Evaluating decontamination and prevention techniques by establishing standardized broad-range microbial testing platforms

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SUMMARY
To find efficient decontamination techniques and preventive measures that create solutions for the future of human travel via public transportation, we established standardized experimental testing platforms that enable us to test the effectiveness of countermeasures against microbial contaminations. Additionally, we characterise the antimicrobial effects of, for example, active copper and copper alloy surfaces, which were topographically modified via Ultrashort Pulsed Direct Laser Interference Patterning (USP-DLIP). The experimental set-up includes a standardized bacterial bioaerosol including abundant microbes found in indoor air as well as an array of multi-drug-resistant bacteria (enterococci) and microbiological sterilization indicators like fungal spores (following the DIN EN 16868 standards). In our research, we e.g., aerosolize authentic bacterial bioaerosols (active or inactive form) and treat them with a given decontamination technique. In laboratory settings, as well as train and airplane cabin mock-ups, and real-life scenarios, we combine flow dynamic, material, and microbiome research.

KEYWORDS
Microbial surface contamination, self-cleaning and antimicrobial surfaces, decontamination, prevention, bioaerosol

1 INTRODUCTION

The challenges posed to modern society by the global SARS-CoV-2 pandemic are seriously novel and complex. However, it is not only since 2020 that there is a fundamental need to address the issue that microorganisms can have a significant impact on human habitats. The potential hazards posed by microbial contamination are complex and the associated issues will shape many scientific sectors in the coming decades. We aim to find short- and long-term technical solutions to solve not only viral - but phylogenetically broad biological problems for microbial contaminations within confined human environments, specifically for aviation and public transport. We are focusing on two essential aspects: First, we are conducting research on finding efficient standards for microbiological detection measures for airplane cabins and other public transport settings. These measures are fundamental to specifically research our second focus area, namely "decontamination and prevention" of microbial contaminants on high-contact surface areas in confined built environments. To this end, we produced a synthetic and defined bioaerosol containing known and widespread microorganisms from the environment (Mora et al., 2016; Tang et al., 2006). With this biological tool, we can optimise and cross-check our detection methods for microbial contaminations, and at the same time we are able to test and evaluate innovative decontamination measures. We are currently testing modified

functional metal surfaces. The antimicrobial effects of the surfaces used are based on the combination of active copper and topographically modified structures. Due to these functionalities of these surfaces, they cause chemical and physical cell damage to microbial life (see **Figure 1**). Some relevant model organisms, such as fungal spores and clinically relevant enterococci, have already been tested and results will be presented at the conference. The clear added value of these research aspects for aviation and public transport sectors is to create an internal reference contact point working in an interdisciplinary way to find targeted solutions to restore a certain "sense of safety" to travelling with public transportation.

