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A new textile-based approach to assess the antimicrobial activity of volatiles

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Abstract

Textiles with antimicrobial properties are required in many areas but it is difficult to assess the effect with current assays based on the cultivation of microorganisms. Using confocal laser scanning microscopy (CLSM) we observed that the complex matrix of textiles provides an ideal niche for the adhesion of microorganisms because polyester fibres showed a high number of irregularly appearing notches. In another experiment with green fluorescent protein-labelled *Staphylococcus epidermidis* cells were found in these notches, tightly bound to the fibres. *Staphylococcus* cells shielded in the textile were not killed by conventional decontamination techniques like UV irradiation. Cultivation-dependent evaluation of bacterial survival after 10 minutes of irradiation indicated a good reduction of more than 99%, whereas fluorescent viability staining of cells, in combination with CLSM, displayed more than 5% survival. This latter sensitive assay can also be used to assess the activity of volatile antimicrobials. It was shown that volatiles produced by plant-associated bacteria (*Pseudomonas chlororaphis* and *Paenibacillus polymyxa)* irreversibly inhibited the growth of human-associated pathogens like *Staphylococcus aureus* and *Candida albicans* on textiles. This new approach enables the detection and evaluation of new volatile antimicrobials for their use in disinfection of garments.

Keywords

Polyester fibre, confocal laser scanning microscopy, decontamination, gaseous antimicrobials

Introduction

During the last few years, the number of biofunctional textiles with antimicrobial effects has increased considerably. Various agents were used for antimicrobial finishing of textiles including metals, quaternary ammonium compounds (QACs), triclosan, chitosan and dyes.[1–3] With the increasing number of antimicrobial fabrics, the demand for appropriate and standardized test systems is arising.

Antimicrobial efficacy of antimicrobial finishing and decontamination of textiles is currently evaluated using either the agar diffusion test or suspension test (challenge test).[4] Agar diffusion tests (EN 14119:2003-12) have a long-standing tradition in microbiology. This qualitative method allows a distinction between active (clear zone of growth around the sample) and passive antimicrobial principles (no zone of inhibition). This semi-quantitative analysis gives only very poor or imprecise conclusions for textile materials other than fabrics. Suspension tests such as the JIS 1902-2002 are based on the adhesion of microbes to the textile and their elution from the fabric. The number of surviving cells is determined by dilution and plating on nutrient agar plates. Finally, the samples are compared with the initial population and appropriate controls.[5] By both test systems only culturable microorganisms can be detected, so non-culturable bacteria, which represent at least more than 95% of all bacteria, will not be evaluated with these test methods.[6]

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Most biocides used for the production of commercial antimicrobial textiles can induce bacterial resistance and can lead to increased resistance to certain antibiotics in clinical use.[7] Therefore, new concepts and active agents for the decontamination of surfaces are needed. Microorganisms from soil and plants have been a great source for the natural-product industry, and they provide sources for the discovery of new antimicrobials.[8] Volatile organic compounds (VOCs) produced by plant-associated microbes are interesting antimicrobials because bacterial volatiles can inhibit the growth of various fungi and influence the metabolism of other bacteria.[9,10] Examples for bacterial volatiles are cyclohexanol, produced by *Pseudomonas chlororaphis*, which inhibits the growth of fungal mycelium.[11] Short-chain volatile fatty acids such as isobutyrate or succinate, produced by *Bacteroides fragilis*, inhibited the sporulation of *Clostridium perfringens*.[12] However, most of the currently used methods that prove antimicrobial efficacy of VOCs are unable to detect gaseous antimicrobials. New test methods are necessary to identify potential producers of volatile substances with an antimicrobial activity.

