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Ramstedt, Madeleine; Burmølle, Mette

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Can multi-species biofilms defeat antimicrobial surfaces on medical devices?
Madeleine Ramstedt and Mette Burmølle

Abstract
Infections deriving from medical devices represent a critical problem in health care causing suffering for patients, prolonged medical care, as well as consuming both human and monetary resources. An attractive solution is physical or chemical surface modifications of devices rendering them antibacterial and/or antifouling. However, the testing of such surfaces and coatings faces a range of challenges where one important is the predictability of in vitro assays for the outcome in vivo and in clinic. In this short review, we discuss what we consider is a major obstacle for design and evaluation of antimicrobial surfaces: microbial interspecies interactions. We give examples from the urinary tract, airways and from the oral cavity of functional consequences of such interactions in microbial communities, their therapeutic application for treatment, and how multi-species biofilms may influence the successful outcome of antimicrobial or antifouling surfaces. Furthermore, we suggest a path forward for in vitro testing taking these complexities into account during research and development.

Addresses
1 Department of Chemistry, Umeå Center of Microbial Research, Umeå University, 901 87 Umeå, Sweden
2 Section of Microbiology, Department of Biology, University of Copenhagen, 2100 Copenhagen, Denmark

Corresponding author: Ramstedt, Madeleine (madeleine.ramstedt@umu.se)

In the context of medical device infection, multi-species biofilms are emerging as an important but sometimes largely overlooked problem. In many environmental compartments, including the human body, microorganisms live in multi-species communities. This has been described for the oral cavity, the digestive system, devices in airways, circulatory systems, urinary tract and several other bodily compartments [4–9]. Microorganisms may also colonize surrounding tissue areas enabling reinfection of replaced devices [10]. The internal organization of these microorganisms within the biofilm may range from being a well-mixed community with high levels of synergies, to separated microcolonies that benefit from microenvironments forming for example at the top or bottom of a biofilm (Figure 1) [11,12]. Cross-kingdom biofilms are also important in this context. However, compared to antibacterial treatment regimes, literature covering work devoted to antifungal ones is sparse and, of these, even fewer cover development of surfaces aiming to prevent cross-kingdom microbial colonization [9,13,14].

Keywords
Multi-species biofilms, Colonization order, Microbial adaptation, Fungi, Bacteria, Antimicrobial, Antifouling.

Introduction
The need for medical devices in health care systems around the world is continuously growing as we live longer and work to advance medical technologies, enabling us to support patients in a wide variety of conditions. However, the medical progress is hampered by the fact that a number of patients acquire device-related infections. These may lead to serious complications causing great suffering for the patient and an increased need of healthcare resources. Device-related infections occur in all types of medical devices with varying rates of infection and severity of outcome [1]. One route to tackle infections of medical devices is to construct surfaces that repel or kill microorganisms. Extensive scientific research is, and has been, invested towards this aim. For such research to be successfully translated into functional medical products there is a large need for in vitro testing schemes that have predictive power for the outcome in vivo and in clinic. A recent review highlighted a number of perspectives relating to this, for devices in the urinary tract, pointing to a number of important considerations that are essential for predictive in vitro models investigating microbial colonization [2]. The considerations outlined can be adapted and applied to a range of areas in the human body enabling testing of antimicrobial and antifouling surfaces in a structured way, giving data with increased predictive power within a specific context (e.g. the urinary tract, the blood stream, the oral cavity or implanted devices) [2,3].
When building microbial models for use in the development of antimicrobial/antifouling surfaces, it is of great importance to understand the microbiology at the site where the device surface will be present. The response from one type of microbial consortium may be dramatically different from another one. Microorganisms in different areas of the body have adapted to the available nutrient levels and conditions of that site. Such conditions include temperature, pH, oxygen level, redox conditions, humidity, salinity, hydrodynamic shear forces, host immune defenses, etc. [4]. As the complexity of the microbial community increases, traits emerge enabling bacteria to benefit from other members via cooperative synergies, where all members benefit, or commensalism where the action of one or several strains promotes the growth of others [4].

