Antimicrobial effects of bacterial binding to a dialkylcarbamoyl chloride-coated wound dressing: an in vitro study

Objective: Wound dressings that inactivate or sequestrate microorganisms, such as those with a hydrophobic, bacteria-binding dialkylcarbamoyl chloride (DACC) surface, can reduce the risk of clinical infections. This 'passive' bioburden control, avoiding bacterial cell wall disruption with associated release of bacterial endotoxins aggravating inflammation, is advantageous in hard-to-heal wounds. Hence, the full scope of DACC dressings, including the potential impact of higher inoculum densities, increased protein load and different pH on antibacterial activity, needs to be evaluated.
Method: The Japanese Industrial Standard (JIS) L 1902 challenge test was used to evaluate the antimicrobial activity of the DACC-coated dressing against several World Health Organization (WHO)-prioritised wound pathogens (e.g., meticillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, microorganisms with extended-spectrum beta-lactamases and *Acinetobacter baumannii*), the effect of repeated bacterial challenge in an adverse wound environment, and antimicrobial performance at wound-related pH.
Results: High antibacterial activity of the DACC-coated dressing against the WHO-prioritised bacteria strains by its irreversible binding and inhibition of growth of bound bacteria was confirmed using JIS L 1902. At increased inoculation densities, compared to standard conditions, the DACC-coated dressing still achieved strong-to-significant antibacterial effects. Augmenting the media protein content also affected antibacterial performance; a 0.5–1 log reduction in antibacterial activity was observed upon addition of 10% fetal calf serum. The pH did not influence antibacterial performance. The DACC-coated dressing also sustained antibacterial activity over subsequent reinfection steps.
Conclusion: It can be assumed that the DACC-coated dressing exerts beneficial effects in controlling the wound bioburden, reducing the overall demand placed on antibiotics, without using antimicrobial substances.
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antibacterial activity • bacteria binding • DACC • dialkylcarbamoyl chloride (DACC)-coating • *Pseudomonas aeruginosa* • *Staphylococcus aureus* • World Health Organization-prioritised pathogens • World Health Organization-relevant pathogens • wound • wound care • wound dressing • wound healing • wound infection

Hard-to heal wounds present a significant clinical challenge.[1] An impaired wound repair process can lead to the spread of an infection with subsequent damage to adjacent tissues or even systemic harm.[1] Wounds of patients with concomitant factors, for instance older age, poor nutritional status, comorbidities or immune deficiencies, are more susceptible to bacterial contamination and infection, which further delays healing.[2–4] Hence, wound infections are still considered to be one of the most significant drivers of high mortality and morbidity.[1] *Staphylococcus aureus*, meticillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* are the most prevalent microbial species occurring in patients with infected wounds.[1] Consequently, treatment of wound infections plays an important role in wound management.

Generally, a combination of topical antiseptics and/or systemic antibiotics is used to battle high bioburden.[1] Antiseptic solutions of polyhexanide (PHMB), octenidine, silver or iodine, as well as wound dressings containing these antimicrobial agents, are most commonly used in the healthcare setting.[5–7] These can disrupt or kill microorganisms by acting on multiple target sites of the microbial cell and thus have a lower risk of inducing bacterial resistance compared with topical antibiotics.[2] Such topical antiseptics are, however, non-selective and concern has been raised regarding potential cytotoxic and damaging effects on the various cell types involved in wound healing, impairing the healing process.[2] Moreover, killing bacteria by such an 'active' mechanism leads to disruption of the bacterial cell wall and the potential release of bacterial endotoxins that act as danger signals and aggravate inflammatory processes in the hard-to-heal wound.[8,9]

The use of 'passive' strategies for bioburden control, which rely on a physical mode of action that does not kill bacteria in the wound and thus the potential release of endotoxins, has been suggested.[2,10] For example, coating fibres with dialkylcarbamoyl chloride (DACC), a fatty acid derivative, conveys a hydrophobic

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Table 1. Comparison of strengths and weaknesses of common antimicrobial in vitro test assays with respect to evaluation of wound dressings

Test method	Agar diffusion test	ASTM E2149	AATCC 100	JIS L 1902
Description	• Agar plates are inoculated with test microorganism • Dressing samples are placed on top of the inoculated surface • Evaluation of the formation of a zone of inhibition (ZOI) after 24 hours	• 50ml of standardised microbial culture is placed into three containers: (1) bacteria only suspension; (2) antimicrobial test substance; and (3) control object • Microbial concentrations are enumerated at 'time zero' • All jars are shaken in a wrist-action shaker for a specified contact time, usually 1 hour • After the specified contact time, the microbial concentration in all jars is determined • Concentration of microorganisms for the antimicrobial product are compared to the microbial suspension alone or the untreated control • 'Antimicrobial' = product that produces a substantial reduction relative to either the inoculum or untreated controls	• Control and test fabric swatches are inoculated with microorganisms (microbial suspension touches only the fabric) • Bacteria levels on both control and test fabrics are determined at 'time zero' by elution in a large volume of neutralising broth, followed by dilution and plating • Inoculated control and test fabrics are allowed to incubate, undisturbed in sealed jars, for 24 hours • After incubation, microbial concentrations are determined • Reduction of microorganisms relative to initial concentrations and the control fabric is calculated (mostly in %)	• Control and test fabrics are inoculated with microorganisms in triplicate, ensuring that the inoculum is only in contact with the fabrics • Initial microbial concentrations are determined at 'time zero' by elution, then dilution and plating of control fabrics immediately after inoculation • Inoculated control and test fabrics are allowed to incubate undisturbed in sealed containers at body temperature for 18 hours • After incubation, microbial concentrations are determined • Reduction of microorganisms relative to initial concentrations and the control fabric is calculated (mostly in log CFU)
Strengths	• 'Treatment' of a surface • Relatively fast • Easy to handle	• Reproducible • Standardised microbial concentrations • Allows experimental flexibility (adaptation to products of various shapes and sizes)	• Quantitative • Reproducible • Tests for both bacteriostatic and bactericidal properties • Standardised microbial concentrations • Provision of nutrients	• Quantitative • Reproducible • Tests for both bacteriostatic and bactericidal properties • Standardised microbial concentrations • Technical replicates 'failed/pass' criteria
Weaknesses	• Qualitative result only • No quantification • Depends on diffusible agent	• Not a realistic situation • Highly dependent on the contact time • No clear success criteria • Removed from actual usage of most antimicrobial products	• Vague success criteria ('the criteria for success must be decided by the interested parties') • Cumbersome if the test fabric does not readily absorb liquids (hydrophobic)	• Generally not accepted by the US Environmental Protection Agency for so-called 'health claims' • Hydrophobic materials might be difficult to test