The aim of this study was to develop a sensitive, textile-based method, which allows the assessment of volatile antimicrobials. Therefore, we combined fluorescent viability staining with confocal laser scanning microscopy (CLSM) to monitor cells within the fibres of the textiles with the aim to evaluate antimicrobial activity of volatile antimicrobials. The bacterial nosocomial pathogen *Staphylococcus aureus* and the yeast-like fungus *Candida albicans* were used as model organisms. Plant-associated strains of the species *Paenibacillus polymyxa* and *Pseudomonas chlororaphis,* originally isolated from oil pumpkins, were tested for their production of volatiles. Two colour fluorescence-based viability staining was used to allow a fast and reliable qualitative and semi quantitative distinction between live and dead cells.

Experimental

Microorganisms and growth conditions

The model pathogens *Staphylococcus aureus* ATCC 25923 and *Candida albicans* H5 were grown aerobically in 5 mL CASO medium on a rotary shaker at 37°C and 120 rpm for approximately 18 h until the late exponential phase. An aliquot of each culture was then transferred into a 5 mL CASO medium and grown to a final OD_{600} of 0.4 to 0.6.

A green-fluorescent protein (GFP)-labelled strain of *Staphylococcus epidermidis* 1457 pASgfp_{hld} was kindly provided by Dr.med. H. Rohde (Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany). Cells were grown in CASO medium (Roth, Karlsruhe, Germany) supplemented with 20 µg/mL tetracycline (Sigma Aldrich, Vienna, Austria) and 1% (w/v) xylose (Roth, Karlsruhe, Germany) on a rotary shaker at 37°C and 120 rpm for approximately 18 h. After incubation, 3 µL of a liquid culture of *S. epidermidis* were applied to a textile piece placed onto a glass slide and covered by a glass slip.

The bacterial antagonists *Paenibacillus polymyxa* GnDWu39 and *Pseudomonas chlororaphis* ÖWuP28 were isolated from the seed and root of Styrian oil pumpkins (*Cucurbita pepo* var. *styriaca*).[13]

For all tests, *Paenibacillus polymyxa* GnDWu39 and *Pseudomonas chlororaphis* ÖWuP28 were incubated in 5 mL of CASO medium overnight on a rotary shaker at 30°C and 120 rpm. Bacterial cultures were then transferred into 5 mL of fresh CASO media and incubated at 30°C under agitation until an OD_{600} of 1.3–2 was reached. Finally, 50 µL of the cultures were plated onto the nutrient agar side of a two–compartment petri dish and the bacteria were co-incubated with the pathogen at 30°C for 20–24 hours.

Effect of UV illumination on the survival of S. aureus

Textile pieces of a defined size (1 cm x 1 cm) were cut out of commercially available polyester-based fabric ION-NOSTAT VI.2 used for the production of clean room garments (Dastex, Muggensturm, Germany). Textiles were autoclaved for 15 minutes and dried at 60°C.

Prior to irradiation, 3 µL of a liquid culture of *S. aureus* ATCC 25923 in the exponential growth phase (10^6 CFU mL^{-1}) were applied to a sterile textile piece and dried for 5 minutes in the dark. The textile pieces were exposed to UV light (SteriAir, Kürten, Germany) for 10 minutes (0.2 $Wm^{-1}s^{-1}$). After illumination, viability of the bacterial cells was either analyzed with fluorescent live/dead staining (see below) or a contact plate testing. Therefore, textile pieces were pressed on CASO agar plates for one minute. Textile pieces were then removed and the agar plates were incubated at 37°C for 24 hours.

Dual-culture assay for evaluation of VOCs on the survival of S. aureus *and* C. albicans

The efficacy of the antimicrobial potential of VOCs produced by antagonistic bacteria was determined using high sensitive fluorescent dyeing.

For the dual-culture assay, 3 µL (10^6 CFU mL^{-1}) were applied onto sterile textile pieces and placed on the non-agar side of a two-compartment petri dish. Textile pieces inoculated with bacteria were stained with the LIVE/DEAD® Baclight™ kit (Invitrogen,

Karlsruhe, Germany) as follows. For the preparation of the Baclight™ stock solution, 15 µL of propidium iodide (red-fluorescent nucleic acid stain) were mixed with 15 µL of SYTO® 9 (green-fluorescent nucleic acid stain), and 3 µL of the stock solution were diluted in 1 mL phosphate buffered saline. 150 µL of this work solution were transferred onto the textiles and incubated for five minutes. After incubation the textile pieces were removed from the staining solution, placed on a glass slide and covered by a cover slip.