In multi-species biofilms, a higher genetic diversity is found enabling the consortium to respond in a more diverse way to environmental challenges and stressors [15]. Thus, the community lifestyle enables e.g. oral bacteria to exploit a larger range of habitats, interact to create more efficient metabolism of resources available, more successfully evade the host immune system and more efficiently invade host tissue [16]. Multi-species biofilms, in general, exhibit increased fitness and higher capacity to withstand different types of stressors due to synergistic interactions and specialization. This may be observed as increased biofilm biomass, increased tolerance to antibiotics, increased capacity to degrade pollutants and/or ability to thrive outside the expected ecological niche (e.g. anaerobic/aerobic environment) [17,18]. For example, cross-kingdom biofilm from Candida albicans and Pseudomonas aeruginosa protected the bacterium from the antibiotic meropenem [19]. Multi-species biofilms with Escherichia coli, Delftia tsuruhatensis and Achromobacter xylosoxidans have also been shown to exhibit higher tolerance to antibiotics [20]. Interactions between microorganisms have been shown to increase the time of healing for infected wounds [21], as well as alter virulence of pathogens [9], exemplified by augmented periodontal disease severity [8,22] and increased bacteremia and mortality rate attributed to catheter-associated urinary-tract co-infections (CAUTI) [23,24].

These examples illustrate that the fitness of one microbial species inside a multi-species community can be dramatically different from a mono-species situation of the same organism. The interactions within multi-species biofilms are, however, complex and may vary with time and with surrounding stressors [18], as these biofilm systems are inherently responsive and dynamic in nature.

**Multi- and dual-species biofilms on medical devices**

In many medical devices, for example urinary catheters, biofilms develop from more than one pathogen and these may interact synergistically or in an antagonistic way [25–27]. On some types of devices, these multi-species consortia may give rise to very specific characteristics greatly influencing the clinical outcome for a patient. For example, in CAUTI, the presence of Proteus mirabilis and other urease producing bacteria, distinctly alter the microenvironment surrounding the device. This leads to alkaline conditions, precipitation of salts and encrustation of medical devices [24]. Apart from this, P. mirabilis may influence the fitness of other bacterial species at the site of the device, both in a negative and positive way [28,29]. For endotracheal tubes, a range of strains have been observed and it has been shown that the clinical outcome generally became worse if Pseudomonas strains were present [30]. These strains also appeared to outcompete strains of Candida and Staphylococcus. Furthermore, it was observed that biofilm quantity on the device or a general presence of bacteria on the tubes was not directly correlated with disease. Instead, the authors found that the composition of the consortium played a more important role for the clinical outcome [30].

Multi-species biofilms have been studied for many years in the oral cavity. This intense research has revealed many interesting aspects of these types of highly
complex, spatially organized and dynamic microbial communities \cite{16,31,32}. After insertion, dental-implant surfaces become covered by a conditioning film from saliva and subsequently are colonized in a very ordered way by initial colonizers such as \textit{Streptococcus} spp. and \textit{Actinomyces} spp. forming a biofilm \cite{16,33}. With time these biofilms develop into poly-microbial communities by inclusion of other species groups through various successional stages \cite{33}. This succession relies heavily on interspecies communication controlling growth and maturation of the biofilm \cite{16,33}, as well as production of extracellular polymeric substances (EPS) \cite{34}.