AATCC 100—American Association of Textile Chemists and Colorists (a challenge test); ASTM E2149—American Society for Testing and Materials (a suspension test); CFU—colony forming units; JIS L 1902—Japanese Industrial Standard challenge test; ZOI—zone of inhibition

surface. Wound bacteria, on the other hand, possess a high cell surface hydrophobicity (CSH).[11] When the hydrophobic, DACC-coated surface comes into contact with the hydrophobic bacterial surface, binding between them occurs through hydrophobic interaction and expulsion of water molecules.[11] This mediates the irreversible binding of microorganisms to DACC-coated surfaces.[2] During dressing changes, the bound microorganisms are subsequently removed.[11] DACC-coated dressings have been successfully employed in the management and prevention of wound infections, and in reducing wound bioburden, as described in many clinical studies.[2,12]

Mechanistic proof for the antibacterial activity is difficult to demonstrate as most test methods have been developed for releasable agents. In vitro standard tests allow direct comparison of the antimicrobial effects of dressings.[13] They are simple, rapid, reproducible, inexpensive and enable handling of a range of sample quantities. Yet the different tests vary distinctly in their properties and, consequently, their outcomes.[13,14] The most commonly used tests are the agar diffusion test (ADT), suspension methods (e.g., the American Society for Testing and Materials (ASTM) E2149), and the direct contact tests, such as Japanese Industrial Standard (JIS) L 1902 or American Association of Textile Chemists and Colorists (AATCC) 100, all of which have unique strengths and weaknesses as regards the evaluation of wound dressings (Table 1). Passive mechanisms may be overlooked in settings employing ADT or suspension tests since they are optimised to test 'active' antimicrobial agents. In contrast, challenge tests

(e.g., JIS L 1902, AATCC 100), where diffusability of the antimicrobial agent is not a prerequisite and the dressing samples come into close contact with the microorganisms, are able to assess effects based on the ability to immobilise microorganisms.[13]

Hence, the aim of this study was to use the challenge test JIS L 1902 to evaluate the antimicrobial activity of a DACC-coated dressing (Sorbact Compress, ABIGO Medical AB, Sweden) against several World Health Organization (WHO)-prioritised wound pathogens, such as MRSA, vancomycin-resistant *Enterococcus* (VRE), extended-spectrum beta-lactamases (ESBL) and *Acinetobacter baumannii*, as well as the effect of repeated bacterial challenge in an adverse wound environment, and performance at wound-related pH.

Methods

Materials

The DACC-coated wound dressing (Sorbact Compress) was obtained from the manufacturer ABIGO Medical AB, Sweden. The silver-containing dressings Acticoat Flex 3 and Mepitel Ag were purchased from Smith+Nephew, UK and Mölnlycke Health Care AB, Sweden, respectively. Gauze bandage was purchased from Akla AB, Sweden.

Staphylococcus aureus ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* DSM 11729 (MRSA), *Enterococcus faecium* DSM 17050 (VRE), *Pseudomonas aeruginosa* DSM 24599 (ESBL), *Enterobacter cloacae* DSM 26481 (ESBL), and *Acinetobacter baumannii* DSM 102929 were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen DSMZ, Germany. *Staphylococcus aureus* CCUG 2354 and CCUG 10778 (corresponding to ATCC 6538), and *Pseudomonas aeruginosa* CCUG 17619 were purchased from the culture collection at the University of Gothenburg, Sweden. For cultivation of bacteria, special peptone and 'lab-lemco' powder for preparation of caso-bouillon were purchased from Oxoid, UK. The 0.9% sodium chloride (NaCl) solution was obtained from Fresenius Kabi Deutschland GmbH, Germany and Tween 20 was purchased from Roth, Germany. Horse blood (HB) agar, Saburoud agar, cation-adjusted Mueller–Hinton agar and phosphate-buffered saline (PBS) were purchased from Sahlgrenska University Hospital Substrate Department, Sweden.

Agar diffusion test (ADT)

To investigate the effect of various dressings on the viability of bacterial cells, an antimicrobial assay based on the zone of inhibition (ZOI) on an agar plate was performed according to the Kirby–Bauer disk diffusion susceptibility test protocol.[15] An aliquot of 100µl of a suspension of approximately 10^8 colony-forming units (CFU)/ml of *Staphylococcus aureus* CCUG 2354 or *Pseudomonas aeruginosa* CCUG 17619 was spread evenly onto cation-adjusted Mueller–Hinton agar using a cotton swab. All wound dressing materials were cut into circular discs (14mm diameter) and placed onto the Mueller–Hinton agar plate. The agar plate was then incubated for 18–24 hours at 37°C before photographing the ZOI. The ZOI was measured using a standard ruler.

