two-way ANOVA statistical tests with Tukey's tests for post hoc analysis were performed using GraphPad Prism version 8.4.2 (GraphPad Software, USA).

Results

Bark cloth directly inhibited growth of *S. aureus* strain USA300

Prior to use, bark cloth was washed (Fig. 3A and B) to determine the durability of the fabric and whether any observed antimicrobial activity was due to loosely incorporated residues. Washing resulted in a dark coloured elution (Fig. 3C), which resembled the distinctive bark cloth colour and suggested evidence of residual leachate. Bark cloth was then used in a surface contact agar diffusion assay and demonstrated antimicrobial activity against Methicillin-resistant *S. aureus* strain USA300. Zones of bacterial growth inhibition were observed with both unwashed and washed derivatives of bark cloth with mean diameters of 7.58 mm and 7.36 mm respectively (Fig. 4, black and shaded bars). One-way ANOVA with a Tukey's test for post hoc analysis demonstrated that there was no significant difference between the diameters of the inhibitory zones produced by either bark cloth derivative ($P > 0.05$), in contrast to the filter paper disc control ($P \leq 0.001$) where no growth inhibition was observed (Fig. 4, no bar). No evidence of antimicrobial activity was observed when bark cloth was exposed to *P. aeruginosa* strain PAO1 (data not shown).

Solvent extracts from bark cloth inhibited growth of *S. aureus* strain USA300

Extracts from both unwashed and washed bark cloth were produced by exposure to PBS, ethanol (95%) or DMSO and 10 µL of supernatant was added to filter paper discs. These were positioned onto MH agar, which was pre-inoculated with *S. aureus* strain USA300. After 24 h incubation, DMSO extracted supernatant from both unwashed and washed bark cloth significantly inhibited bacterial growth ($P \leq 0.001$) and produced inhibitory zones of 7.40 and 7.14 mm respectively (Fig. 5, black and shaded bars), compared to 0 mm for the DMSO solvent alone (Fig. 5, no bar). The DMSO extract from unwashed bark demonstrated significantly greater antimicrobial activity than the extract from the washed cloth ($P \leq 0.05$). Ethanol and PBS extracts had no observable effect on the growth of *S. aureus* strain USA300 (data not shown).

Bark cloth significantly reduced the viability of *S. aureus* strain USA300

Unwashed bark cloth was incubated with *S. aureus* strain USA300 and bacterial viability was determined over a 24 h period. After 4 h incubation, a significant one-log reduction in viability was observed ($P \leq 0.01$) in bacteria exposed to bark cloth compared to the unexposed control (Fig. 6, dashed compared to solid line). After 24 h, bacterial viability decreased by approximately four logs when compared to the starting culture viability at 0 h (10^7 to 10^3 CFU mL^{-1}). However, when compared to the unexposed

bacterial culture control, at 24 h there was a significant ($P \leq 0.0001$) seven-log reduction (10^{10} to 10^{3} CFU mL^{-1}) in bacterial viability in the culture exposed to bark cloth. No bacterial growth was observed when bark cloth was incubated in MH broth without bacterial inoculation which demonstrated the sterility of the cloth (data not shown).

Bark cloth exposure resulted in morphological changes to the cell ultrastructure of *S. aureus* strain USA300

Scanning electron microscopy (SEM) was used to examine the effects of exposure to bark cloth on *S. aureus* strain USA300 and to help elucidate the mechanisms of antimicrobial activity. Bark cloth samples from the time-kill kinetics assay were subjected to visual analysis by SEM after 24 h. Bacterial attachment and biofilm formation was visible on the fibrous structure of the bark cloth (Fig. 7A) compared to the un-inoculated control (Fig. 7C). Distinct morphological changes in the bacterial cellular ultrastructure, including invaginations, perforations, pits, and holes were observed in *S. aureus* strain USA300 cells which were exposed to bark cloth (Fig. 7A). Such morphological changes were indicative of antimicrobial activity against the cell ultrastructure when compared to unexposed bacterial cells (Fig. 7B), which had intact cellular features.