Confocal laser scanning microscopy (CLSM) and image analysis

CLSM was performed with a Leica TCS SPE confocal microscope (Leica Microsystems, Mannheim, Germany). Fluorescent dyes SYTO® 9 and propidium iodide (contained in the LIVE/DEAD® Baclight™ kit) were sequentially excited with 488 and 532 nm laser beams, respectively. The emitted light was detected in the range of 496−560 nm and 600−680 nm, respectively. An additional channel (excitation at 405 nm; emission range 422–477 nm) was applied for acquiring the autofluorescence of the textile. GFP-labelled cells were excited with a 488 nm laser beam and the emitted signal was detected in the range between 500 535 nm. The GFP and LIVE/DEAD® Baclight™ kit staining were not applied together, because of the overlapping of the emitted fluorescent signals of GF-protein and SYTO® 9, respectively. Photomultiplier gain and offset were individually optimized for every channel and every field of view, in order to improve the signal/noise ratio. Confocal stacks were acquired with both the Leica ACS APO 40X OIL CS objective (NA: 1.15) and the Leica ACS APO 63X OIL CS objective (NA: 1.30) by applying a Z-step of 0.25–1.2 µm. Three-dimensional reconstructions were created with the software Imaris 7.0 (Bitplane, Zurich, Switzerland). Statistical comparison of the LIVE/DEAD® cells was performed with the software ImageJ (National Institutes of Health, Bethesda, USA).[14]

Results and discussion

Surface analysis of the textile fibre

Clean room textiles used in this study consisted of polyester-based fabrics. The micro-dimensional structure of polyester fibres was described more or less as flat; only small bulges were detected when analysed by scanning electron microscopy (SEM).[15] Surface analysis of the synthetic fibres using CLSM displayed a completely different picture: the images showed a complex structure with irregularly appearing notches. Surprisingly, these irregular structures were of the same size as bacterial cells (2–5 µm in diameter). The Imaris derived reconstruction of the fibre matrix confirmed that *S. epidermidis* cells were able to use these fibres micro-structures as niches to attach closely to the yarn. Bacterial cells were observed preferably accumulated in the space between the fibres (Figure 1). Therefore, contact plate methods will not capture cells closely attached to the fibres or in the internal parts of the fabric. This observation will definitely influence results obtained by the contact-based test methods and should be considered in further analyses.

Effect of UV illumination on the survival of S. aureus

The reconstruction of CLSM derived pictures from the textile surface showed the microbial cells closely attached to the notches and accumulated in the space between the fibres. Cells arranged themselves in a multilayer, which shielded cells that are inside this layer.

UV radiation of flat surfaces is a very suitable and common application broadly used in pharmaceutical applications.[16] Techniques for the decontamination of surfaces such as irradiation with high energy UV light result not always in a complete disinfection of textiles. In complex and irregular structures, illumination is hindered by the structure and shadowing effects, which can dramatically reduce the efficacy of this method. Other limitations for the decontamination of textiles are caused by the fact that light cannot penetrate into deeper parts of the fabric. This study compared contact testing and fluorescent dyeing in combination with CLSM. We chose UV illumination for the proof of concept. The level of decontamination after the contact plate assay was defined as follows: 1 to 5 colonies, strong reduction; 6–10 colonies, significant reduction; 11–15 colonies, good reduction; 16–20 colonies, weak reduction; and 21–uncountable number of colonies, no reduction. Textiles pieces which were incubated with *S. aureus* and not exposed to UV light showed no reduction of viable cells after contact plate assay, no single colonies were visible. After 10 minutes of irradiation we counted a maximum of 15 colonies per piece of textile (1 cm x 1 cm), which corresponded to a good reduction (Figure 2).

