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# New strategies for prevention of central venous catheter colonization by biofilm-associated microorganisms

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## Abbreviations

**ATCC** American Type Culture Collection

**BSI** Bloodstream infection

**CLSI** Clinical and Laboratory Standards Institute

**CRBSI** Catheter-related bloodstream infection

**CV** Crystal violet

**CVC** Central venous catheter

**EPS** Extracellular polymeric substance

**HCRI** Healthcare related infection

**ICU** Intensive Care Unit

**LMWC** Low molecular weight chitosans

**LOS** Length of stay

**MDR** Multidrug resistance

**MIC** Minimal inhibitory concentration

**MLC** Minimal lethal concentration

**MRSA** Methicillin-resistant *Staphylococcus aureus*

**MSSA** Methicillin-sensitive *Staphylococcus aureus*

**MW** Molecular weight

**SEM** Scanning electron microscopy

**XTT** 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

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# Introduction

## General considerations

Patients admitted at Intensive Care Units (ICUs) often require central venous catheterization for multiple purposes, namely administration of medication (such as vasopressors, chemotherapy or total parenteral nutrition), infusion of large amounts of fluids or blood products, hemodynamic monitoring, dialysis or transvenous cardiac pacing. However, infectious complications may follow, ranging from simple catheter colonization (significant growth of a microorganism in a culture of the catheter tip, its subcutaneous segment or its hub), to local catheter infection (exit-site, tunnel or pocket infection and phlebitis) or to sepsis (generalized bloodstream infection, which is considered severe when it is associated with organ dysfunction, hypoperfusion or hypotension). Ultimately, mortality may result from septic thrombophlebitis, endocarditis, metastatic infection (such as lung or brain abscess) and septic shock with multiorgan failure.

Bloodstream infections may be related to the catheter itself (at least, one positive culture result obtained from a peripheral vein, clinical manifestations of systemic infection and no other detectable source for the bloodstream infection than the intravascular catheter) or to the infusate (whenever there is growth of the same microorganism both from the infusate and the percutaneously obtained blood samples). Nevertheless, the vast majority of catheter-related bloodstream infections (CRBSIs) are associated with central venous catheters (CVCs).[1] The laboratorial confirmation of the diagnosis of CRBSI demands one of the following criteria: (1) a positive result of a semiquantitative (≥15 colony forming unit per catheter segment) or quantitative ($\geq 10^2$ cfu per catheter segment) catheter culture, while the same organism is isolated from a peripheral blood sample; (2) simultaneously positive quantitative cultures of blood samples with a ratio of ≥5:1 (central *vs* peripheral venous catheter) or (3) differential time to positivity (a positive result from a CVC culture obtained at least 2 hours earlier than a positive result from the culture of peripheral blood).[2,3]

The socio-economic impact of CRBSIs is considerable. In a recent European review, data from France, Germany, Italy and the United Kingdom revealed an additional length of stay (LOS) of 1.9 to 14 days and extra costs of €4,200 to €13,030 per CRBSI episode.[4] This economic burden is in line with data from the United States: additional LOS of 2.41 to 8 days and costs that may reach $11,971 to $56,167 per episode.[5-7]

## Epidemiology and microbiology

The risk factors for infectious complications of central venous catheterization are related to co-existing diseases (such as malignancy, neutropenia and shock), to the procedure (use of an aseptic technique during insertion, site of venous access, type of catheter material and number of lumens) and to the infused therapy (composition, adequate solution preparation and delivery, catheter care during manipulation and indwelling time).[8,9]

In a recent European review, the incidence rate of CRBSIs was estimated at 1.23 to 4.2 per 1000 catheter days.[4] A slightly higher incidence rate of 5.0 CRBSIs per 1000 catheter days was reported from the US.[10] No reliable data has been available regarding Portugal.

The microbial agents most frequently isolated from CRBSIs are coagulase-negative staphylococci, *Staphylococcus aureus*, gram negative bacilli and *Candida* species.[2] Among a multitude of coagulase-negative staphylococci, *Staphylococcus epidermidis* inhabits the human skin and mucous membranes and leads not only the ranking of etiological agents of CRBSIs, but also of contamination of blood cultures.[3] *S. epidermidis* is able to form biofilm easily and, therefore, it is frequently associated with colonization of indwelling foreign medical devices such as intravenous catheters.[11] Moreover, healthcare related strains of coagulase-negative staphylococci became increasingly resistant to antibiotics; vancomycin resistant strains have already been reported.[12]

*S. aureus* may be found colonizing the nares and skin of humans. It is usually associated with more serious infections, ranging from skin and soft tissue infections to pneumonia, meningitis, endocarditis and bloodstream infections.[2,13,14] Currently, more than 50% of isolates obtained from ICUs are methicillin-resistant *Staphylococcus aureus* (MRSA).[10] Infections caused by MRSA increase the mortality, length of hospitalization and costs compared with methicillin-sensitive *Staphylococcus aureus* (MSSA) infections.[15] Therefore, preventive efforts are adopted in order to decrease the incidence of MRSA infections, either by widespread decolonization with nasal mupirocin or by contact isolation of patients.[16]

Gram-negative bacilli have also been widely documented as agents of CRBSIs. The most frequent isolates are *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* spp., *Enterobacter* spp. and *Acinetobacter baumannii*.[16,17] Infection by Gram negative

bacilli more often afflicts patients with a compromised immune system, receiving contaminated infusates or placed with tunnelled indwelling devices, while the proportion of antibiotic resistant strains is increasing.[18] *A. baumannii* is an emerging organism responsible for severe CRBSIs and pneumonia, with high rates of multidrug resistance (MDR).[19] Besides being ubiquitous and a frequent patient colonizer, the increase in the number of healthcare related infections (HCRIs) caused by *A. baumannii* may be explained by its ability to persist for months in undisturbed surfaces of healthcare equipment. Hence, outbreaks in hospital settings are difficult to contain because of the environmental contamination by such an agent.[20-22]