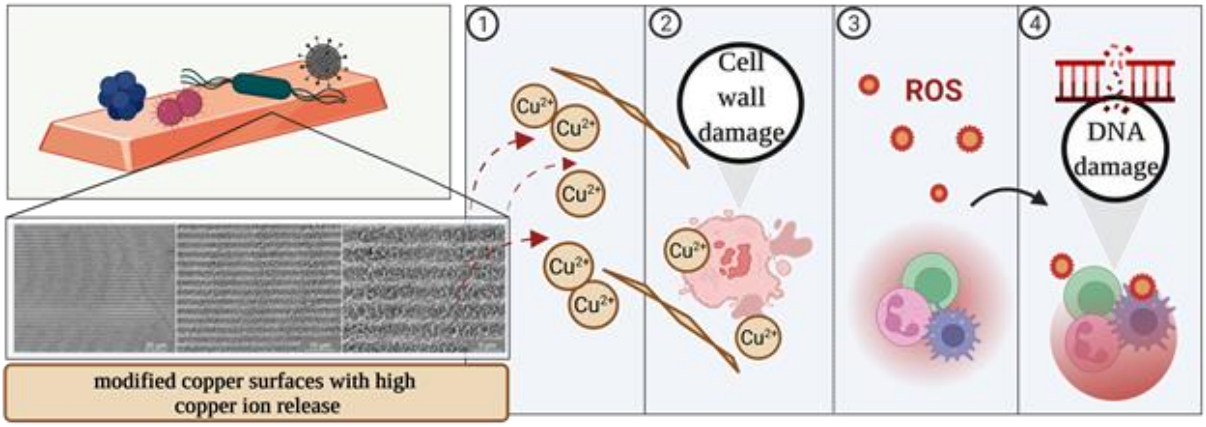


Figure 1 Visualization of the contact killing concept with modified copper structures. (1) The functionalized surfaces (provided by Daniel Müller, Material Engineering Center Saarland (MECS)) release a high number of copper ions. (**2)** These ions cause cellular stress onto the microorganisms which reach the corresponding surfaces. The copper ions lead to cell wall damage due to unbalanced copper homeostasis and by (**3)** inducing the production of "reactive oxygen species" (ROS) and creating oxidative stress which causes (**4)** DNA damage in the microbial cells which causes cell death.

The properties of copper and corresponding alloys (composition of metals) are recognized and officially registered at the U.S. Environmental Protection Agency as the first solid antimicrobial material (Monk et al., 2014). An alloy of copper (for example brass) needs to contain a specific proportion of the antimicrobial active metal (in terms of copper alloys, they need to possess a minimum of 55% of copper). Correspondingly, antimicrobial metals and alloys are already generally applied in environments that need special hygiene standards, like health care institutions or the food industry. Yet, up until now, this research area is starting to evolve and needs to be further explored, because there are only limited studies that prove copper for its antifungal properties or help against bacterial clinical isolates (e.g. *Enterococci sp.*), which are known to be more resistant towards disinfection techniques. Another aspect that could prevent microbial growth, and has been a topic of material science studies, is the design of nanostructured surfaces (Rosenkranz et al., 2016). Several studies proved that not only chemical modifications of material surfaces could help in preventing microbial growth but also physically change the surface topography (Tripathy et al., 2017). The inspiration behind the antimicrobial surface structures comes from naturally occurring nanostructured surfaces like the wings of cicadas or gecko skin (Hasan et al., 2013; Watson et al., 2015).

2 MATERIALS/METHODS

2.1 Design of the synthetic bioaerosol

Based on thorough literature research on abundant bacteria in public transportation, a synthetic microbial community with biosafety level 1 bacteria was designed. Each bacterial strain of this microbial community was cultivated and then mixed evenly in artificial saliva (based on Woo

et al., 2010). After inactivating the microbial community by X-ray irradiation, samples were embedded in fixative for scanning electron microscopy (SEM), which was performed at the Robert-Koch-Institute in Berlin. For scanning electron microscopy, particles from the suspension were adsorbed onto special coverslips. The samples were dehydrated and air dried. Subsequently, the samples were coated ("sputter coating"). Evaluation was performed on the Zeiss "Leo 1530" scanning electron microscope. To stain cells of the images, Photoshop CS2 was used.

2.2 Testing innovative/novel functionalized surfaces

To determine whether specific metal surfaces have antimicrobial properties, two different types of metals were examined as sample plates. Steel was used as a reference material since it is not known to have antimicrobial properties. Pure copper was tested as an antimicrobial surface. The sample plates had a size of 10 mm x 25 mm. In addition to testing smooth surfaces, structured surfaces, which were generated by direct laser interference patterning (DLIP) (Rosenkranz et al., 2016), were tested as well. The structures were 3 µm in depth. All sample plates were provided by the Department of Material Sciences and Engineering, Chair of Functional Materials at Saarland University. The experimental set-up used is named “wet-contact killing”. After sterilizing the surfaces, the bacterial cell suspension and the fungal spore suspensions were prepared. The cell/spore suspensions were applied in triplicates on each surface for 0 min, 30 min, 60 min and 120 min of surface contact time. After each time point, samples were taken off for a serial dilution. To calculate the Colony Forming Units per mL (CFU/mL) all dilutions were streaked out on corresponding growth agar and incubated under optimal conditions for each organism. Finally, the colonies were counted to determine the CFU/mL (survival fraction). Additionally, parameters of survivability were tested and additionally fluorescence microscopy of fungal colonies that grew on the surfaces was performed. The contaminated materials were observed with a Fluorescence Microscope from Axio Zeiss (Imager). A LIVE/DEAD staining was performed to assess fungal spore viability.