For other types of surfaces, it has been shown that weak colonizers may be enabled to colonize in succession (Figure 2). For example, \textit{E. coli} unable to form biofilm could integrate into an already formed biofilm by \textit{P. aeruginosa} \cite{35}. Another example is \textit{P. mirabilis} and \textit{Enterococcus faecalis} that are often co-isolated from urinary catheters. Careful studies using clinical isolates from long-term catheterized patients revealed that the deposition of fibrinogen (a host factor) on the catheter enhanced \textit{E. faecalis} adhesion at the sites where this host-derived protein had deposited \cite{17}. \textit{P. mirabilis} thereafter co-colonized the catheter preferentially where \textit{E. faecalis} had already settled, but could also settle on bare or fibrinogen-coated areas of the catheter. The colonization order was only in a few cases found to be reversed, with \textit{P. mirabilis} being the pioneer. In both cases, the resulting consortium was found to remain stable, with the two strains closely co-localized in space, as well as with enhanced EPS production, altering both biofilm architecture and stability compared to mono-species films. Thus, clearly illustrating a case where both species appear to benefit from a close co-localization \cite{17}.

The composition of the fluid surrounding the biofilm has also been shown to play a role. Interactions between consortium members were shown to be dependent on medium composition, and different biofilm phenotypes were observed in medium simulating body fluids compared to standard culture media \cite{36}. For co-cultures of \textit{P. aeruginosa} and \textit{P. mirabilis}, it was shown that artificial urine medium led to a competitive disadvantage for \textit{P. aeruginosa} that could be reduced if the medium was buffered to reduce development of alkalinity and mineralization \cite{25}. The same study also showed that \textit{P. aeruginosa} preferentially resided in the bottom of the biofilm whereas \textit{P. mirabilis} was positioned at the top of the biofilm in unbuffered conditions. This was discussed from the perspective of \textit{P. aeruginosa} being the pioneer colonizer. With time, these bacteria were prevented from spreading by the onset of alkalization in the surroundings medium creating a more hostile “outside” environment, but still enabling them to reside in the biofilm close to the material surface.

Supernatant from \textit{P. aeruginosa} has been shown to cause \textit{Staphylococcus epidermidis} strains to detach and leave already formed biofilms, an effect hypothesized to be related to secreted polysaccharides \cite{37}. Polysaccharides have also been shown to play a role in the interactions in dual-species biofilms of \textit{P. aeruginosa} and \textit{Staphylococcus aureus} increasing the volume and spread of their biofilms \cite{38}. In dual species cultures of \textit{P. aeruginosa} and \textit{S. aureus}, a multitude of capabilities have been observed by which \textit{P. aeruginosa} can

Figure 2

Illustration of how successive colonization may take place (inspired by Gaston et al., 2020). \textit{E. faecalis} settles as primary colonizer, starting to build biofilm. At later time points, \textit{P. mirabilis} joins and colonizes the biofilm forming a dual-species biofilm with increased biofilm volume and mass of EPS compared to mono-species variants. As \textit{P. mirabilis} induces alkalization of the urine, salts precipitate out of solution transforming the biofilm into a crystalline biofilm with time.
outcompete *S. aureus* [39]. For example, *P. aeruginosa* has been shown to sense peptidoglycan shed from *S. aureus* and switch to increased virulence as well as production of gram-positive antimicrobials [40]. However, these species can coexist under certain conditions. For example, in biofilms dominated by the exopolysaccharide Psl, the two species formed isolated microcolonies, whereas they were intermixed in a biofilmominated by the Pel exopolysaccharide [39].

This illustrates that there are a range of factors controlling spatial and temporal distribution of microbial species in multi-species biofilms, including those formed on medical devices. For further reading please consult for example the reviews by Stacy et al. [22] and Bai et al. [8].

**Multi-species biofilms in the context of antimicrobial surfaces**

The fungal opportunistic pathogen *C. albicans* has been reported to form a large variety of multi-species as well as cross-kingdom biofilms [7]. Yeasts from the genus *Candida* are the main fungal pathogens causing device-related infections with subsequent systemic infection and very high mortality rates, the second most common genus being *Aspergillus* [14]. When these fungal pathogens form cross-kingdom biofilms with bacteria, they are more difficult to treat than their mono-species counterparts, and can cause much more severe infections [9,15,27].