The incidence of invasive fungal HCRIs has risen markedly over the last 10 years.[23] The presence of a central venous catheter, prolonged LOS, broad-spectrum antibiotic use, burns, ICU stay, administration of parenteral nutrition and neutropenia are risk factors for invasive candidosis.[16,24] Concerning bloodstream infections, *Candida* species are invariably associated with a high mortality rate (39.3 to 44%). *C. albicans* is the most frequent isolate,[25-27] standing out as the fourth leading cause of CRBSI.[9] Among non-*albicans* species, *C. parapsilosis* is the most common isolate in Europe.[25] It is often associated with the presence of intravascular catheters or breaks in infection control, since *C. parapsilosis* is most often an exogenous pathogen. *C. glabrata* usually ranks second and its isolation has been related to azole selection pressure.[25,26]

**Pathogenesis of CRBSIs**

Contamination of CVCs may occur from several routes: (1) migration of skin organisms from the insertion site along the external surface of the catheter, which is the most common infection route for short-term CVCs (duration of placement <1 week);[28,29] (2) intraluminal spread from the manipulation of the catheter hub, which may be the dominant route for long-term CVC infection (duration of placement >1 week);[30] rarely, (3) haematogenous seeding of the catheter tip from a distant focus of infection[31] or (4) infusate contamination, which may ultimately lead to CRBSIs.[32]

Among the most relevant pathogenic determinants of CRBSIs, the intrinsic virulence factors of microbial agents, human host factors and the material of the catheter by itself must be taken into consideration:

(1) Microorganisms such as coagulase negative staphylococci,[33] *S. aureus*[34] and

*Candida* spp[35] produce extracellular polymeric substance (EPS), which is an exopolysaccharide that contributes to the formation of a biofilm layer.[36,37] Metallic cations from the blood circulation may add to this biofilm and form a dense matrix in which microbial agents may stay embedded.[38]

(2) Some host adhesion proteins, such as fibrin and fibronectin, form a sheath around the catheter that promote adherence of microorganisms. This is the case with *S. aureus*, which expresses the clumping factors ClfA and ClfB that bind to host adhesins.[39-41]

(3) Catheter materials that are more thrombogenic or hydrophobic display higher vulnerability to microbial adhesion.[42,43] Moreover, the extent of colonization appears to increase with surface roughness because shear forces are locally diminished and the adhesion area is higher.[37] Polyurethane and polytetrafluoroethylene (Teflon®) catheters have been associated with less infectious complications than catheters made of polyvinyl chloride or polyethylene.[44,45] At present, polyurethane catheters are the most frequently used worldwide in medical care.

**The role of microbial biofilms**

Microbial biofilms are sessile communities of organisms that are embedded in a matrix of extracellular polymers and exhibit an altered phenotype concerning growth rate and gene transcription.[36] The basic structural unit of the biofilm is the microcolony, which is encased in the matrix and separated from other colonies by water channels where oxygen and nutrients may diffuse.[37]

In general, biofilm organisms are more resistant than their planktonic counterparts: (1) penetration of antimicrobial agents through the matrix is delayed because it works as a diffusional barrier; (2) growth of biofilm organisms is slower since nutrient limitation and accumulation of toxic metabolites within the biofilm may delay bacterial growth, hindering the uptake of antimicrobial agents.[36] Moreover, biofilms that develop on indwelling medical devices such as CVCs are correlated with CRBSI by the following suggested mechanisms: (1) detachment of microbial cells by shear stress or changes in substrate concentration in the matrix; (2) production of endotoxin by gram-negative bacteria; (3) resistance to the host immune system response

by inhibition of macrophage phagocytosis or antibody activity and (4) promotion of antibiotic resistance by plasmid exchange within the niche of microcolonies.[33,36,37,46,47]

Several comprehensive strategies have been proposed to control biofilms in CRBSIs, either by (1) preventing medical device contamination during insertion steps; (2) inhibiting microbial cell attachment to the surface of devices; (3) destructing the formed biofilm by killing the microorganisms embedded in the matrix or by (4) removing the infected device.[36]

## Current strategies for prevention of CRBSIs

### *Essentials of CVC management*

Several strategies are strongly recommended and supported by current literature aiming an effective reduction of CRBSIs:

1. Education of healthcare personnel regarding CVC insertion and maintenance, with adherence to a catheter care protocol involving strict aseptic technique. Trained staff should be periodically assessed for knowledge and commitment to guidelines.[48-50]
2. Benefits of each placement site must be weighed against the risk of mechanical and infectious complications: femoral vein catheters have the higher colonization rate and the higher risk for deep venous thrombosis; the use of the subclavian site is associated with the lowest risk of infection, although mechanical complications (such as pneumothorax, subclavian artery puncture, subclavian vein laceration or stenosis) should be considered.[51-59]
3. The use of polyurethane and polytetrafluoroethylene (Teflon®) CVCs is associated with less infectious complications, as is the use of the minimum number of ports and lumens.[44,60-62]
4. Superfluous catheters must be promptly removed; replacement of CVCs within 48h should occur whenever asepsis cannot be ensured.[63-67]
5. Cleaning the skin with >0.5% chlorhexidine preparation with alcohol and using maximal sterile barrier precautions (cap, mask and sterile gown, gloves and full body drape) for the insertion of central catheters, with sterile gauze or

transparent dressing to cover the catheter site. Hand hygiene procedures (with soap or alcohol-based hand rubs) should be undertaken before and after manipulation.[66,68-74]