Discussion

As society approaches a post antibiotic era, new antimicrobial interventions are urgently needed, both as treatment options and infection prevention strategies. Wound infections caused by multidrug-resistant bacterial pathogens are a major cause of morbidity and mortality, and result in significant socio-economic burdens to the healthcare system (Leaper *et al.* 2004). Current wound dressing technology predominantly focuses on utilising synthetic antimicrobial agents to assist with clinical wound management. However, natural products from floral and arboreal origins have been used in medicinal applications for centuries (Mahady *et al.* 2008) and there is an ever-increasing need for greener, more sustainable healthcare technologies.

Antimicrobial compounds derived from extracts of flora, herbs, leaves and trees are widely reported and reviewed elsewhere (Cowan 1999; Khameneh *et al.* 2019). Many studies rely on solvent extraction to identify key antimicrobial mediators and rarely use whole products for antimicrobial applications. Here, we reported the first example of an unmodified, natural fabric product derived from the tree bark of *F. natalensis*, which could

be used as an antimicrobial textile or wound dressing. The bark cloth inhibited growth of a well-characterised MRSA clinical isolate and this activity was retained despite washing the fabric. One of the biggest challenges for antimicrobial fabric production is ensuring that activity is retained after standard washing procedures; therefore the bark cloth presents an interesting material for further research into potential commercial fabric uses.

For clinical wound dressing applications, it is important that antimicrobial activity is retained for a designated time, such as the period between dressing and reapplication (Negut *et al.* 2018). Antimicrobial releasing properties are also a factor when considering novel wound dressing development, in order to ensure local dissemination of the active compound(s). A 99% reduction in bacterial viability after 4 h was observed when bark cloth was exposed to MRSA during time-kill kinetic assays, compared to the unexposed bacterial control strain. After 24 h, this increased to a 99.99999% (seven-log) reduction in bacterial viability. Such potent and long lasting antimicrobial activity would be highly beneficial for use within wound care management. The time-kill kinetics assay demonstrated that the active antimicrobial compound(s) might have diffused from the bark cloth, but visualisation of the fabric using SEM showed biofilms of cells with significant morphological changes in cellular ultrastructure. Bacterial cells became irregular in shape, with invaginations, holes and perforations, which is consistent with bacterial cytoplasmic membrane damage (Côté et al. 2019). Furthermore, there was clear visual evidence of cytoplasmic leakage that is consistent with perturbation of the bacterial cytoplasmic membrane (Otto *et al.* 2010), which further demonstrated the mechanism of antimicrobial activity of the bark cloth (and associated active antimicrobial compound(s)). Combined, these indicated that bark cloth has both slow releasing and contact killing antibacterial properties, which are highly desirable characteristics of novel wound dressings.

The preferable characteristics of an ideal wound dressing include, control of moisture around the wound, good gaseous transmission, elimination of excess exudates, antimicrobial activity, biocompatibility, reduced wound surface necrosis, mechanical protection, easily exchangeable, biodegradable and cost-effective (Rezvani Ghomi *et al.* 2019). The bark cloth utilised throughout this study has previously demonstrated good porosity (Fig. 2; Venkatraman *et al.* 2020), which would facilitate gaseous exchange between the wound site and environment, and potentially self-disinfecting against

contamination with Gram-positive *S. aureus* bacteria, which are also key characteristics of ideal wound dressings (Dhivya *et al.* 2015).