Eradication of fungal infections requires different sets of antimicrobials and, as fungal cells are eukaryotes, these treatments may have more severe effects on the host tissue compared to antibacterial agents targeting mechanisms unique to prokaryotes. Treatment regimens targeting cross-kingdom biofilms require eradication of both pathogens, and high failure rates have been reported [9,14]. If a cross-kingdom biofilm is treated only with antibacterial agents, the fungal part of the biofilm will continue to flourish. Antibacterial treatment regimens may even select for fungal pathogens and facilitate their colonization, as any competition is eliminated. Thus, these cross-kingdom biofilms present additional challenges with respect to prevention and eradication of biofilms on medical devices [9,14]. Furthermore, the presence of fungal cells may facilitate bacterial colonization of various surfaces, and vice versa [7,9,14] (Figure 3). For example, *Streptococcus gorondii* has been shown to attach directly to *C. albicans* to form dual-species consortia with increased fitness and tolerance to antimicrobial agents [36] and co-infections with *C. albicans* and *S. aureus* enhanced the bacterial invasion of epithelial layers presumably by attachment of bacterial cells to the fungal hyphae [15,41]. Exopolysaccharides from *C. albicans* have shown to increase biofilm formation and colonization of *Streptococcus mutans* [34]. Thus, it is not an uncommon medical problem that fungal infections evolve into multi-species cross-kingdom biofilms that are very challenging to treat.

Another way that the effect of antimicrobial surfaces can be circumvented by microorganisms is by triggering events forming a conditioning layer covering the active surface rendering it harmless or adhesive. For example, *P. aeruginosa* has been shown to have active attachment processes involving flagella that enable attachment to crevices in non-wetting antifouling surfaces and alter

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**Figure 3**

a) Illustration of *C. albicans* primary colonization and biofilm formation on an antibacterial or antifouling surface leading to subsequent co-colonization of bacteria (here, *S. gorondii* is shown). b) The same biofilm after intense and “successful” antibiotic therapy removing bacteria but leaving the fungal biofilm more or less intact.
surface wettabiliy. Flagella have also been described to enable attachment on surfaces exhibiting electrostatic repulsion against the bacterial cell. Furthermore, extracellular polysaccharide trails deposited on a surface by a bacterium were shown to enhance further surface interactions by other bacteria [42–44]. Another example of preconditioning is performed by P. mirabilis that induces alkaline conditions in urine leading to salt deposition and encrustation of medical devices [25]. Once formed, these secondary materials induced by the microorganisms can efficiently cover any surface functionality designed to prevent them from attaching. A clear general hypothesis arising from these observations is that microbial strains that have some advantage enabling them to colonize a seemingly “hostile” surface by preconditioning it, can pave the way for other strains and enable sequential co-colonization. Thus, to mimic the challenge antimicrobial surfaces may face clinically, model strains should preferentially be chosen that have a very pronounced ability to colonize and form robust biofilms on a wide range of surfaces of relevance for the medical device group. Isolates that are weak colonizers are most likely not pioneer colonizers at a site and, therefore, not suitable to mimic the challenge the device surface would be exposed to. However, these strains should still be included in multi-species model communities, since low-abundant strains with poor biofilm-formation capabilities can impact biofilm biomass and spatial organization significantly [43]. Furthermore, strains should be selected from (infections in) the area of the body intended for the device, and the in vitro challenge should be made in fluids resembling that of the site, for increased predictability.

Many good critical reviews have recently been published outlining important aspects of performing testing of material surfaces for antimicrobial effect. For example: biofilm testing [46], important considerations for fungal systems [14], increasing predictability of assays aiming for the urinary tract [2]. For further reading on multi-species systems and interactions in multispecies model biofilms including cross-kingdom ones please consult previous reviews [8,9]. Several of the referenced critical reviews highlight the great need for in vitro assays that can predict outcome in vivo and suggest different approaches that should be considered towards that goal. With respect to testing multi-species consortia, a key aspect is microscopy or other methods enabling spatial characterization of the biofilm in 3D. Only such methods reveal where in the biofilm the different species are located and if they are in proximity to each other or live in isolated clusters [47] (Figure 1). Such methods may also shine light on what types of processes are enabling microorganisms to colonize antibacterial surfaces, and give clues how to best counteract these processes [8].