6. At present, there are no recommendations to routine use of systemic antimicrobial prophylaxis, periodical replacement of CVCs or guidewire exchanges in order to prevent CRBSIs.[75,76]
7. Replacement of administration sets should occur within 96-hour to 7-day intervals.[77,78] For blood products or lipid emulsions, tubing should be replaced within a maximum of 24 hours.[79,80]

Other approaches aiming to reduce CRBSIs are available, but are only supported by suggestive clinical or epidemiologic studies or by a theoretical rationale:

1. The use of a prophylactic antimicrobial lock solution may be advisable in patients with long term CVCs and a clinical record of multiple CRBSIs, despite total adherence to the aseptic technique.[81,82]
2. Anticoagulant therapy should not be routinely used to decrease the risk of CRBSIs, even though it may reduce the risk of catheter-related thrombosis.[83]

***The role of impregnated CVCs***

Among strategies to prevent microbial cell attachment in a more effective way, CVCs impregnated with antiseptics and antibiotics have been proposed. However, the reduction of the infection rate was achieved with variable success and toxicity:

1. Catheters impregnated with chlorhexidine-silver sulfadiazine can be effective in reducing colonization and infection,[84] but hypersensitivity reactions have been documented.[85] Although these are more expensive than standard catheters, the use of chlorhexidine-silver sulfadiazine catheters may lead to cost savings whenever the risk for CRBSIs is high and no success has been achieved with other preventive strategies, such as maximal barrier precautions and aseptic techniques.

2. Catheters impregnated with minocycline and rifampin decreased significantly the incidence of CRBSIs,[86] even though some concern may still exist regarding

the development of antimicrobial resistance.[87] Its use may be cost effective if other preventive measures fail to control the incidence of CRBSIs.

3. Silver-impregnated catheters are not associated with a lower rate of colonization or infection when compared to standard CVCs.[88]

**Novel strategies for prevention of CRBSIs**

A large number of approaches targeting the different stages of biofilm formation are currently in study:

- silver nanoparticles display good antimicrobial activity and may allow a constant supply of $Ag^+$ ions at the coated surface;[89]
- phages displaying bactericidal activity can be used in the local control of biofilms;[90]
- peptides, such as β-defensins, are a potential new class of antimicrobial agents with a broad activity spectrum and a relative selectivity towards microbial membranes;[91]
- enzymes targeting EPS, namely dispersin B and *N*-acetyl-*L*-cysteine, are able to reduce bacterial adhesion and, furthermore, to disrupt and detach mature biofilms;[92,93]
- drug delivery carrier systems, such as liposomes, polymeric microspheres, micelles and hydrogel-type materials may target matrix or biofilm bacteria by specific attachment and release of drugs in close vicinity of microorganisms;[94]
- nitric oxide is an hydrophobic free-radical gas that may be impregnated in medical devices, showing antibacterial properties;[95,96]
- electrical current may be used against biofilms either by prolonged exposure to low intensity direct electrical current (electricidal effect) or by enhancement of the activity of antimicrobials (bioelectric effect);[97,98]

- ultrasound has been demonstrated to enhance the activity of antimicrobial agents by inducing cavitation within the biofilm (bioacustic effect);[99]
- light-activated antimicrobial agents generate reactive oxygen species when excited with the appropriate wavelength light, which are toxic to microorganisms growing on the surface of medical devices.[100,101]

However, concerns about biosafety, microbial resistance and cost-effectiveness of such approaches still remain to be clarified.[102-104]

Concerning other alternatives, biocompatible and inexpensive compounds already available may help to prevent microbial colonization. Cerium ($^{58}$Ce) is the most abundant of the rare earth elements and belongs to the lanthanide group. It is extracted from minerals such as monazite and bastnaesite. Cerium has no known physiological role and does not penetrate mammalian cell membranes; however, it may form salts of medical interest. Cerium nitrate (Figure 1), by itself or in combination with silver sulphadiazine, has been extensively used in the management of burns, with a reduction in patient morbidity and mortality.[105] In spite of the assumed reversion of the post-burn cell-mediated immunosuppression, its antimicrobial properties and mechanism of action still remain controversial and yet unexplained.[105,106] Nevertheless, the uptake of cerium into the cytoplasm of microbial cells was described to inhibit cellular respiration, glucose metabolism or to disrupt the cell membrane.[107]

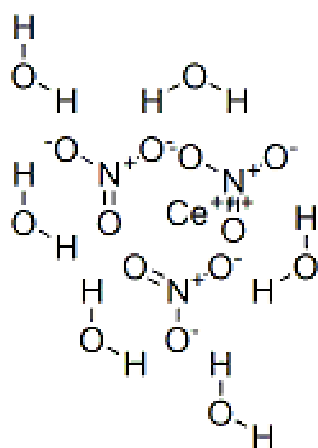

**Figure 1.** Structure of cerium(III) nitrate hexahydrate.

Chitosans are hydrophilic polyaminosaccharides obtained by *N*-deacetylation of the crustacean chitin. Considerable attention has been dedicated by the pharmaceutical, food and cosmetic industries to these nontoxic and biocompatible polymers. In the medical field, chitosans are being investigated regarding its immunomodulating, antitumoral and antioxidant properties.[108,109] In addition, chitosans exhibit a broad spectrum antimicrobial activity that is dependent on molecular weight, degree of deacetylation and type of substitution. High molecular weight chitosans cannot cross through cell membranes, forming a film because of its viscosity. In contrast, low molecular weight chitosans (LMWCs) can enter into the cell, being more amenable for a wide variety of biomedical applications due to its higher solubility in water (Figure 2). Hence, the mechanism of action of chitosans may be explained by: (1) external barrier formation and metal chelation with inhibition of microbial growth; (2) interaction between cationic chitosan derivatives and anionic microbial surface, resulting in cell wall leakage; (3) binding with teichoic acid and extraction of membrane lipids and (4) interference with mRNA and protein synthesis after nucleic penetration.[110,111]