The active antimicrobial compound(s) from bark cloth remains to be determined. However, a study conducted previously by Venkatraman *et al.* (2020) revealed the elemental composition of the *F. natalensis* bark cloth, where elements such as chlorine, calcium and magnesium were reported on the surface of the bark cloth. All three of these elements have known antimicrobial activity, in particular in ionic forms due to their binding affinity for functional groups (*i.e.* thiol groups) in bacterial cells (Stiefel *et al.* 2015; Slate *et al.* 2019). Chlorine affects bacterial cell viability by chlorinating the lipid protein in the cell wall, which results in macromolecule leakage from the cell (Kim *et al.* 2008). Calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions are physiologically essential to almost all living organisms (Cutinelli and Galdiero 1967). However, such ions can bind with cardiolipin, a major lipid component in the *S. aureus* cytoplasmic membrane, where binding complexes form a negative curvature which can result in a variety of membrane-destabilisation processes and subsequent cell death (Short and White 1971; Rand *et al.* 1972; Schmidt *et al*. 2011; Tsai *et al.* 2011; Xie and Yang 2016). This could explain the observed antimicrobial activity against MRSA and morphological changes in bacterial cell ultrastructure. Interestingly however, when washed, the bark cloth retained antimicrobial activity, therefore it is likely that combinations of antimicrobial factors are responsible.

Other potential antimicrobial compounds within the bark cloth may include triterpenoids and quinones such as tectoquinone, which were previously identified from crude *F. natalensis* extracts by vacuum column chromatography (Awolola *et al.* 2017). However, in this study, extracts from other components of the tree, such as the leaves and fruit were more effective antimicrobial and anti-biofilm agents than those isolated from the bark. These compounds demonstrated broad-spectrum antimicrobial activity against both Gram-positive and negative bacteria (Awolola *et al.* 2017), but required a solvent extraction process. In contrast, in our study the unmodified and natural bark cloth was bactericidal against the Gram-positive *S. aureus* strain USA300 and was not active against the Gram-negative *P. aeruginosa*. However, this suggests that combining other components of *F. natalensis* into the bark cloth may produce a final product with enhanced broad-spectrum activity.

To the authors' knowledge, this is the first report where an unmodified, sustainable natural whole bark cloth made by traditional methods has been employed as an antimicrobial fabric against MRSA. The potential clinical and commercial use of bark cloth derived exclusively from *F. natalensis* represents a significant advance in the search for novel antimicrobial products which could help prevent healthcare associated and wound infections. Isolation, purification and identification of the active antimicrobial compound derived from bark cloth may help further understand the efficacy and mechanisms of antimicrobial activity. Furthermore, understanding the immunological role of bark cloth in mediating the healing process would also provide a useful insight into the potential for use in clinical applications. Overall, this study suggests the current antimicrobial properties and physical characteristics of natural bark cloth are ideally suited for use in wound care management.

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Declaration of competing interest

The authors report no conflict of interest.

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Author contributions

J.A.B. devised the project, performed laboratory experimentation and wrote the manuscript. A.J.S., N.A.H. and P.D.V. contributed to experimental design, analysis and drafting of the manuscript. D.B.T., D.A. and M.H. substantially contributed to the design and acquisition of data. K.S. provided experimental materials and details/figures of the fabric production process. All authors discussed the results and contributed to the final drafting and revising of the manuscript. All authors approved the final submitted version.

Figures

Figure 1. Stages of bark cloth production including harvesting (A), fabrication (B), large sheets dried under sunlight (C) and quality inspection by Bukomansimbi Organic Tree Farmers Association representatives (D).

Figure 2. Analysis of bark cloth demonstrated the direction of the fabric grain (A, white lines) and Scanning Electron Microscopy revealed the fibrous nature of the cloth (B).

Figure 3. Bark cloth before (A) and after washing (B). Coloured leachates showing a characteristic bark cloth terracotta colour were observed in both the washing and rinsing stages (C).

Figure 4. Contact assays demonstrated the antimicrobial activity of unwashed bark cloth (black bar) and washed (shaded bar) against *S. aureus* strain USA300. There was no significant difference in the size of inhibitory zones produced between these samples, whereas both produced significantly larger inhibitory zones compared the filter paper control (no bar). $n = 3$; Error bars represent SEM; **** denotes $P \leq 0.0001$.