Scanning electron microscopy (SEM) imaging

An 8×10^7 CFU/sample of *Staphylococcus aureus* was inoculated onto 14mm-diameter circular discs of DACC-coated wound dressing and incubated in capped tubes for 24 hours at 37°C under aerobic conditions. Half of the samples were immediately fixed in 2.5% glutaraldehyde (EMSdiasum, US) in 0.1M piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) buffer, and the other half were washed in 8ml 0.9% NaCl solution with Tween 80 (Merck, Germany) by vortexing at maximum speed for 5×5 seconds before fixing. The samples were further processed by washing several times with 0.1M PIPES, and postfixation was carried out in 1% osmium tetroxide (OsO_4) (EMSdiasum, US) in 0.1M PIPES for 1.5 hours at 4°C; the samples were then washed several times with Milli-Q water (Merck, Germany) and dehydrated at room temperature in a graduated ethanol (Solveco AB, Sweden) series (30, 50, 70, 85, 95 and 4×100%). The 100% ethanol was subsequently replaced by hexamethyldisilazane (HMDS, Supelco #52619, Merck KG, Germany), which was removed after a three-minute incubation. Samples were air-dried overnight, mounted on aluminium stubs using silver glue (Pelco #16062, Ted Pella Inc., US) and coated with 20nm of gold (Quorum Technologies, UK) the following day. The SEM images–were aquired with a ZEISS Gemini II 450 (Carl Zeiss Microscopy, Germany) at an operating voltage of 2kV.

Testing of antimicrobial activity according to JIS L 1902

Antibacterial activity was determined according to the Japanese Industrial Standard (JIS L 1902:2008, 'Testing method for antibacterial activity of textiles') as reported previously.[16,17] In brief, an appropriate culture medium was inoculated with the test microbes and cultivated for 24 hours at 37°C under aerobic conditions. For experiments, 400mg samples of the wound dressings were incubated with 200µl of each test microbe solution (10^5 CFU/ml) for 24 hours at 37°C under aerobic conditions. For higher inoculum densities, microbe concentrations were raised to 10^6, 10^7 and 10^8 CFU/ml. Experiments with increased protein content were performed using culture medium containing 10% and 50% fetal calf serum (FCS; Promocell, Germany). To evaluate the impact of pH, the culture medium (approximately pH 7) was adjusted to pH 6 with 0.1M hydrochloric acid (HCl, Roth, Germany), and pH 8 and pH 9 using 0.1M sodium hydroxide (NaOH, Roth, Germany). Polyester (TITK, Germany) was used as the reference material since it is also hydrophobic. For bacterial number determination, the incubated samples were extracted in 10ml 0.9% NaCl solution with Tween 20. Serial dilutions were plated on Columbia agar plates (BioMerieux, France), incubated for 24 hours at 37°C and colonies subsequently counted. The

CFU/ml value and the total microbial count of the samples (in CFU) were calculated. The growth reduction compared with the starting value was determined using the following equation and rated according to the JIS L 1902:2008:

growth reduction [log CFU] =
$\log(^{24h}\text{MW [CFU]}_{\text{control(polyester)}}) - \log(^{24h}\text{MW [CFU]}_{\text{sample}})$

with growth reduction expressed as log difference of mean values (MW) of CFUs at 24 hours.

Rating:
- no antimicrobial activity=<0.5 log microbial growth reduction
- slight antimicrobial activity=0.5–1 log microbial growth reduction
- significant antimicrobial activity=>1–≤3 log microbial growth reduction
- strong antimicrobial activity=>3 log microbial growth reduction

Investigation of the sustained antimicrobial effect of DACC-coated wound dressing

The sustained antimicrobial effect of the DACC-coated wound dressing was investigated over seven days using a direct incubation method with repeated reinoculation of the material. Therefore, caso-bouillon was inoculated with *Staphylococcus aureus* ATCC 6538 and cultivated for 24 hours at 37°C under aerobic conditions. For experiments, 400mg samples of the wound dressings were incubated with 200µl of the prepared *Staphylococcus aureus* suspension according to JIS L 1902 at 37°C under aerobic conditions. Polyester was used as reference material. The inoculation step was repeated every day for seven days in total. Inoculated test samples were kept at 37°C in a humidified atmosphere. After 24 hours (day 1), 48 hours (day 2), 72 hours (day 3), 96 hours (day 4), 120 hours (day 5), 144 hours (day 6) and 168 hours (day 7) of incubation, dressing samples were extracted in 10ml 0.9% NaCl solution with Tween 20 for bacterial number determination. Serial dilutions were plated on Columbia agar plates, incubated for 24 hours at 37°C and colonies subsequently counted. The CFU/ml value and the total microbial count of the samples (in CFU) were calculated.

Statistical analysis

In all cases, two independent experiments were performed, each including three technical replicates. All values were expressed as mean±standard deviation (SD). One-way analysis of variance (ANOVA) was carried out to determine statistical significance (Excel 2000, Microsoft Corp., US). Differences were considered statistically significant at a level of $p<0.05$.