A completely different result was obtained with fluorescent viability staining. It was shown, that only a small number of surviving bacteria was captured by the contact plate testing. According to the above stated criteria for the level of decontamination, software-based counting of green spots (viable cells) reflected no reduction after 10 minutes of irradiation since the number of green spots dramatically exceeded the chosen criteria (Figure 3). After 10 minutes of irradiation a total number of green spots of 437 single cells were calculated for an area of 1 mm x 1 mm.

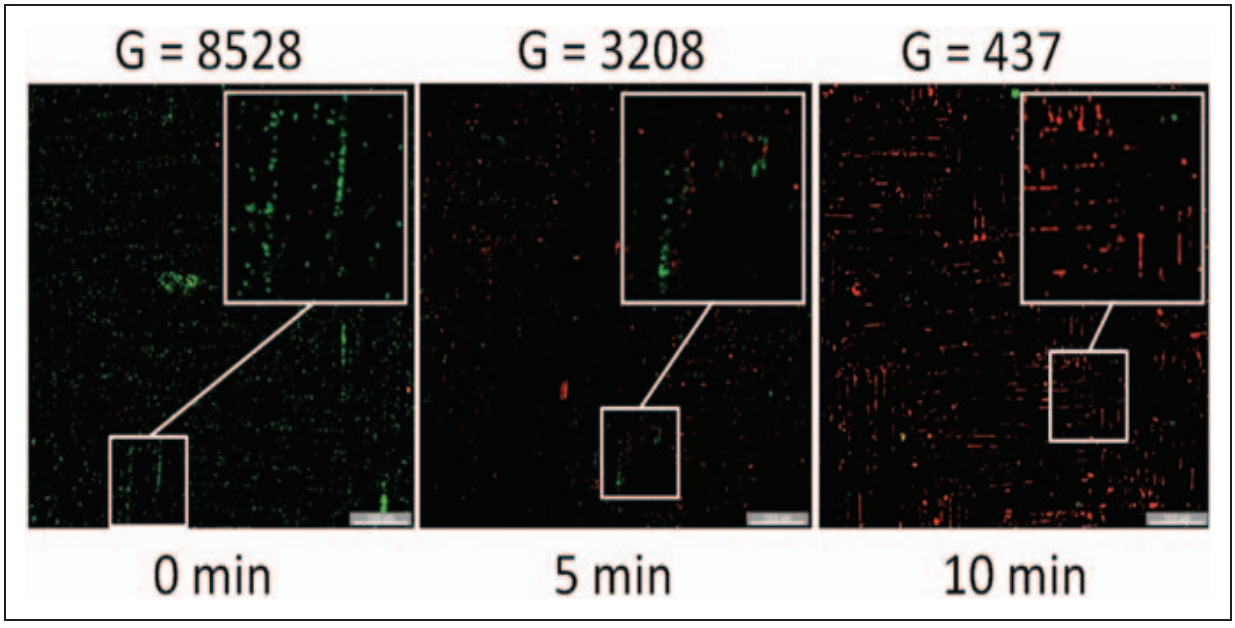

Figure 1. Three-dimensional reconstruction of polyester fibres (grey) colonized by GFP-marked *S. epidermidis* 1457 pAS*gfp*$_{hld}$ (green) obtained with the Imaris software, showing the texturing of the surface and the associated bacteria.