Figure 5. Disc diffusion assays demonstrated the antimicrobial activity of DMSO solvent extracts from unwashed bark cloth (black bar) and washed (shaded bar) against *S. aureus* strain USA300. Extracts from unwashed bark cloth produced significantly larger zones of inhibition compared to extracts from pre-washed fabric. Both produced significantly larger zones compared to the DMSO solvent control (no bar). *n* = 3; Error bars represent SEM; * denotes P ≤ 0.05 and **** P ≤ 0.0001.

Figure 6. Time-kill kinetic assay of *S. aureus* strain USA300 after 24 h after constant exposure to bark cloth (-●- , dashed line) compared to *S. aureus* strain USA300 alone (-■- , solid line). *n* = 3; Error bars represent SEM (not visible); ** denotes P ≤ 0.01 and **** P ≤ 0.0001.

Figure 7. Scanning Electron Microscopy analysis of (A) *S. aureus* strain USA300 in contact with bark fabric, (B) unexposed *S. aureus* strain USA300 and (C) un-inoculated bark fabric. Micrographs are representative examples. Scale bars are shown in μm.

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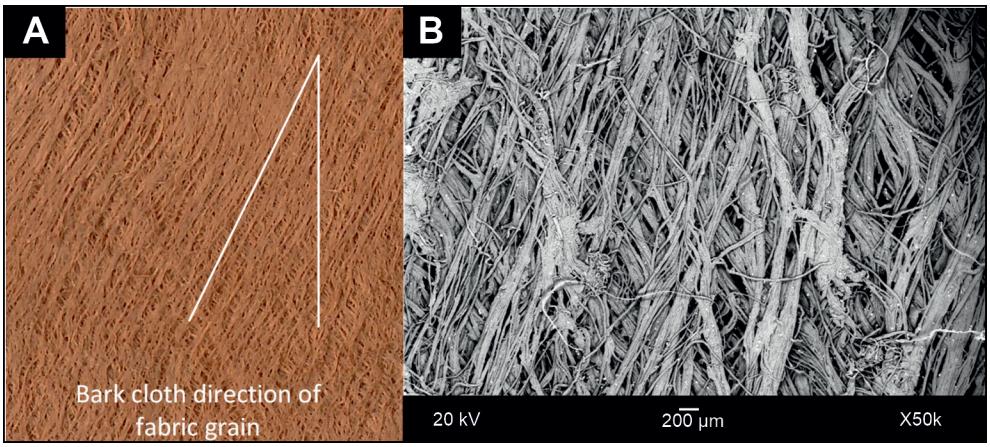

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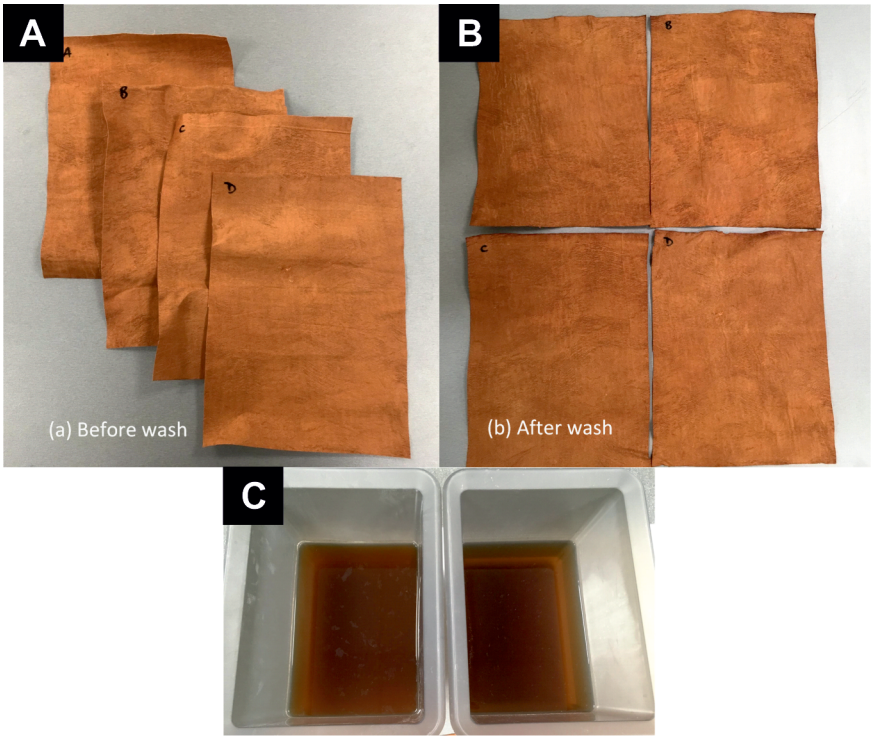