Conclusions and outlook

This short review highlights the importance of emerging properties of multi-species consortia in the perspective of colonization and biofilm formation on medical devices. This has long been established for materials in the oral cavity, where continuous development of in vitro models has been conducted through the years [3]. However, we here propose that multi-species interactions may be relevant to consider for most devices that are, in some way, linked to the external environment of a patient, and therefore may be co-colonized by several microorganisms. The emergent traits observed for multi-species biofilms have been shown to lead to increased fitness, pathogenicity and can seriously influence microbial colonization of foreign materials in the body. In the title we posed the question: Can multi-species biofilms defeat antimicrobial surfaces on medical devices? The combined literature seems to suggest that the answer to that question is: “Yes.”

One reason (most likely not the only) why several decades of research aiming to construct antimicrobial surfaces have yet not shown promising results in clinic, may be that the test conditions used to mimic what actually happens at a medical device in vivo have been too simplified. The approaches used may have failed to account for crucial parameters, including microbial aspects, host factors and site-specific conditions, reducing their predictability for clinical effect. We here propose that this could be enhanced by careful consideration of the site where the surfaces should be used. Tailoring in vitro assays to align with challenges faced in vivo, should result in enhanced predictability, and help to bring the field of antimicrobial surfaces forward.

We hope the above review has illustrated the importance of targeted in vitro testing from the aspect of the microbial composition. If assays are designed with the clinical function and location in mind, antimicrobial surfaces can be tested using clinically relevant growth medium, preconditioning of relevant host factors [1,48], bacterial challenge, consortium composition etc. Furthermore, research should ideally be made to identify “enablers” in the microflora at the site of interest, addressing questions such as:

1) Is it likely that fungi such as C. albicans may invade an antibacterial surface and facilitate for subsequent bacterial adhesion?
2) If the device will be used long term, how well is the antifouling surface preventing late colonizers, and do these have the ability to alter the microenvironment close to the surface? An illustrative example from the urinary tract is P. mirabilis with its enhanced swarming capacity. Would surface functionalities tailored for preventing CAUTI be easily destroyed or buried by...
alkalinity or precipitation produced in the urinary tract by late colonizers such as _P. mirabilis_?

3) Are surfaces triggering microorganisms to secrete increased amounts of extracellular substances in their biofilm, thereby making them less vulnerable to treatments?

If microbial testing is performed targeting the most challenging conditions and microorganisms, it is possible that the technical solutions identified will be better prepared for the challenges facing the device surface _in vivo_. Another important point to consider is what type of _selection pressure_ is posed on the highly dynamic microbial systems by the antimicrobial surface design? Are surfaces producing a selection pressure that may worsen the outcome for a patient? As was exemplified by Giles et al. [14], where fungal infections appeared to have been a result of strict antibacterial treatment.

For the future, we identify a need for a range of stable multi-species model consortia to be developed. They would enable site-specific and predictive _in vitro_ testing of antimicrobial and antifouling surface for different body compartments, in line with what is being done for the oral cavity [3]. Due to the large complexity and the dynamic environments in different body sites, it is not likely that one single _in vitro_ assay can give an overall prediction of global antibacterial or antifouling efficacy. Several different sets of experiments would need to be performed. To have predictive power, these must also mimic the site where the device will be placed. Furthermore, such sets of _in vitro_ assays should be optimized so that their results have some level of predictability for effect _in vivo_. Thus, defined model systems need to be compared _in vitro_ and _in vivo_. Possibly several types of consortia should be constructed for each device area where the challenge can stepwise be increased, with some of the consortia including “enabling” strains as primary colonizers. All with the aim to achieve increased predictive power of the _in vitro_ testing of antimicrobial surfaces and enhance the research progress in this important field.

**Credit statement**

Both authors have been involved in all aspects of the manuscript preparation and writing.

**Declaration of competing interest**

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

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