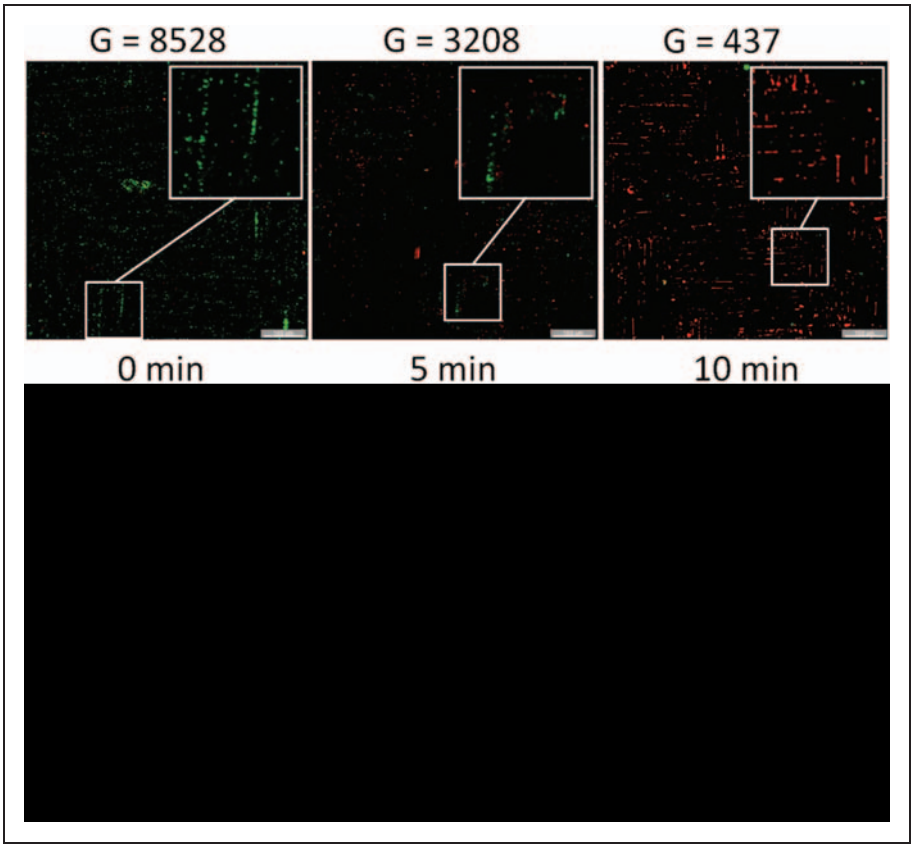

Figure 2. Comparison of the contact plate technique to fluorescent microscopy for the detection of viable cells of UV light treated textile samples (fluorescent blue) incubated with *S. aureus* ATCC 25293. Samples were exposed to UV light for 10 minutes. After illumination, viability was tested either by contact plate testing (b, d) or staining with fluorescent dyes (a, c) where viable cells appeared fluorescent green, dead cells appeared fluorescent red. Scale bar 100 μm.

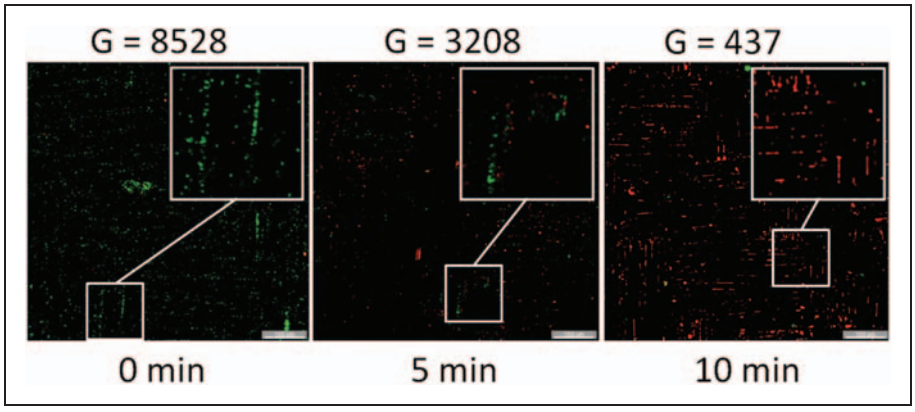

Figure 3. Fluorescence microscopy images of clean room textiles inoculated with *S. aureus* ATCC 25293. Textile pieces (1 cm^2) were exposed to UV light for 10 minutes. Dead cells stained fluorescent red, whereas vital cells stained fluorescent green. The total number of viable cells (green spots, G) were counted using Imaris 7.0 software. Scale bar represents 100 μm.