3 RESULTS

The main findings which we want to present at the conference and in this paper, is the accomplishment of producing a stable composition of a bacterial synthetic bioaerosol which can be used as a tool in broad-range aerosol and decontamination research campaigns in the future as well as testing antimicrobial surfaces against our established model organisms. For the development of an authentic and standardized biological bioaerosol, a lot of different parameters needed to be taken into consideration (see scheme in **Figure 2**).

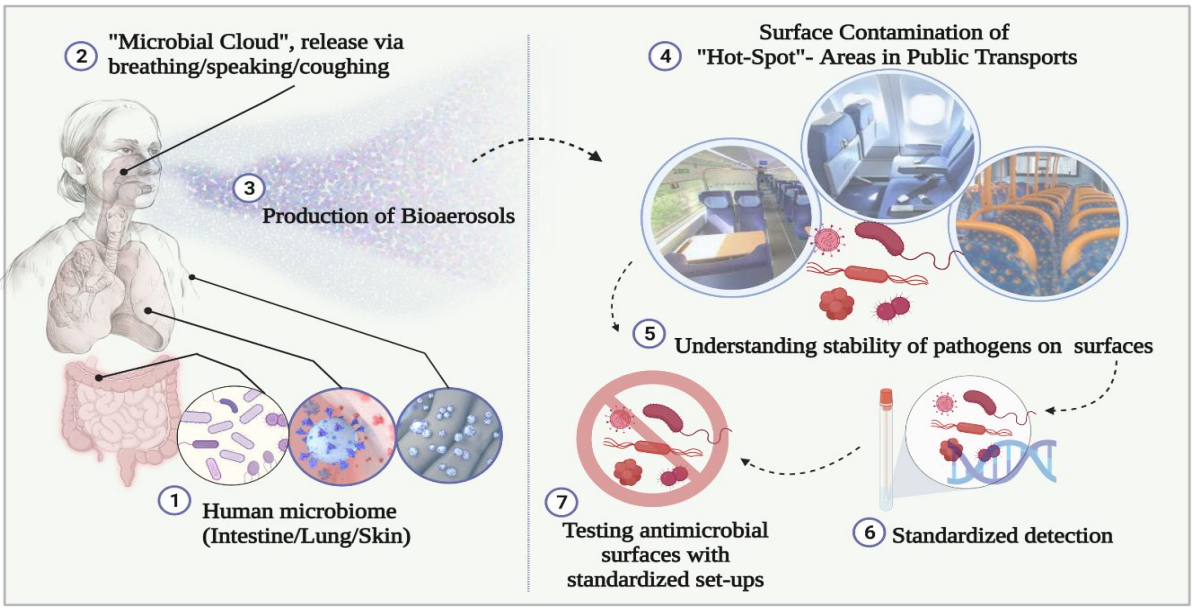
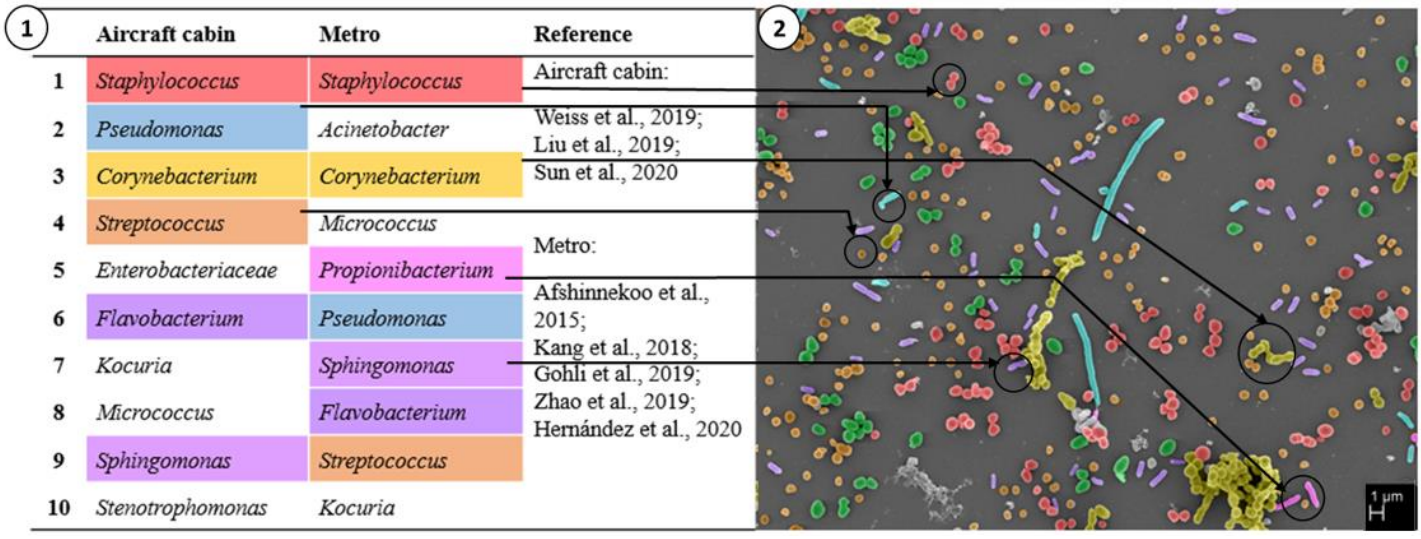