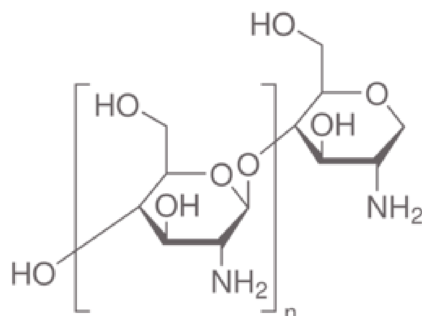


**Figure 2.** Structure of low molecular weight chitosan.

Hamamelitannin is a natural compound found mainly in the bark and leaves of *Hamamelis virginiana* (witch hazel), indigenous to the woods of the Atlantic coast of North America and scarcely cultivated in Europe. It belongs to the family of tannins, which are plant polyphenols. Hamamelitannin is the ester of D-hamamelose (2-hydroxymethyl-D-ribose) with two molecules of gallic acid, each containing three phenolic functional groups (Figure 3). Although scientific data supporting consistent efficacy are still limited, patients currently use hamamelis preparations in eye cleansing and in the treatment of eczema, varicose vein symptoms and minor inflammation of the skin and oral cavity.[112] Hamamelis distillate may promote wound healing via anti-

inflammatory effects.[113] Furthermore, hamamelitannin inhibits tumor necrosis factor α-mediated endothelial cell death,[114] protects against cell damage induced by peroxides[115] or UVB radiation,[116] while it suppresses staphylococcal infections *in vivo* (including methicillin-resistant *S. aureus* and *S. epidermidis*) by inhibiting the quorum sensing system of such bacteria.[117]

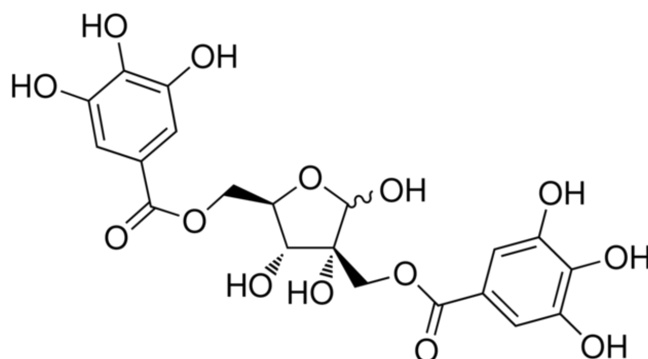


**Figure 3.** Structure of hamamelitannin.

# Study aims

This investigation has three main goals:

**1-** To determine the aetiology of CVC colonization among intensive care patients at a national University Hospital. Attending to the ecological niches occupied by the distinct microbial agents and to the patterns of antibiotic susceptibility found, the selection of preventive attitudes aiming to reduce CVC colonization in this hospital is also to be expected.

**2-** To study *in vitro* biofilm formation by different organisms (*S. epidermidis*, *S. aureus*, *A. baumannii* and *C. albicans*), commonly associated to CVC colonization and CRBSI, at the surface of cerium, chitosan and hamamelitannin treated polyurethane catheters.

**3-** To evaluate the *in vivo* efficacy of cerium, chitosan and hamamelitannin in the prevention of biofilm formation by *S. epidermidis*, *S. aureus*, *A. baumannii* and *C. albicans* on treated polyurethane catheter segments, using a mouse foreign body infection model. The validation of this model to study CRBSI is also to be expected.

Such goals were addressed according to the three tasks that follow.

# Task 1

## Aetiology of CVC colonization

## Materials and methods

### Study population

A retrospective study of the clinical and laboratorial data base of Hospital de S. João, Porto, was performed. A 12-month period (from 1st January to 31st December, 2007) was considered. The results were collected from all the ICUs in this Hospital, corresponding to 2 General ICUs, 1 Neurocritical ICU, 1 Infectious Diseases ICU and 1 Burn Unit, totalizing 45 beds. All positive results from the culture of the tips of CVCs were taken into account.

### Definitions

Positive results from culture of CVC tips were assumed as CVC colonization since no data from associated blood infection neither the number of days of catheterization could be consistently recovered from the data source.

### Microbial characterization

Microorganisms were isolated after removal of the CVCs from patients and culture of its distal tips, using the semiquantitative roll plate method described by Maki *et al.*[118] Whenever more than 15 colonies were visualised, the isolates were characterized and identified using the appropriate Vitek identification cards (bioMérieux, Paris, France), with the exception of fungal colonies (every colony was identified). Staphylococci were identified and tested for antimicrobial susceptibility with Vitek GP and GP-549 cards, respectively; Enterobacteriaceae and *A. baumannii*, with Vitek GN and GN-037 cards; *P. aeruginosa*, with Vitek GN and GN-022 cards. *Candida* isolates were characterized with Vitek 2 identification cards or API 20C galleries. Susceptibility testing to fluconazole, voriconazole, posaconazole, amphotericin B and caspofungin was assessed accordingly to the CLSI protocol M27-A3.

## Results

Globally, from 1482 CVC distal tips recovered from ICU patients, 647 were found to be positive during the study period. The microorganisms found are listed in Table 1.