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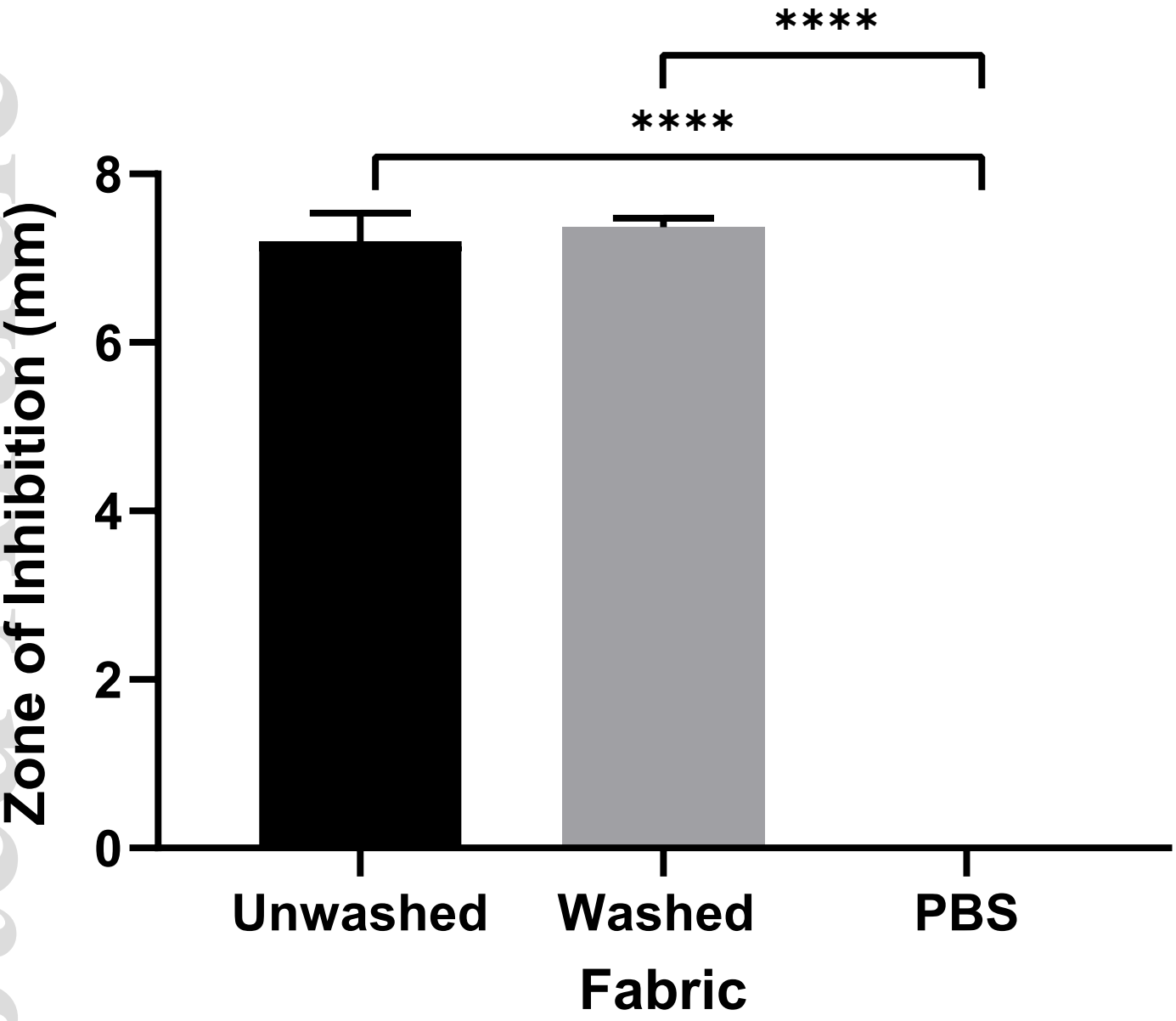