Figure 2 Scheme of experimental set-up and development of the experimental workflow. **(1)** Design of bioaerosol composed of abundant human-associated microorganisms. **(2)** and **(3)** Mimicking the release of a "microbial cloud" which can be released via different "emitting events". After the release of human-associated microorganisms into the direct environment via droplets, these droplets **(4)** will reach surfaces in built environments (like trains, buses or the airplane cabin). After understanding how stable/desiccation resistant the released microorganisms are on certain touch - surfaces **(5)**, we detect them via DNA-based methods **(6)** and test potential antimicrobial surfaces in standardised lab-conditions **(7)**. Figure created with Biorender.

As model organisms, we have selected a set of bacteria, based on their high abundance in public transportation and clinical relevance, which we were able to build upon a complex literature research referring to the microbiome data from e.g., subways, airplane cabins or other sampled public transport (see **Figure 3**). According to the findings, the microbiome in public transportation is composed of microorganisms derived from humans, especially human skin, and the environment (Leung et al., 2021). The human skin acts as the first contact surface to the environment and shapes it accordingly (Kang et al., 2018). Additionally, we are undertaking sampling campaigns in-flight to further substantiate those datasets. In order to have an end product for technical use, we needed to take more parameters into account to develop a stable consortium of microorganisms. These include most importantly the safe and easy handling of the bacteria. Therefore, non-pathogenic bacteria were selected, each referring to human pathogens that occur in public spaces. Other requirements that the end product must meet are the stability in the liquid medium (half-shelf life) and knowledge of resistance to stress conditions e.g. desiccation and inactivation through high dose irradiation. After selecting the bacteria, they first had to be cultivated individually to a high yield and the above mentioned parameters had to be tested. The focus of ongoing research is testing the effectiveness of decontamination methods against the developed microbial community, in direct combination with flow dynamic research of aerosol distribution. For these studies, however, preliminary experiments are still needed to optimize the detection of the bioaerosol, e.g. by sequencing. This should improve the understanding of the modes of action of decontamination methods, as well as their evaluation of our product transferred to environmental microorganisms and pathogens.