Results

Antimicrobial activity exerted by bacteria binding

The agar diffusion test demonstrated that the DACC-coated wound dressing did not contain a releasable antimicrobial agent (Fig 1a, b). While a silver-containing dressing sample demonstrated distinct formation of a ZOI, no ZOI was found for the wound dressing with DACC-coating or the control fabric. Nonetheless, significant antimicrobial activity was found in contact tests, such as the JIS L 1902 against *Staphylococcus aureus* (Fig 1c) and *Pseudomonas aeruginosa* (Fig 1d), indicating efficacy through a binding mechanism. Binding of bacteria to the DACC-coated dressing was so strong that no detectable bacteria were released even after extensive washing in the presence of a surfactant. The SEM images after washing verify the presence of bacteria on the DACC-coated fibres of the wound dressing (Fig 2). Moreover, anchoring points between the bacteria and the fibre surface were observed, indicating strong binding to the latter. It was further found that bound bacteria were still viable, as confirmed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining, but appeared to be impeded in reproduction, as no multiplication of bound bacteria was observed.

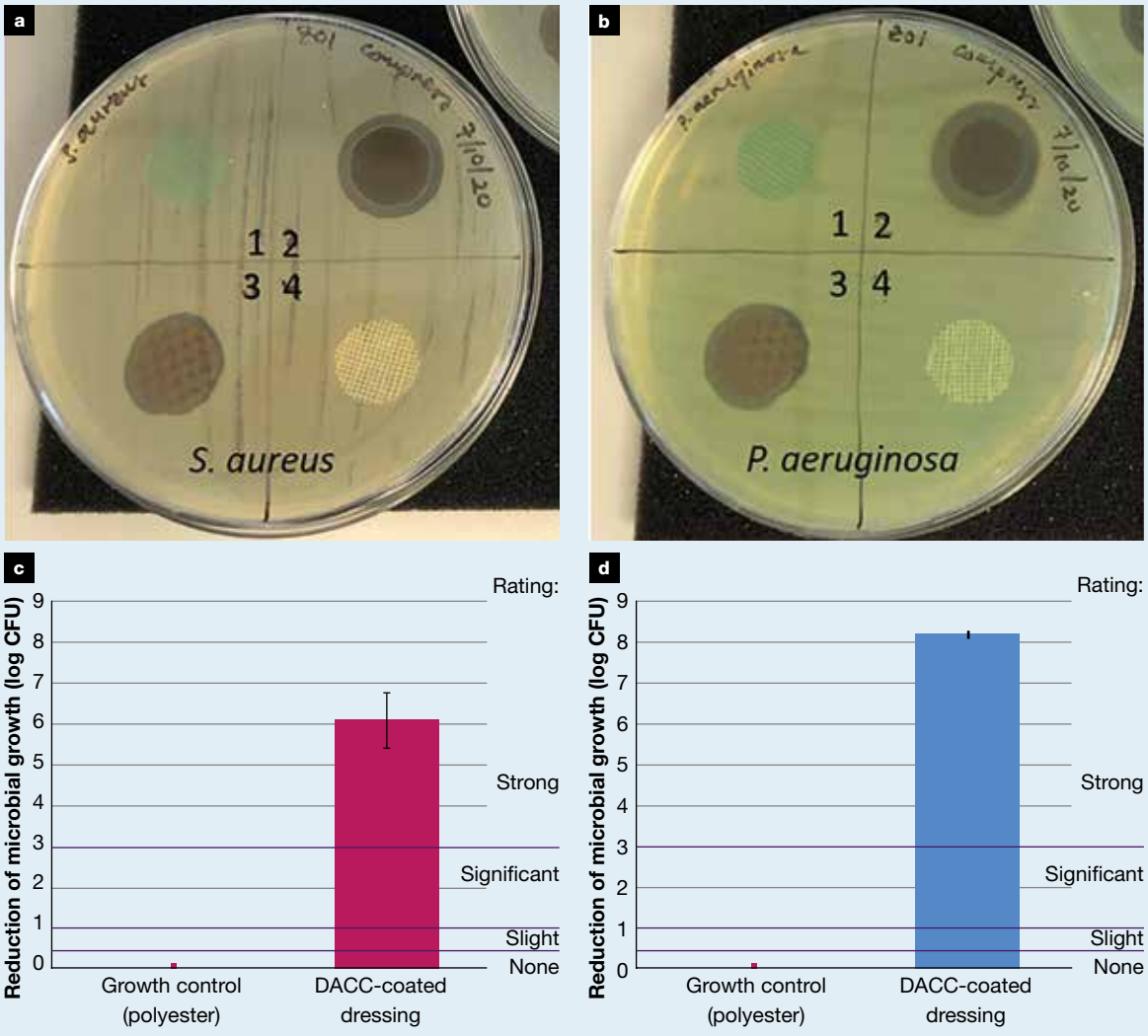
Antimicrobial activity against WHO-prioritised pathogens

Different aspects of the binding-based antimicrobial activity of the DACC-coated wound dressing were explored. The DACC dressing was found to effectively inhibit growth of various bacterial species. Testing of antibacterial activity showed a strong reduction of all tested resistant, WHO-prioritised bacteria strains according to JIS L 1902 (Fig 3). Moreover, the DACC-coated dressing was able to completely inhibit the growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterobacter cloacae* and *Acinetobacter baumannii* in the test. It was confirmed that the DACC-coated dressing exerted this high antibacterial effect over a prolonged period of time despite repeated inoculation in the in vitro experiment (Fig 4).