**Table 1.** Microbial isolates from CVC tips.

| **Gram-positive cocci** | **n** | **%** |
|---|---|---|
| *Staphylococcus epidermidis* | 260 | 40.2 |
| *Staphylococcus aureus* | 83 | 12.8 |
| *Staphylococcus haemolyticus* | 41 | 6.3 |
| *Enterococcus faecalis* | 24 | 3.7 |
| *Staphylococcus hominis* | 22 | 3.4 |
| Other coagulase-negative staphylococci | 19 | 2.9 |
| *Staphylococcus capitis* | 7 | 1.1 |
| *Enterococcus faecium* | 7 | 1.1 |
| *Staphylococcus warneri* | 4 | 0.62 |
| *Kocuria kristinae* | 4 | 0.62 |
| *Streptococcus sanguis* | 1 | 0.15 |
| *Streptococcus pneumoniae* | 1 | 0.15 |
| *Streptococcus agalactiae* | 1 | 0.15 |
| *Staphylococcus simulans* | 1 | 0.15 |
| **Total** | 475 | 73.4 |

| **Gram-negative bacilli** | **n** | **%** |
|---|---|---|
| *Pseudomonas aeruginosa* | 40 | 6.2 |
| *Acinetobacter baumannii* | 18 | 2.8 |
| *Proteus mirabilis* | 12 | 1.9 |
| *Escherichia coli* | 11 | 1.7 |
| *Klebsiella pneumoniae* | 10 | 1.5 |
| *Enterobacter cloacae* | 8 | 1.2 |
| *Morganella morganii* | 6 | 0.93 |
| *Stenotrophomonas maltophilia* | 4 | 0.62 |
| *Enterobacter aerogenes* | 3 | 0.46 |
| *Serratia marcescens* | 3 | 0.46 |
| *Burkholderia cepacia* | 2 | 0.31 |
| Other *Klebsiella species* | 1 | 0.15 |
| *Klebsiella oxytoca* | 1 | 0.15 |
| *Citrobacter braakii* | 1 | 0.15 |
| **Total** | 120 | 18.5 |

| **Gram-positive bacilli** | **n** | **%** |
|---|---|---|
| *Corynebacterium species* | 6 | 0.93 |
| *Corynebacterium jeikeium* | 1 | 0.15 |
| **Total** | 7 | 1.1 |

| **Yeasts** | **n** | **%** |
|---|---|---|
| *Candida albicans* | 32 | 4.9 |
| *Candida glabrata* | 6 | 0.93 |
| *Candida tropicalis* | 4 | 0.62 |
| *Candida parapsilosis* | 3 | 0.46 |
| **Total** | 45 | 7.0 |

The antibacterial susceptibility pattern of gram-positive cocci and gram-negative bacilli is detailed in Tables 2 and 3, respectively, for the most commonly tested and prescribed drugs in Hospital de S. João. The antifungal susceptibility pattern of *Candida* isolates is detailed in Table 4.

**Table 2.** Antimicrobial susceptibility of gram-positive cocci. S, susceptible; R, resistant; I, intermediate.

| | **Coagulase-negative staphylococci** | | | | | | ***Staphylococcus aureus*** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **S** | | **R** | | **I** | | **S** | | **R** | | **I** | |
| **Antibacterial agents** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **N** | **%** |
| Linezolid | 288 | 100 | | | | | 55 | 100 | | | | |
| Oxacillin | 42 | 14.7 | 243 | 85.3 | | | 11 | 20 | 44 | 80 | | |
| Teicoplanin | 271 | 94.4 | | | 16 | 5.6 | 54 | 98.2 | | | 1 | 1.8 |
| Vancomycin | 287 | 99.7 | | | 1 | 0.3 | 55 | 100 | | | | |
| | ***Enterococcus faecalis*** | | | | | | ***Enterococcus faecium*** | | | | | |
| | **S** | | **R** | | **I** | | **S** | | **R** | | **I** | |
| | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **N** | **%** |
| Ampicillin | 12 | 100 | | | | | | | 7 | 100 | | |
| Linezolid | 12 | 92.3 | | | 1 | 7.7 | 7 | 100 | | | | |
| Penicillin | 12 | 92.3 | 1 | 7.7 | | | | | 7 | 100 | | |
| Quinupristin/Dalfopristin | | | 5 | 100 | | | 2 | 100 | | | | |
| Teicoplanin | 12 | 92.3 | 1 | 7.7 | | | 2 | 28.6 | 5 | 71.4 | | |
| Vancomycin | 12 | 92.3 | 1 | 7.7 | | | 1 | 14.3 | 6 | 85.7 | | |