	Aircraft cabin	Metro	Reference
1	*Staphylococcus*	*Staphylococcus*	Aircraft cabin:
2	*Pseudomonas*	*Acinetobacter*	Weiss et al., 2019; Liu et al., 2019; Sun et al., 2020
3	*Corynebacterium*	*Corynebacterium*	
4	*Streptococcus*	*Micrococcus*	Metro:
5	*Enterobacteriaceae*	*Propionibacterium*	
6	*Flavobacterium*	*Pseudomonas*	Afshinnekoo et al., 2015; Kang et al., 2018; Gohli et al., 2019; Zhao et al., 2019; Hernández et al., 2020
7	*Kocuria*	*Sphingomonas*	
8	*Micrococcus*	*Flavobacterium*	
9	*Sphingomonas*	*Streptococcus*	
10	*Stenotrophomonas*	*Kocuria*	

Figure 3 (1) List of the most abundant bacteria in public transport. Most abundant bacterial genera of each environment were determined by comparing respective studies on the airplane cabin and metro microbiome. **(2). Scanning electron microscopy (SEM) of the microbial**

community based on assembled data collected in **(Fig. 3 (1)),** not all coloured species from **(1)** are displayed in the microscopic picture **(2)**. All organisms are surrogates of pathogenic bacteria and unharmful in handling. Most bacteria are environment-associated and resemble the microbial composition in public transport. Imaging with SEM (5kV, WD: 3.1 mm, aperture size: 30.000 µm) after adsorption of bacteria from liquid culture onto glass slides (5.000-fold magnification).

Nevertheless, while now having an established biological tool which facilitates a more standardized and microbial authentic base for testing countermeasures against microbial contaminations, we performed the first experimental campaigns for the surfaces with bacterial and fungal model organisms. First, we wanted to test singular species on these materials before using the actual synthetic bioaerosol (because we expect some protective effects applying co-cultures of microorganisms on surfaces). Therefore, we used *Enterococcus* (*sp.*) as a clinically relevant bacterial species and spores of *Aspergillus* (*sp.*) as first test organisms for the functionalized materials (see **Figure 4**).