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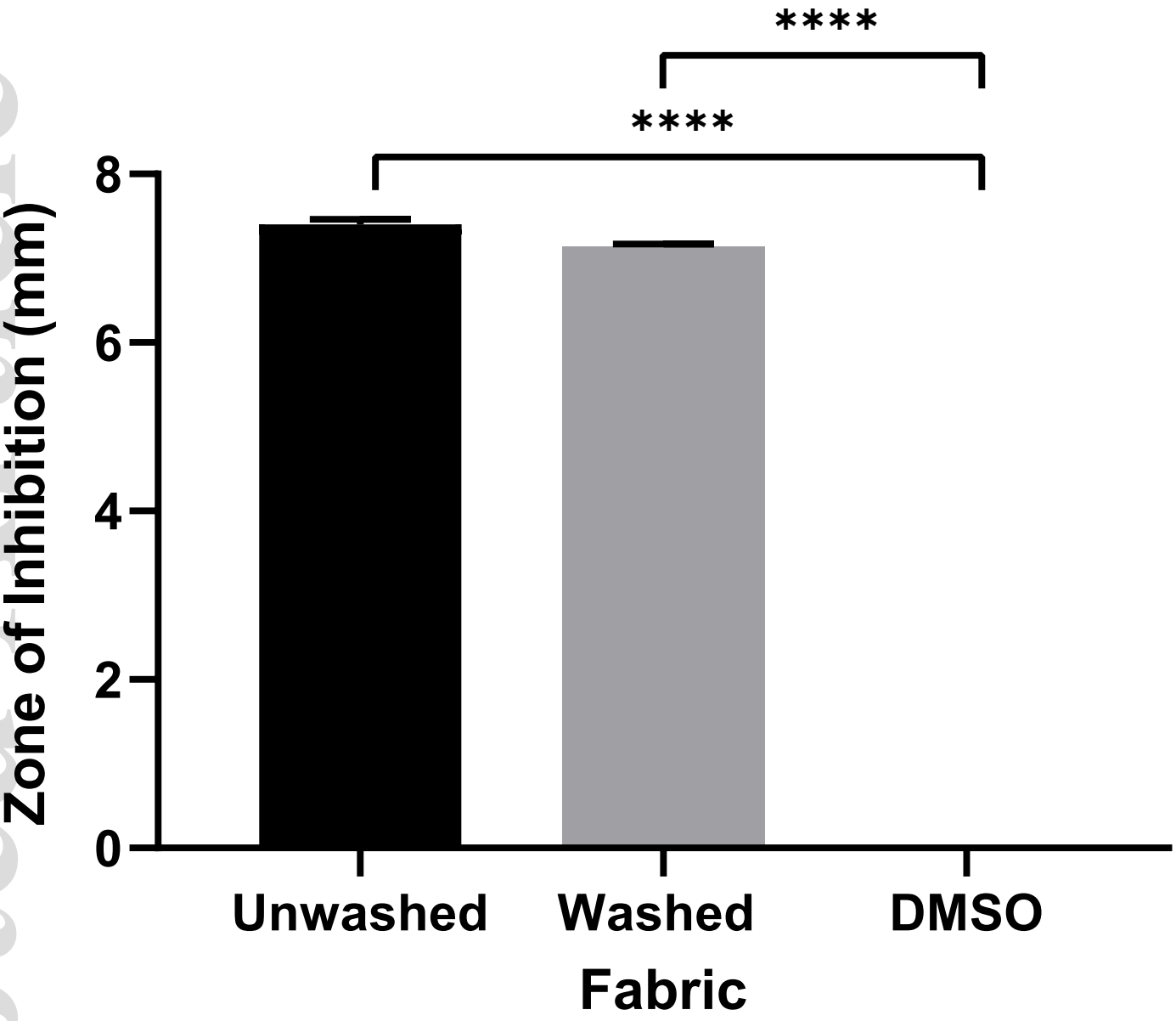