**Table 3.** Antimicrobial susceptibility of gram-negative bacilli.

| | **Enterobacteriaceae** | | | | | | ***Pseudomonas aeruginosa*** | | | | | | ***Acinetobacter baumannii*** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **S** | | **R** | | **I** | | **S** | | **R** | | **I** | | **S** | | **R** | | **I** | |
| **Antibacterial agents** | **n** | **%** | **n** | **%** | **n** | **%** | **N** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **N** | **%** | **n** | **%** |
| Amikacin | 29 | 80.6 | | | 7 | 19.4 | 16 | 69.6 | 7 | 30.4 | | | 17 | 94.4 | 1 | 5.6 | | |
| Cefepime | 2 | 100 | | | | | 11 | 50 | 7 | 31.8 | 4 | 18.2 | | | | | | |
| Cefotaxime | 34 | 97.1 | 1 | 2.9 | | | | | 1 | 100 | | | | | 18 | 100 | | |
| Ceftazidime | 3 | 100 | | | | | 10 | 43.5 | 12 | 52.2 | 1 | 4.3 | 1 | 5.6 | 17 | 94.4 | | |
| Cefoxitine | 3 | 100 | | | | | | | | | | | | | | | | |
| Cefuroxime | 27 | 79.4 | 7 | 20.6 | | | | | 1 | 100 | | | | | 18 | 100 | | |
| Ciprofloxacin | 4 | 100 | | | | | 12 | 52.2 | 5 | 21.7 | 6 | 26.1 | 1 | 5.6 | 17 | 94.4 | | |
| Colistin | 1 | 100 | | | | | 21 | 95.5 | 1 | 4.5 | | | 13 | 100 | | | | |
| Gentamicin | 33 | 94.3 | 2 | 5.7 | | | 11 | 47.8 | 9 | 39.1 | 3 | 13 | 16 | 88.9 | 2 | 11.1 | | |
| Imipenem | 33 | 91.7 | 3 | 8.3 | | | 7 | 30.4 | 15 | 65.2 | 1 | 4.3 | 1 | 5.6 | 17 | 94.4 | | |
| Levofloxacin | 4 | 100 | | | | | 1 | 100 | | | | | 1 | 9.1 | 9 | 81.8 | 1 | 9.1 |
| Meropenem | 36 | 100 | | | | | 9 | 39.1 | 8 | 34.8 | 6 | 26.1 | 1 | 5.6 | 17 | 94.4 | | |
| Piperacillin | | | 2 | 66.7 | 1 | 33.3 | 18 | 78.3 | 4 | 17.4 | 1 | 4.3 | 1 | 5.6 | 17 | 94.4 | | |
| Piperacillin/Tazob. | 29 | 80.6 | 2 | 5.6 | 5 | 13.9 | 9 | 69.2 | 3 | 23.1 | 1 | 7.7 | | | 17 | 100 | | |
| Tobramycin | 1 | 100 | | | | | 13 | 59.1 | 9 | 40.9 | | | | | | | | |

**Table 4.** Antifungal susceptibility of *Candida* isolates.

| | ***C. albicans*** | | | | | | ***C. glabrata*** | | | | | | ***C. parapsilosis*** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **S** | | **R** | | **I** | | **S** | | **R** | | **I** | | **S** | | **R** | | **I** | |
| **Antifungal agents** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** |
| Fluconazole | 27 | 100 | | | | | 5 | 100 | | | | | 2 | 66.7 | | | 1 | 33.3 |
| Voriconazole | 27 | 100 | | | | | 5 | 100 | | | | | 3 | 100 | | | | |
| Posaconazole | 27 | 100 | | | | | 5 | 100 | | | | | 3 | 100 | | | | |
| Amphotericin B | 27 | 100 | | | | | 5 | 100 | | | | | 3 | 100 | | | | |
| Caspofungin | 27 | 100 | | | | | 5 | 100 | | | | | 3 | 100 | | | | |

# Discussion

Taken globally, the epidemiological data concerning the aetiology of CVC colonization in the ICUs under evaluation was similar to previous publications. Although catheter colonization does not invariably lead to CRBSI, it usually represents its initial step.

As expected, coagulase-negative staphylococci (n=354; 54.7%), mainly *S. epidermidis* (40.2%), were the most frequent species isolated from CVC tips. *S. aureus* (12.8%) was the second most common organism. These microbial agents are thought to gain access to distal tips of CVCs by contiguous spread from the patient skin and

mucous membranes along the external surface of catheters or, intraluminally, from contamination of catheter hubs. High susceptibility levels to linezolid, teicoplanin and vancomycin were found for these cocci. However, methicillin resistance among *S. aureus* (80%) and coagulase-negative staphylococci (85.3%) was higher than expected from similar ICU data (55.3% and 75%, respectively) which, by itself, is associated with an increased length of hospitalization, cost and mortality.[119]

*Enterococcus faecalis* (3.7%) and *Enterococcus faecium* (1.1%) were the next most common gram-positive cocci, following staphylococci. These enteric bacteria are found mainly in the intestinal tract of patients. Besides skin contamination with bowel microorganisms during daily hygiene care, such organisms most probably reach CVC hubs through the hands of hospital personnel. Although a small number of enterococci have been tested in this study, *E. faecalis* displayed high susceptibility to penicillin, ampicilin, linezolid, teicoplanin and vancomycin; as expected, quinupristin/dalfopristin lacks activity against this species; conversely, both isolates of *E. faecium* tested were susceptible. The single other antibacterial with activity against *E. faecium* was linezolid; high resistance levels were found to vancomycin and teicoplanin. As there is a global concern about conjugated transfer of the *van*A gene from enterococci to *S. aureus*,[120] routine surveillance for resistance patterns of such organisms is of paramount importance.

Among gram-negative bacilli, Enterobacteriaceae (n=56; 8.6%), *P. aeruginosa* (6.2%) and *A. baumannii* (2.8%) predominated. Enterobacteriaceae, as part of normal intestinal flora, may contaminate indwelling devices during hygiene care, similarly to what was described for enterococci. Except for cefuroxime, a second-generation cephalosporin, Enterobacteriaceae demonstrated high susceptibility to the other tested cephalosporins, which seems to exclude this hospital from the international trend towards third and fourth-generation cephalosporin resistance.[121] Carbapenems also showed good activity against Enterobacteriaceae.

*P. aeruginosa* is frequently a HCRI organism that thrives in moist environments. It may assume a pathogenic pattern of behaviour whenever the mucous membranes or the skin are disrupted, as is the case with central venous catheterization. Since *P. aeruginosa* often displays MDR, it may become dominant when more susceptible bacteria belonging to the indigenous microbial population are suppressed, as usually happens in the ICU setting. Among *Pseudomonas* isolates, resistance levels found to meropenem (34.8%) and imipenem (65.2%) were unexpectedly high, as was also the

case for ceftazidime (52.2%) and tobramycin (40.9%).[122] Drug resistance to piperacillin (17.4%), ciprofloxacin (21.7%), gentamicin (39.1%) and amikacin (30.4%) was in accordance with international data. Colistin showed excellent activity against *P. aeruginosa*.