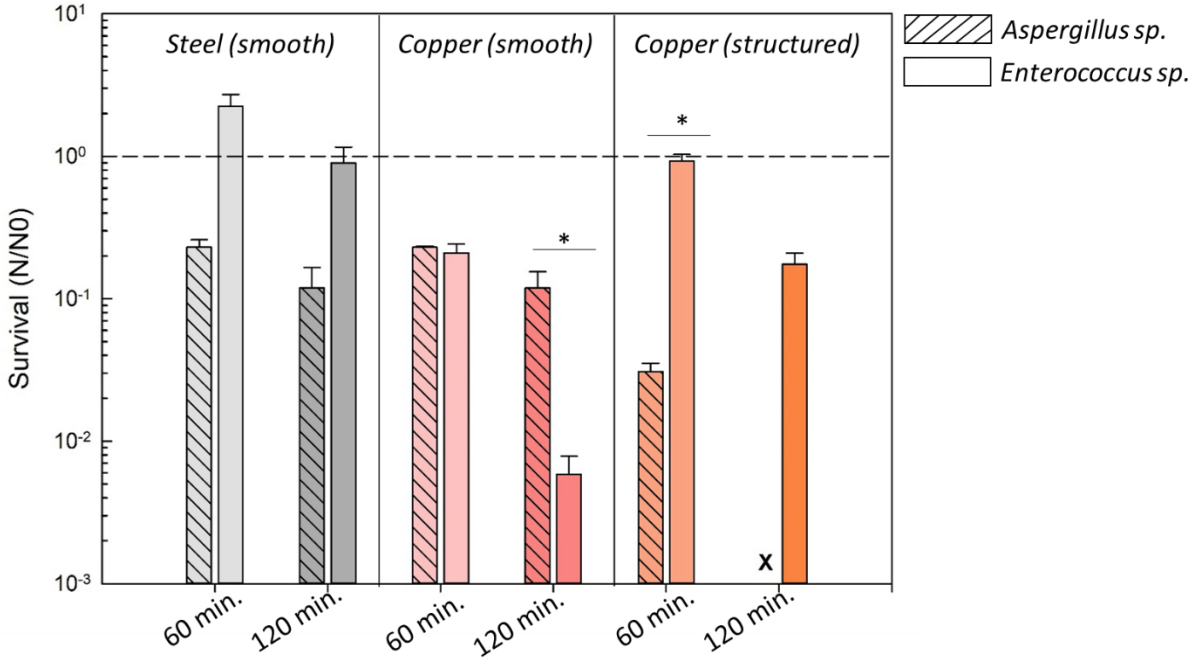


Figure 4 Bacterial and fungal survival on functional innovative DLIP – structured materials, data is shown in logarithmic scale. Survival rate (N/N0) of *Aspergillus sp.* and *Enterococcus sp.* after exposure to the following surfaces: copper (smooth, structured); reference: steel (smooth, structured (structured steel data not shown in this graph)). The survival was determined after 60 min and 120 min of contact respectively. The symbol "X" indicates survival below the threshold of detection. The dashed line resembles the normalized untreated control sample (=100%). The figure shows the average values out of n=3. Data was normalized to 0 min controls and error is represented as standard deviation. Not visible error bars account for a standard deviation < than 0.01. The difference in the mean values of the two groups *Aspergillus/Enterococcus* on copper smooth after 120 min and between *Aspergillus sp.* and *Enterococcus sp.* on copper structured after 60 min is greater than would be expected by chance; there is a statistically significant difference between the input groups (P-value = 0.017 and 0.001).

The survival rate of *Aspergillus sp.* showed a only slight reduction of survival after exposure to the smooth copper surface from 60 min to 120 min, which is not surprising since *Aspergillus sp.* spores are known to be very resistant to oxidative stress (Wiemann et al., 2017). Therefore, the extensive reduction in survival rate of fungal spores on the structured (DLIP processed) copper is a really promising finding. Here no viable cells were detected after 120 min which indicates that the structured copper surface is the most effective against fungal *Aspergillus sp.* spores, which proves the hypothesis, that the combination of chemical and physical functions of the surface created antifungal properties. Additionally, we could detect a high resilience towards copper of the tested clinical enterococci isolates. Surprisingly, the highest reduction of viable cells was observed after 120 min after being in contact with smooth copper.