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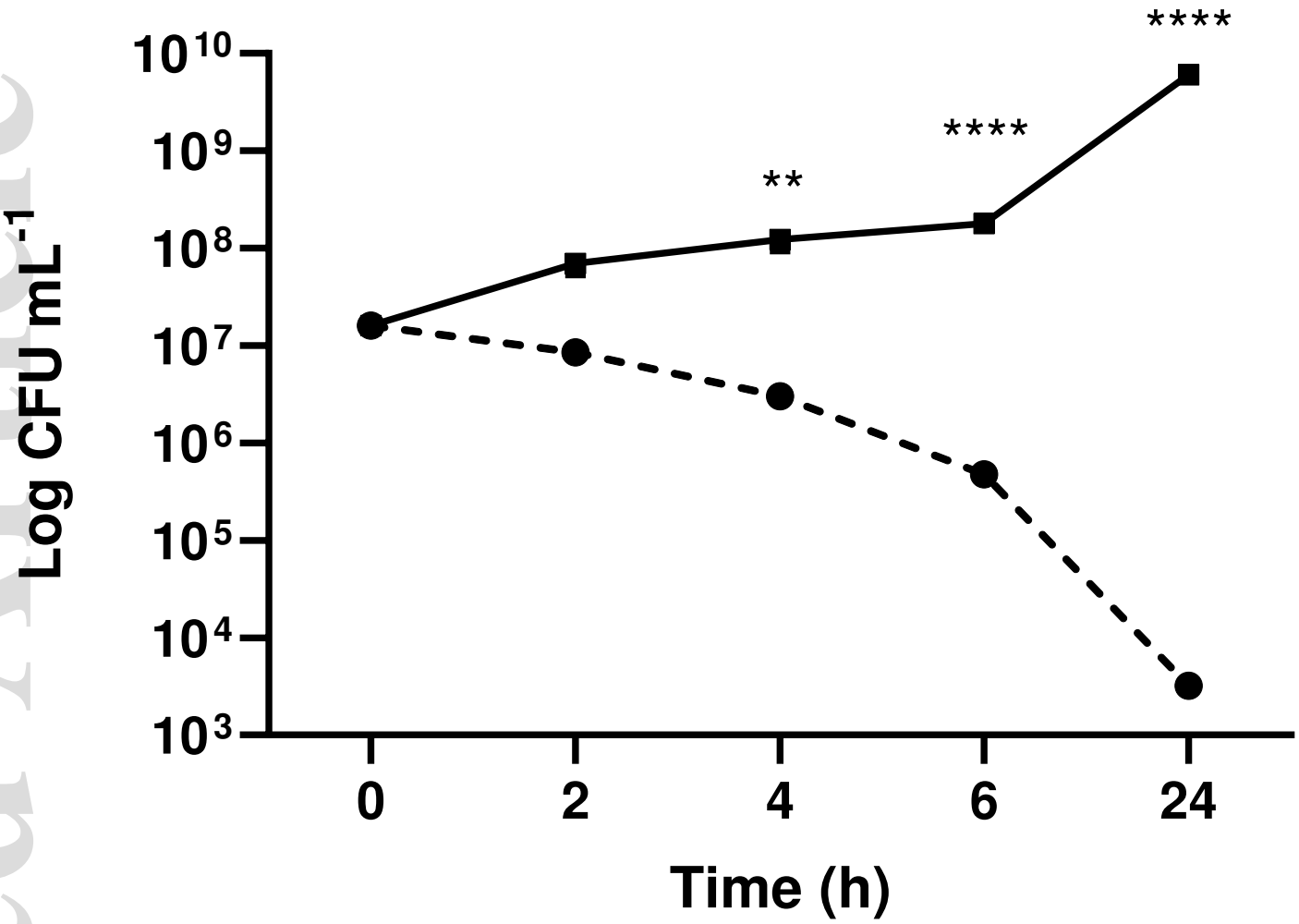