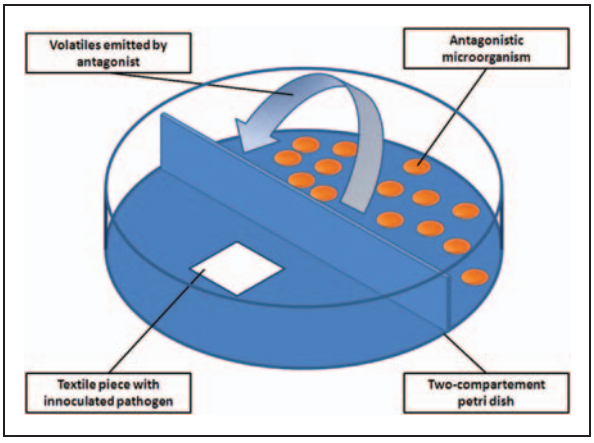

Figure 4. Two-compartment petri dish showing a dual-culture assay for the detection of volatile antimicrobials, produced by bacteria. The textile pieces were inoculated with pathogenic microorganisms. Co-incubation with plant-associated bacteria allowed VOCs to diffuse into the chamber containing the pathogen-inoculated textile piece.

Fluorescent viability staining represented a very accurate monitoring tool for surviving cells that adhere to the textiles. It allowed a fast and reliable interpretation of the chosen decontamination method and beyond that it offered the possibility of a semi-quantitative test method.

Dual-culture assay for evaluation of VOCs on the survival of S. aureus *and* C. albicans

The screening for new antimicrobial substances with standard dual-culture testing was based on the use of two-compartment petri dishes, where both sides are filled with nutrient agar. The determination of gaseous substances which inhibit the growth of other microorganisms was restricted here to visual analyses of the growth behaviour of the target organisms. The development of a textile-based dual-culture test represents a new approach compared to standard testing. In the newly developed assay, one agar side was replaced by a piece of textile inoculated with the pathogen (Figure 4). The surface of the fabric was an ideal basic material as the pathogens were able to attach and survive for at least 24 hours. The application of textiles strongly increased the sensitivity of the test and allowed both, a qualitative and semi-quantitative interpretation of the growth limiting effect of UV irradiation and the detection of volatile compounds. In this study, the plant-associated bacteria *P. polymyxa* GnDWu39 and *Ps. chlororaphis* ÖWuP28 showed their ability to produce VOCs, which efficiently killed the two tested human-associated pathogens on the textile surface. The modified dual-culture assay in combination with fluorescent staining will facilitate both, the screening for new antimicrobials and the testing of antimicrobial textiles. Fluorescent staining allowed also the detection of non-culturable bacterial cells, which form the majority of bacteria. More than 95% of all bacteria do not grow on standard media and can only be detected with molecular biological tools.[6] Fluorescent staining of bacterial cells is based on the penetration of two nucleic acid-binding stains into bacterial cells and allows a rapid simultaneous enumeration of viable and dead cells.[17]

VOCs produced by *P. polymyxa* GnDwu39 and *Ps. chlororaphis* ÖWuP28 were monitored for disinfection of textiles inoculated with *S. aureus* ATCC25293 and *C. albicans* H5. Co-cultivation of the selected antagonist with the pathogen resulted in an inhibition of growth for both target organisms, which clearly demonstrated the production of volatile antibiotics.

Volatiles produced by *P. polymyxa* GnDwu39 showed that this plant-associated antagonist was the most effective isolate to inhibit the growth of *S. aureus* ATCC25293 (Figure 5). CLS-microscopy in combination with fluorescent dyeing revealed an influence of the very complex textile structure. It was proven that gaseous antibiotics could penetrate deeply into the textile matrix and volatiles produced by *P. polymyxa* GnDwu39 killed the cells attached to the fabric. It was shown that *P. polymyxa* GnDwu39 is a strain isolated from the Styrian oilseed pumpkin (*Cucurbita pepo* var. *styriaca*), which produced compounds that are particularly active against gram-positive bacteria. *P. polymyxa* strains are well-known to be effective in the control of plant pathogenic fungi and bacteria.[18,19] Recent studies suggest that volatile compounds produced by antagonistic bacteria and fungi can inhibit the growth of plant pathogens.[20,21] Pathogen suppression is a process, where the presence of living bacteria is a prerequisite.