*A. baumannii* may colonize extensively the hospital environment. Occasionally, it can be found on human skin, mucous membranes and secretions as a saprophyte. Whenever it causes HCRIs, *A. baumannii* is often associated with medical indwelling devices such as endotracheal tubes or CVCs and is highly resistant to antimicrobial agents.[121,123] Invariably, the most disturbing fact in the isolated strains was an extremely high resistance level to carbapenems (94.4%). Susceptibility was found only to gentamicin (88.9%), amikacin (94.4%) and colistin (100%). Moreover, *A. baumannii* has been responsible for the closure of some of the aforementioned ICUs, for several days, in order to hygienize and eradicate the organism from the environment, as it is highly transmissible during an outbreak. Besides the additional costs, the inconvenience and medical risk of patient transfer to other hospital ICUs during these periods must be taken into account.

The yeast most frequently recovered from CVCs was *C. albicans* (4.9%), at the fourth position of the ranking of agents, in conformity with literature. *Candida* spp are the most common opportunistic fungal pathogens. They usually colonize the human skin, mucous membranes and gastrointestinal tract and may cause fungemia following intestinal wall translocation or contamination of vascular catheters. Against expectations from European data, *C. glabrata* (0.93%) was second to *C. albicans*.[25] No remarkable levels of resistance were found among *Candida* spp; in fact, all strains were susceptible to fluconazole, voriconazole, posaconazole, amphotericin B and caspofungin. This might be explained by the limited exposure to antifungal agents in this hospital. Continued surveillance on species distribution and antifungal susceptibility profile is needed, since a low number of *Candida* isolates was tested.

A problem often arising in the interpretation of positive catheter segment cultures is the false positivity that may result from adhesion of skin organisms to CVC during its removal. Such finding may be responsible particularly for cases attributed to coagulase-negative staphylococci. Conversely, the roll plate method may be inadequate for the recovery of organisms from the intraluminal portion of catheter segments, underestimating its colonization and, therefore, leading to false negative results.

All the described organisms share the capacity to form biofilm on indwelling devices. Sessile cells in such a matrix exhibit a promoted ability to colonize catheters and overcome the host immune system response as well as the effects of antimicrobial drugs in circulation, comparing to their planktonic counterparts, from which they are phenotypically distinct.

Regarding that the microbial agents most frequently isolated are usual patient colonizers or hospital environmental contaminants, it seems to be a clear benefit to promote the aforementioned essential elements of CVC use in this hospital, in order to reduce CRBSIs. Furthermore, the design of therapeutic protocols based on the local patterns of antimicrobial susceptibility might help to pursue a more strict selection of antimicrobials. As a policy to control the undesired high levels of resistance found, rotational antimicrobial use could be highly advisable.[124-127]

While new approaches to estimate the true incidence of CVC related infections are needed, devoted attention to measures aiming the reduction or the complete blockade of its colonization is mandatory, particularly in the ICU setting.

# Task 2

## *In vitro* biofilm formation on treated catheters

## Materials and methods

### Chemicals

Cerium nitrate [cerium(III) nitrate hexahydrate, Sigma-Aldrich], chitosan [low-molecular-weight chitosan 107 kDa, 75-85% deacylated; Sigma-Aldrich; stock solution of 4% (w/v) prepared in 1% acetic acid, pH 4.0] and hamamelitannin [hamamelofuranose 2′,5-digallate, 2-C-(Hydroxymethyl)-D-ribofuranose 2′,5-digallate; Sigma-Aldrich] were used in the experiments.

### Microbial strains

A type strain from the American Type Culture Collections (ATCC) and a clinical isolate of each of four microbial species were used: the type strains were *S. aureus* ATCC 29213, *S. epidermidis* ATCC 155, *A. baumannii* ATCC 19606 and *C. albicans* ATCC 90028; the clinical strains of *S. aureus* (SA1), *S. epidermidis* (SE1), *A. baumannii* (AB1) and *C. albicans* (CA1) had been previously isolated from cultures of CVCs removed from critical care patients admitted at Hospital S. João, Porto, Portugal. All clinical isolates had been identified by Vitek system (bioMérieux, Vercieux, France).

Bacterial strains were kept frozen in Luria-Bertani broth (LB; Difco Laboratories, Detroit, MI, USA) supplemented with 20% glycerol and the yeast strains in yeast potato dextrose medium (YPD; Difco Laboratories) supplemented with 40% glycerol at -70ºC until testing. For each experiment, the microorganisms were subcultured twice on LB agar, 37ºC, 24 h for bacteria or Sabouraud agar, 35ºC, 24 h (Difco Laboratories) for yeasts to assess the purity of the culture and its viability.

### Antimicrobial activity

The minimal inhibitory concentration (MIC) of cerium nitrate, LMWC and hamamelitannin was evaluated accordingly to the Clinical and Laboratory Standards Institute (CLSI) microdilution reference protocol M07-A8 for bacterial strains and protocol M27-A3 for yeast strains. The tested concentrations ranged from $5.4 \times 10^3$ to $3.3 \times 10^2$ mg/L for cerium nitrate, $1.0 \times 10^4$ to $1.6 \times 10^2$ mg/L for LMWC and 100 to 10 mg/L for hamamelitannin. MIC end point was defined as the lowest drug concentration that completely inhibited the growth of microorganisms in microdilution wells.

To determine the minimal lethal concentration (MLC), 20 µL from each microdilution well were plated in LB agar (for bacteria) or Sabouraud agar (for yeasts).