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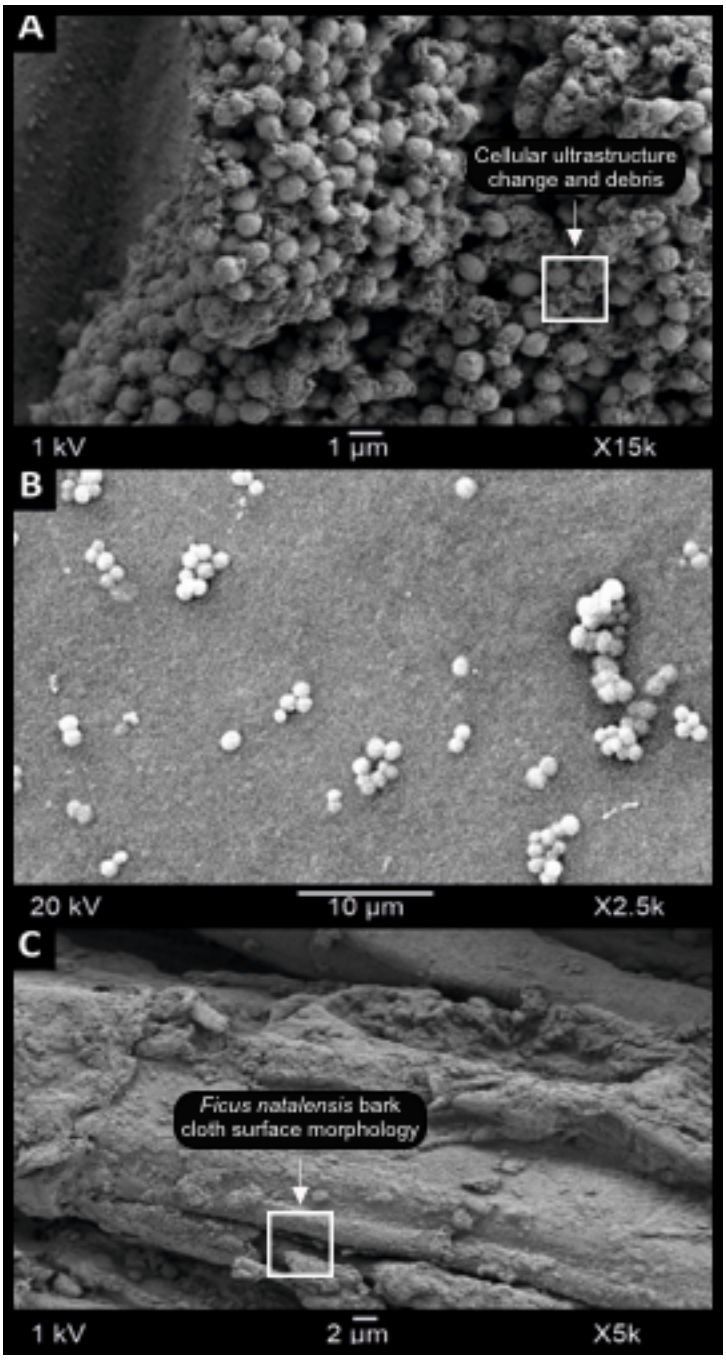

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