Impact of environmental factors on antimicrobial activity

To assess whether or not the efficacy of the DACC-coated dressing against bacteria was influenced by three factors usually not considered in standard test methods, the potential impact of higher inoculum densities, increased protein load and different pH on antibacterial activity were investigated. Higher inoculum densities led to a reduction in antimicrobial efficacy in the contact test (Fig 5a). Nonetheless, at a tenfold increased inoculation density the DACC-coated dressing still achieved a >3 log reduction of bacteria and a 100-fold increase still exerted a strong effect on *Pseudomonas aeruginosa* and a significant effect on *Staphylococcus aureus* according to JIS L 1902. Moreover, augmenting the protein content of the media affected antibacterial performance of the DACC-coated wound dressing against both *Staphylococcus aureus* and *Pseudomonas aeruginosa* in the contact test (Fig 5b). Upon the addition

Fig 1. Agar diffusion test showing the zone of inhibition of different wound dressings against the two microorganism strains *Staphylococcus aureus* **(a)** and *Pseudomonas aeruginosa* **(b)**. Samples shown are: (1) Sorbact Compress; (2) Acticoat Flex 3; (3) Mepitel Ag; and (4) gauze bandage. Testing of the dialkylcarbamoyl chloride-coated dressing Sorbact Compress according to the JIS L 1902 yielded a strong antibacterial activity against *Staphylococcus aureus* **(c)** and *Pseudomonas aeruginosa* **(d)**. CFU—colony forming units



of 10% FCS, an antibacterial activity of 0.5–1 log reduction was observed. Bacteria agglomerates were found to form in the fibre grates under experimentally increased protein conditions, easily discernible in the untreated samples (Fig 5c). Washing of specimens resulted in an observable decrease of bacteria agglomerates in the grates. After extensive vortexing (as required by JIS L 1902) no bacteria were found in the grates of the vortexed specimen, although they were apparent on the dressing fibres. This indicated that under increased protein content, bacteria released into the extraction media were derived from agglomerates stuck in the grates while those bound directly by the DACC-coated fibres were bound tightly and not liberated. Moreover, the DACC-coated dressing

Fig 2. Scanning electron microscopy images of *Staphylococcus aureus* binding to dialkylcarbamoyl chloride-coated fibres. Samples shown are: unwashed **(a)** and washed with 0.9% NaCl with Tween 20 **(b)**, including a higher magnification of the washed sample **(c)**.
Electron high tension voltage (EHT): 2.5kV; magnification 2500× **(a, b)**, 100,000 **(c)**; working distance: 7.2mm

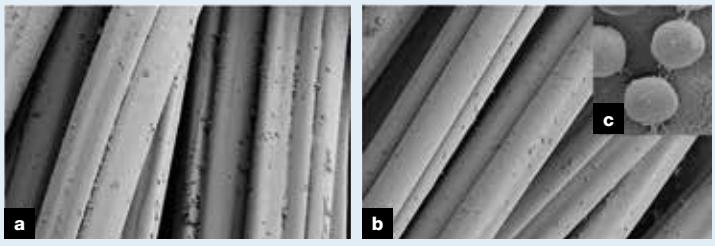


Fig 3. Testing of the antibacterial activity of the dialkylcarbamoyl chloride-coated dressing according to JIS L 1902 showed a strong antibacterial activity against the WHO-prioritised bacteria strains. CFU—colony forming units; *S. aureus*—*Staphylococcus aureus*; *P. aeruginosa*—*Pseudomonas aeruginosa*; *E. cloacae*—*Enterococcus cloacae*; *A. baumannii*—*Acinetobacter baumannii*; ESBL—extended-spectrum beta-lactamase; MRSA—meticillin-resistant *Staphylococcus aureus*; VRE—vancomycin-resistant *Enterococcus*

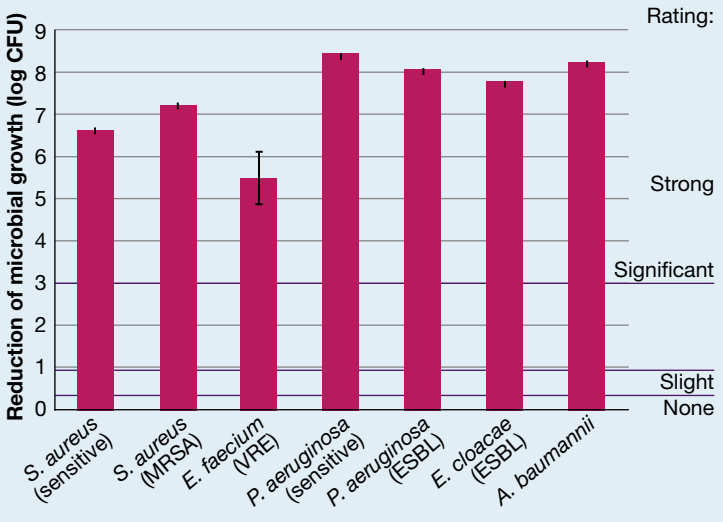
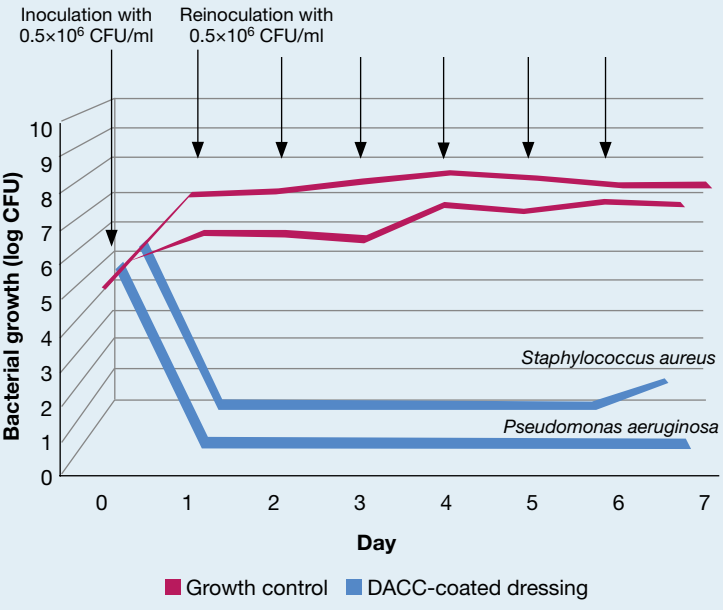