The second human pathogenic organism tested was *C. albicans* H5, which was significantly inhibited by volatiles of *Ps. chlororaphis* ÖWuP28 (Figure 6). Also *Paenibacillus* strains are known for their function as important biological components of agricultural soils suppressiveness to soil-borne pathogenic fungi.[22] The secretion of gaseous antimicrobials by *Pseudomonas sp.* plays a major part in biocontrol and can significantly inhibit the growth of pathogenic bacteria and fungi.[23] Secondary metabolites such as 2,3-butanediol or mixtures of volatile lactones and terpenoids have been shown to function not only in communication (info chemicals)[24] but also in inhibiting the growth behaviour of pathogens.[21,25] Small organic molecules produced by antagonists can act over a wide range of distances via diffusion in air and can not only penetrate soil pores[26] but can actively penetrate into the complex structure of textiles. Irradiation with high energy light will only affect mono-layers of bacterial cells. Wherever multi-layers are involved, shadowing effects can occur and can dramatically reduce the effect of the sterilization technique because the top layers shadow the UV light for underlying microorganisms. Textiles are characterized by their complex three-dimensional structures which makes decontamination difficult. In the life circle of reusable clean room textiles, they become sterilized by washing and autoclaving. In the production process an active decontamination is hard to achieve and if contamination occurs garments have to be refused.