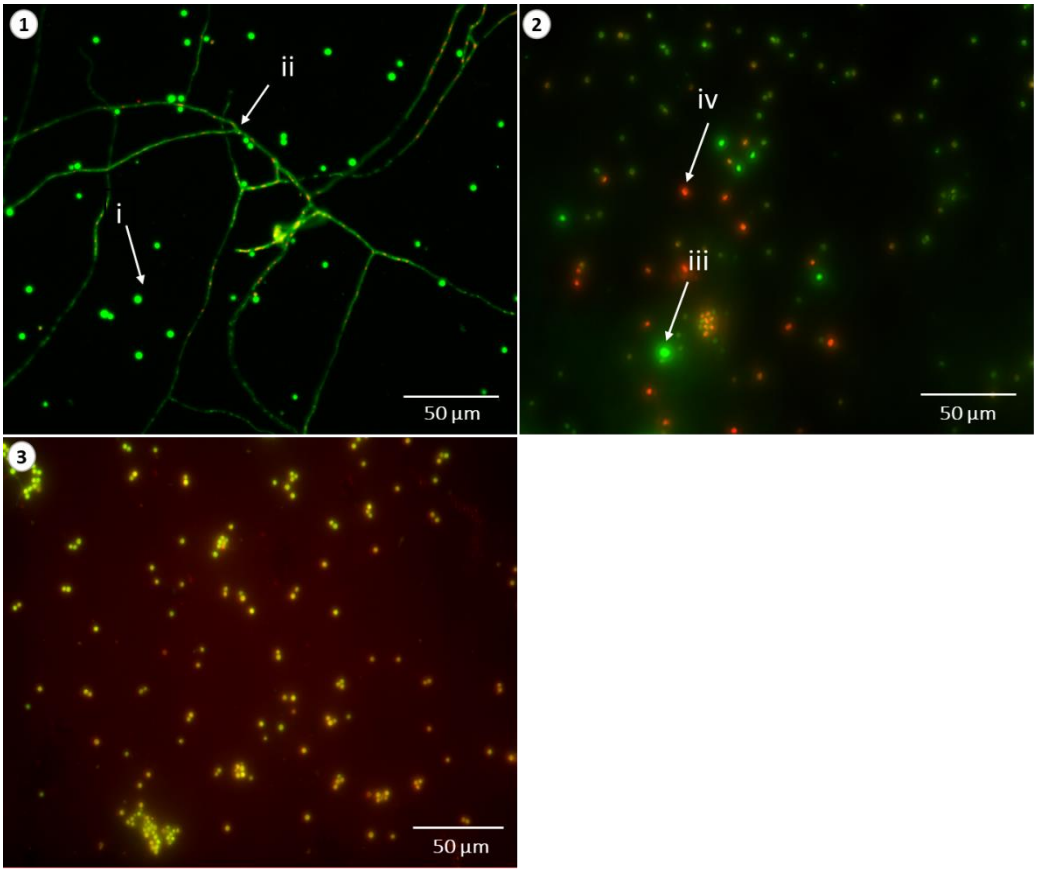


Figure 5 Fluorescent microscopic images of fungal spores, stained with two different fluorescent dyes; Sybr Green and Propidium Iodide (LIVE/DEAD). In **(1)** vital *Aspergillus* fungal spores on steel surfaces are presented. They display growth hyphal networks (ii). Whereas in **(2)** some fungal spores on smooth copper surfaces show damaged membranes (iv) and are only swollen, not germinating (iii). In **(3)**, only damaged fungal spores can be detected being in contact with structured copper. Sybr Green (green) stains all cells and appears strong green when cells have a fully intact cell membrane. Propidium Iodide (red) can only stains damaged/dead cells. Non-viable cells **(3)** appear "orange" since both fluorescent stains are overlaying each other in the composite fluorescent pictures.

Only, *Aspergillus sp.* displayed a sufficient reduction in viable cells after exposure to structured copper, that could be resembled as "sterilization effect" (see **Figure 5** for visualization of viable and dead fungal spores on tested surfaces). To be categorized as a "disinfection a cleaning process", a given method must reach at least a 6-log microbial reduction. Which was not reached with neither the bacterial nor with the eukaryotic organism. This first experiment was exemplary and proved that the antimicrobial effect of copper surfaces will need to be further investigated. It needs to be considered that we tested highly resilient model organisms and we

want to continue with research applying the authentic environmental bacterial composition (synthetic bioaerosol) on these surfaces. Additionally, several clinical isolates of *Enterococcus faecium* strains with different antibiotic resistance behaviour will be tested.

4 CONCLUSIONS

The COVID-19 pandemic displayed how vital preparedness and international cooperation are in a crisis with over 5.000.000 deaths worldwide. Now is the opportunity to prevent future microbial pandemics and respond with innovative research and new decontamination methods. The results of our work support previous research on the effectiveness of (modified) copper surfaces against microorganisms. By first testing functionalized surfaces against fungal spores and antibiotic-resistant bacteria, we have expanded the range of medically relevant model organisms in this research field and will continue using the designed synthetic communities for standardized high-throughput screenings of more functional surfaces in close cooperation with material scientists. Future field test campaigns and environmental applications will take place in public restrooms, trains, airplanes, airports or hospitals. With applying antimicrobial surfaces on highly touched surfaces in closed indoor environments or hospital settings, the transmission of microbial pathogens can be drastically reduced and therefore decrease the spread of infection risks. With the wide range of model organisms and standardised microbial communities, even more decontamination methods can be tested and evaluated, contributing to an increase in indoor air quality and safety including public transportation. Ultimately, this work contributes to the prevention and mitigation of infectious disease pathogen transmission caused by microbial surface contamination. In addition, the standardized bioaerosol can be used as a reference tool for any decontamination measures and can also be used to enhance the understanding of bioaerosol distribution in closed indoor environments. Connecting microbiology and research on aerosol distribution will help to develop useful technologies against the transmission of airborne pathogens.

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