Fig 4. The dialkylcarbamoyl chloride (DACC)-coated dressing demonstrated a statistically significant ($p<0.001$), prolonged antibacterial effect against *Staphylococcus aureus* and *Pseudomonas aeruginosa* over seven days according to JIS L 1902 with repeated inoculation during the experiment. CFU—colony-forming units



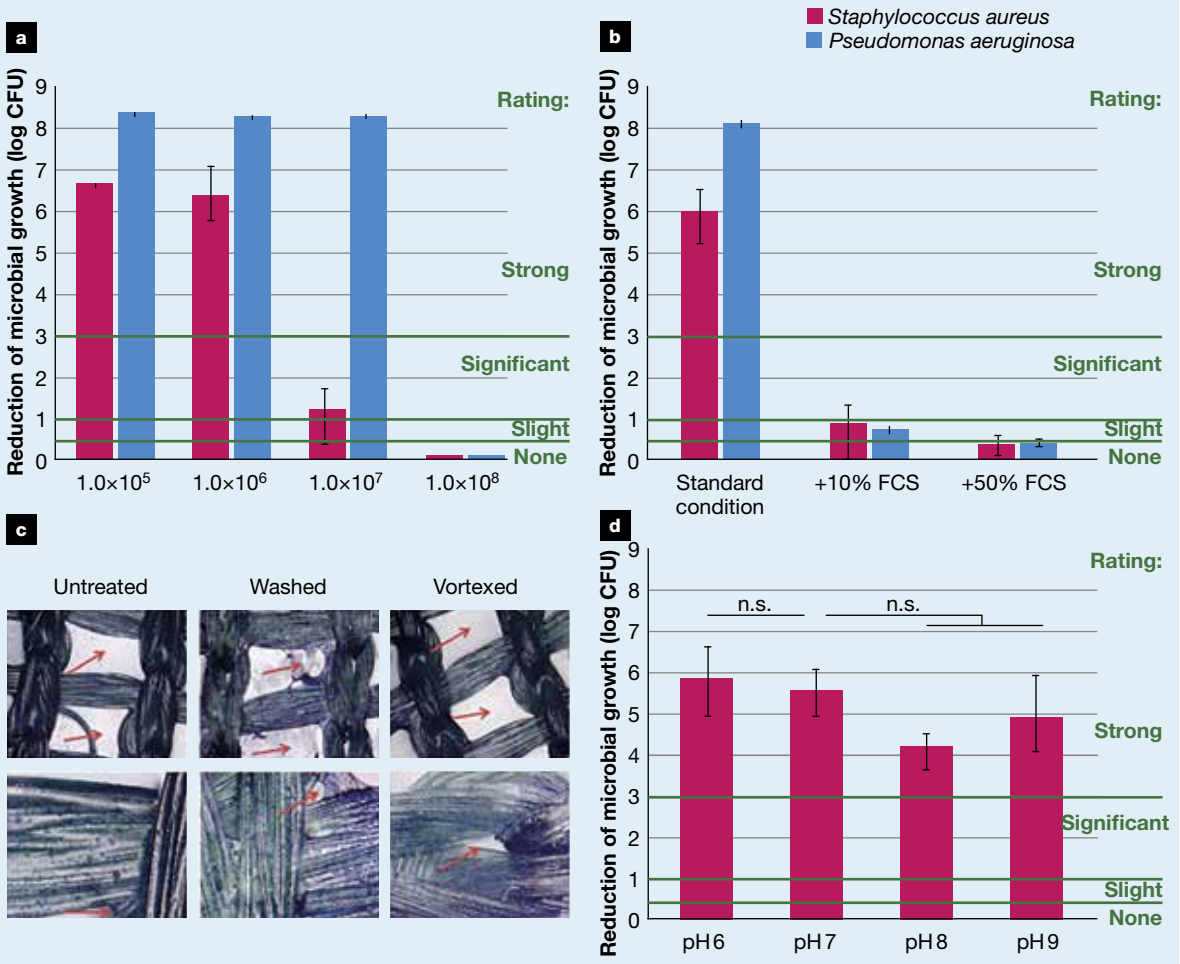
exhibited the same strong antibacterial effect against the model microorganism *Staphylococcus aureus* according to JIS L 1902 at pH 6, as well as pH 8 and pH 9, the alkaline range being typical for hard-to-heal wounds (Fig 5d).