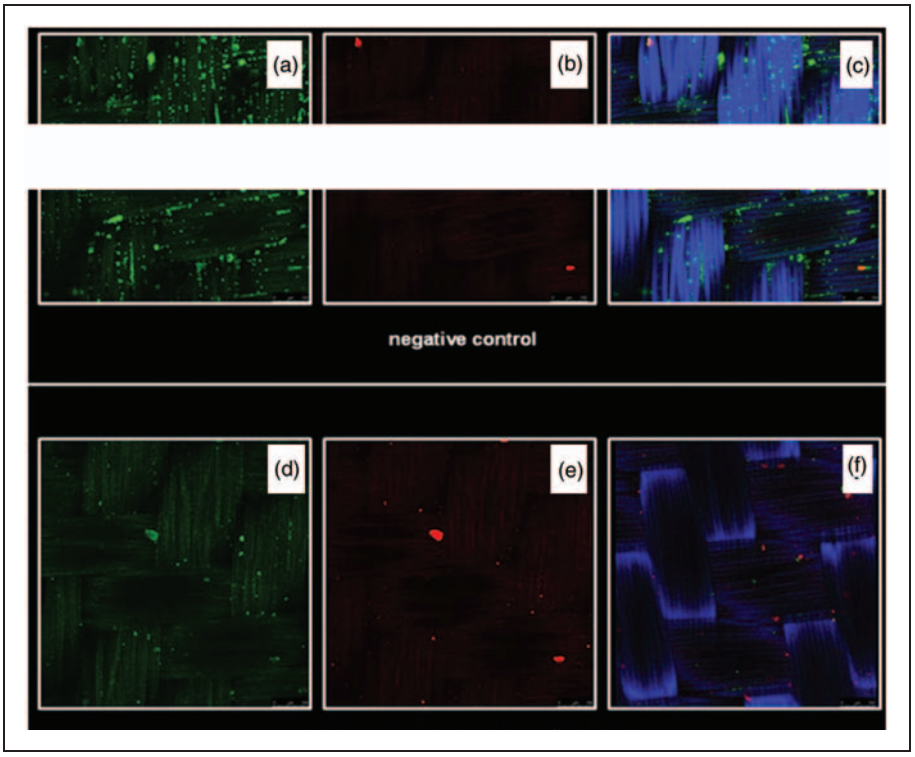

Figure 5. CLSM images of clean room textiles (fluorescent blue) inoculated with *S. aureus* ATCC 25923. Textile pieces were exposed, for 24 hours, to volatile antimicrobials produced by *P. polymyxa* GnDWu39 (d, e, f). For the negative control (a, b, c) the agar side of the two-compartment petri dish was not inoculated with the bacterial antagonist. Dead cells stained fluorescent red (b, e), whereas vital cells stained fluorescent green (a, d). Scale bar represents 100 μm.

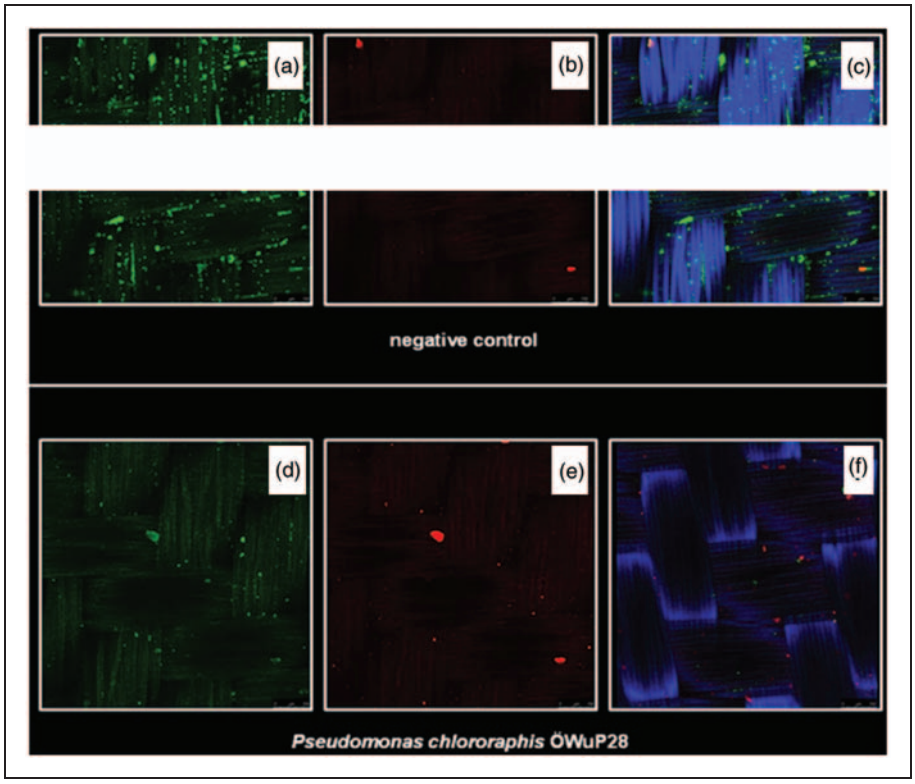

Figure 6. CLSM images of clean room textiles (fluorescent blue) inoculated with *C. albicans* H5. Textile pieces were exposed, for 24 hours, to volatile antimicrobials produced by *P. chlororaphis* ÖWuP28. For the negative control (a, b, c) the agar side of the two-compartment petri dish was not inoculated with the bacterial antagonist. Dead cells stained fluorescent red (b, e), whereas vital cells stained fluorescent green (a, d). Scale bar represents 100 μm.

The use of a textile-based dual-culture assay for the detection of antimicrobially active VOCs produced by plant-associated microbes successfully demonstrated an ideal base for the subsequent identification of the active compounds with GC/MS headspace analysis.

Conclusion

This study showed an efficient method of monitoring the effects of VOCs produced by bacteria. The produced VOCs demonstrated a high antimicrobial effect on clinically relevant pathogens which were applied on textiles. Future work will focus on the identification of the active antimicrobials compounds with GC/MS and their use in clean room environment for the decontamination of textiles and surfaces.

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