Discussion

In a number of clinical studies, DACC-coated dressings have shown promising results in both the prevention and treatment of wound infections, often in combination with improved wound healing or reduction in wound size.[5,18–26] The clinical experience of DACC-coated wound dressings was also summarised recently in two reviews, demonstrating their usefulness in the management of a variety of acute and hard-to-heal wounds.[2,12] For instance, DACC-coated dressings reduce the risk for surgical side infections,[21,22] being most effective in the early postoperative period by preventing bacterial access to the wound.[2,19] Furthermore, infection in high-risk patients could effectively be prevented using this bacteria-binding dressing.[23] In addition, reduction of the bacterial load helped with successful treatment of infected hard-to-heal wounds using DACC-coated dressings.[5,27] There is also evidence that supports the use of DACC-coated wound dressings as an alternative approach to preventing and/or treating infection in both acute and hard-to-heal wounds by not exacerbating antimicrobial resistance (AMR) and supporting antimicrobial stewardship (AMS).[10] The DACC conveys microbial binding properties primarily by hydrophobic interactions.[27] The first step is initially non-specific, involving the hydrophobic DACC coating and the hydrophobic surface of microorganisms.[11] In this study, the presence of bacteria on the DACC-coated fibres of the wound dressing was confirmed in the experiments with SEM images. In the second step, interactions between bacterial adhesion proteins and the DACC surface are thought to become predominant.[11,27] Accordingly, anchoring points between the bacteria and the fibre surface were observed in the SEM images, suggesting a strong binding of the bacteria to the DACC-coated fibres. Moreover, a significant antimicrobial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* according to JIS L 1902 was observed, indicating efficacy through a binding mechanism and thereby controlling the overall bioburden. This could explain the reduced bioburden and improved wound healing[5,18–26] found in clinical practice with DACC-coated dressings.

We found that a prerequisite for antibacterial activity is the close contact of bacteria to the DACC-coated dressing. No chemically or pharmacologically active substances are released from the DACC-coated dressing.[2] This was verified using the agar diffusion test, where no ZOI was found for the wound dressing with the DACC coating while a silver-containing dressing sample demonstrated distinct formation of a ZOI. The sole physical binding mechanism is considered advantageous as the dressing can be expected not to adversely affect the wound bed or cells involved in the wound healing process,[2] and clinical studies have confirmed an excellent safety profile for the DACC-coated dressing.[2,5] In contrast, silver may exert a local cytotoxic effect on fibroblasts and keratinocytes at higher concentrations,

Fig 5. Assessment of the influence of higher inoculum densities **(a)** and increased protein load **(b)** on the antibacterial activity of the DACC-coated dressing according to JIS L 1902 against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Bacteria agglomerates stained with MTT were found in the fibre grates under experimentally increased protein conditions, which decreased with washing of the samples and were removed upon vortexing **(c)**. Different pH did not affect the antibacterial activity of the DACC-coated dressing according to JIS L 1902 against *Staphylococcus aureus* **(d)**. CFU—colony-forming units; DACC—dialkylcarbamoyl chloride; FCS—fetal calf serum; MTT—3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; n.s.—not significant



resulting in delayed healing.[17,28] In addition, bacterial resistance to silver has been reported previously[29,30] and could be experimentally induced in *Staphylococcus aureus*.[31] Because of the 'passive' antibacterial mechanism of the DACC coating by simple hydrophobic interaction, development of resistance is unlikely.[2] Nonetheless, it should be noted that bacteria could adapt by changes in surface hydrophobicity. Larkö et al.,[32] considered this a possible limitation in infections caused by bacteria expressing a hydrophilic cell surface, which they observed for their *Staphylococcus aureus* strain.

No distinct reduction of antimicrobial efficacy against *Staphylococcus aureus* was observed in this study. This is also reflected in the studies of Ljungh et al.,[11] who showed that most wound pathogens are likely to express a higher CSH in wounds and simulated wound environments than in conventional in vitro culture. Moreover, several studies noted increased CSH in resistant bacteria strains as this, together with expression of pigment, haemolysin, lipase, protease, gelatinase, rhamnolipids and biofilm formation, constitute the virulence factors.[33–35] Accordingly, it was found that the DACC-coated dressing can effectively inhibit growth of various bacterial species, including the resistant, WHO-prioritised bacteria strains tested in this study according to JIS L 1902. Importantly, the physical binding of bacteria to the DACC surface means that the bacterial cell walls remain intact, avoiding the release of endotoxins[2] that subsequently act as aggravating factors for wound inflammation.[36,37]

It has become increasingly clear that standard test conditions might not adequately reflect the wound environment, where dressings may be challenged with

higher bacteria loads, elevated protein content and alkaline pH. Hence, in this study we investigated how higher inoculum densities, increased protein load and different pH affect the antibacterial activity of the DACC-coated dressing according to JIS L 1902. Larkö et al.,[32] reported a potential saturation of the DACC-coated surface with bacteria that allowed remaining nonbound *Pseudomonas aeruginosa* to multiply. In contrast, Ljungh et al.,[11] demonstrated much higher DACC-coated dressing binding capacities for bacteria in the range of 10^8 CFU/ml, 5.01×10^6 CFU/ml, 10^6 CFU/ml and 3.16×10^7 CFU/ml for *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacteroides fragilis* and *Fusobacterium nucleatum*, respectively. This difference is likely to be related to the different experimental designs of the two studies. In our study, we also observed a reduction in antimicrobial efficacy in the contact test with higher inoculum densities due to overgrowth of the samples during the incubation period. In contrast, when the samples were challenged repeatedly with low amounts of microorganisms, a consistently high antibacterial activity was observed. Extensive overgrowth, meaning that bacteria not in close, direct contact with the DACC surface retain their ability to proliferate, probably leads to protein deposition and biofilm formation around the fibres. Increasing the protein content of the media also affected the antibacterial performance of the DACC-coated dressing against *Staphylococcus aureus*, as well as *Pseudomonas aeruginosa*. Bacteria agglomerates were found to form in the fibre grates under these conditions. Washing decreased the bacteria agglomerates in the grates while extensive vortexing, as required by JIS L 1902, removed them completely. In contrast, bacteria bound directly to the DACC-coated fibres were bound tightly and could not be liberated in the elution procedure.

These results raised two questions:

i. How does the term irreversible binding translate into daily practice and dressing handling?
ii. What protein content is needed to reflect hard-to-heal wound status?

Cooper et al.,[38] demonstrated that DACC-coated dressing samples exposed to *Pseudomonas aeruginosa* biofilm rapidly and extensively retained biofilm, which could be removed effectively from the surface afterwards. Ljungh et al.,[11] also used a relatively gentle procedure for enumeration of bacteria bound to the DACC surface. Similarly, it can be expected that bacteria will remain stuck to the wound dressing upon removal from the wound, aiding reduction of the bioburden. Hence, these studies demonstrate the high retention capacity. In contrast, the JIS L 1902 enumeration process is distinctly harsher, resulting in loss of all bacteria only loosely bound to the DACC surface (possibly in the second or third layers), as well as that of bacteria only absorbed by the material. Contrary to this, bacteria in direct contact with the DACC surface are bound irreversibly. It should be acknowledged, however, that the term 'irreversible' is also dynamic, as Sjollema et al.,[39] for example, describe bacterial adherence to substratum surfaces as multiple, reversibly-binding tethers that detach and successively re-attach without ever detaching all at once, resulting in irreversible bacterial adhesion.

Generally, the total protein content in hard-to-heal wounds is reported to range from 10mg/ml to approximately 55mg/ml,[40,41] with 40mg/ml tending to be the most widely accepted value.[42] We recently conducted a survey on hard-to-heal wounds presenting at the wound clinic of the Department of Dermatology in Jena, and found a protein content of 0.2–4600µg/ml in a total of 303 wounds with a median value of 242µg/ml and a mean value of 428±573µg/ml (unpublished data). These results are consistent with a study by Moseley et al.,[43] showing a mean protein concentration in hard-to-heal wound fluids of 0.644±0.153mg/ml. Discrepancies can be explained by the different sampling techniques; while the former studies used aspirated wound fluid after collection beneath airtight film dressings,[40–42] we sampled the wound surface by a washing procedure, whereas Moseley et al.,[43] extracted the protein from filters after placing them on the wound surface. Similar results were obtained by Broszczak et al.,[44] who employed both methods. The different protein loads used in this test reflect the different potential protein levels in hard-to-heal wounds. The standard condition yielded a protein load of 265±7µg/ml, which represented the lowest possible protein amount; addition of 10% FCS corresponded to 2463±77µg/ml, representing the middle range, while supplementation of 50% FCS with 14183±1330µg/ml protein was intended to confer the high protein load found in some wounds. Under the latter conditions, we observed bacteria agglomerates forming in the fibre grates and which flaked off during vortexing in the standard test elution procedure. In contrast, under low protein conditions, bacteria bound tightly to the DACC-coated fibres and could not be liberated.

Braunwarth et al.,[45] showed that silver-containing dressings possessed similar bacteriostatic effects over a pH range of 5.5–9.0. Another study on the performance of antiseptic solutions at different pH demonstrated that chlorhexidine and octenidine feature mainly pH-independent bactericidal activity; in contrast PHMB exhibited increased efficacy while povidone-iodine showed a distinct reduction in antibacterial effect over a pH range of 5.0–9.0.[46] Here it was shown that the DACC-coated dressing demonstrated comparable antibacterial effects against the model microorganism *Staphylococcus aureus* according to JIS L 1902 at different pH.

Hard-to-heal wounds most commonly have a pH range of 6.5–8.5, and in individual cases even higher pH values have been observed.[47,48] This 'alkaline shift' in hard-to-heal wounds compared to acute wounds is thought to be due to tissue necrosis, as well as the presence of microorganisms.[47,48] Therefore, it can be

concluded that DACC-coated dressings will exert antibacterial activity regardless of the pH of the treated hard-to-heal wound.

Limitations

The antibacterial effect of the DACC-coated dressing was evaluated using a standard in vitro test method and investigated the effect on several Gram-positive and Gram-negative bacteria species. Although the selection was based on relevant wound pathogens, not all species detected in wounds were included. Moreover, yeast species were not included in the study and no conclusions about the effect of the DACC-coated dressing on yeast can be drawn from these results.

This study further focused on the elucidation of the mechanism of bacteria binding by the DACC-coated dressing and no clinical study was performed. We attempted to create conditions in vitro common to hard-to-heal wounds such as pH, increased protein content and high bacteria loads to be able to translate the findings into clinical practice.

Conclusions

Previous clinical studies have demonstrated that DACC-coated dressings can aid reducing bacterial burden in critically colonised or locally infected wounds (as reviewed by Chadwick et al.,[2]). Here, the antibacterial effect by irreversible binding and inhibition of growth of bound bacteria was confirmed using a standard in vitro test method, the JIS L 1902. Antibacterial performance of the DACC-coated dressing was not affected over a pH range typically found in hard-to-heal wounds, and could be sustained over subsequent reinfection steps.

In conclusion, it can therefore be assumed that this type of dressing is beneficial in controlling wound bioburden. There is also a growing body of clinical evidence illustrating that the DACC-coated dressings can be used successfully to prevent and treat wound infections, reducing the overall consumption of antibiotics.[10] Thereby, DACC-coated dressing do their part to support the AMS strategy. **JWC**

Reflective questions

- Why is it important to accurately evaluate different mechanisms of antibacterial activity?
- What does this study show with regard to the versatility of dialkylcarbamoyl chloride-coated dressings in wound management?
- How can wound dressings with passive inactivation mechanisms help to reduce the spread of resistant bacteria?

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notes