The plates were incubated at 37ºC for 24 h (bacterial strains) or 35ºC for 48 h (yeast strains) with subsequent enumeration of colony forming units. MLC was defined as the lowest drug concentration that killed at least 99.9% of the final inoculum.

Furthermore, to study the effect of cerium nitrate against *C. albicans*, flow cytometric analysis was performed with two fluorescent markers: FUN-1 (to evaluate cell metabolic activity) and propidium iodide (PI; a marker of cell death that only penetrates cells with severe membrane lesions).[128] After cerium nitrate treatment, yeasts were collected, washed twice in distilled water and resuspended in FUN-1 0.5 μM or PI 1 μM. The yeast suspensions were incubated for 30 min at 37ºC in the dark; following incubation, the suspensions were centrifuged and the supernatant transferred to flow cytometry propylene tubes. As controls for FUN-1 staining, untreated cell suspensions and cells treated with 1 mM sodium azide (Sigma) for 1 h and stained under the same conditions were used. Controls for PI staining included suspensions of untreated and killed cells (90ºC for 30 min), stained with PI under the same conditions. For each experimental condition, 20,000 events were analysed in a standard flow cytometer (FACSCalibur BD Biosciences, Sydney) with three PMTs equipped with standard filters (FL1: BP 530/30 nm; FL2: BP 585/42 nm; FL3: LP 670 nm), a 15 mW 488 Argon Laser and operating with Quest Pro software (version 4.0.2, BD Biosciences, Sydney). Results were expressed as a staining index (SI) for FUN-1, defined as the ratio between the mean fluorescence of treated cell suspensions and the corresponding value for the non-treated cells at FL2 (values ≥ 1 represent treatment effect) and, for PI, as the percentage of positive cells showing high fluorescence at FL3.[128]

**Biofilm formation on polyurethane catheters**

The microbial strains were grown overnight in LB broth at 37ºC and 180 rpm, for bacteria, or Sabouraud broth at 35ºC and 180 rpm, for yeasts. Cells were harvested by centrifugation (10000 *g*, 5 min), washed with phosphate buffered saline (PBS), counted in a Newbauer chamber and standardized to $1x10^7$ cells/mL in LB broth for bacteria and $1x10^6$ cells/mL in RPMI (Sigma-Aldrich) for yeasts. The anti-biofilm effect of the 3 different test compounds was evaluated in the presence of four concentrations of cerium nitrate ($1.7x10^3$, $1.3x10^3$, $6.5x10^2$ and $3.3x10^2$ mg/L) and three of hamamelitannin (100, 80 and 50 mg/L); regarding LMWC, concentrations tested corresponded to 625, 160 and 78 mg/L for bacteria and $1.0x10^4$, $5.0x10^3$ and 625 mg/L for yeasts. One single fragment of polyurethane intravenous catheters (BD Vialon™ 16G 1.7x45 mm), with 1

cm length, was placed in each well of 12-well microplates, containing 1 mL of the standardized microbial suspensions.

After incubation (24 h for bacteria; 24 and 48 h for yeasts), at 37ºC, catheter fragments were removed, gently washed with PBS and placed in new microplates for biofilm quantification with 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), as previously described [129]. Briefly, 1 mL of the XTT solution was added to each well containing the CVC fragment with the prewashed biofilm and to control wells containing a sterile CVC fragment (for the measurement of background XTT reduction levels). The microplates were incubated for 5 h at 37ºC, in the dark. Finally, the optical density was measured at 492 nm. All the assays were performed in triplicate.

### Data analysis

Biofilm metabolic activity in the presence of cerium nitrate, LMWC and hamamelitannin was expressed as the percentage in relation to the positive control. Values were divided by 1000 and arcsine square root transformed to achieve a normal distribution and homoscedasticity. For each compound, each concentration and each microorganism, biofilm formation was compared by one-way ANOVA, followed by a Dunnett's test to identify significant effects; $p$ value $<0.05$ was considered significant.

## Results

### Antimicrobial activity

Cerium nitrate MIC ranged between $3.3 \times 10^2$ to $1.3 \times 10^3$ mg/L for bacterial strains. For yeasts, concentrations up to $2.6 \times 10^3$ mg/L were needed. Globally, MLC corresponded to the double or more the MIC values for all microbial species (Table 5).

For LMWC, MICs ranged between $1.6 \times 10^2$ and $3.1 \times 10^2$ mg/L for all bacteria. A higher concentration ($1.0 \times 10^4$ mg/L) was needed to inhibit *C. albicans* growth. MLC values nearly doubled the MIC values for all bacterial species. MLC and MIC were similar for *C. albicans* (Table 5).

Hamamelitannin produced no inhibition of microbial growth, at concentrations ranging between 10 to 100 mg/L.

**Table 5.** Minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) of cerium nitrate and low molecular weight chitosans (LMWC).

| | **MIC** | | **MLC** | |
|---|---|---|---|---|
| | **Cerium nitrate (mg/L)** | **LMWC (mg/L)** | **Cerium nitrate (mg/L)** | **LMWC (mg/L)** |
| **Bacterial strains** | | | | |
| *S. aureus* (SA1) | $3.3x10^2$ | $1.6x10^2$ | $5.4x10^3$ | $3.1x10^2$ |
| *S. aureus* ATCC | $3.3x10^2$ | $1.6x10^2$ | $2.6x10^3$ | $1.25x10^3$ |
| *S. epidermidis* (SE1) | $1.3x10^3$ | $1.6x10^2$ | $2.6x10^3$ | $3.1x10^2$ |
| *S. epidermidis* ATCC | $6.5x10^2$ | $1.6x10^2$ | $2.6x10^3$ | $6.3x10^2$ |
| *A. baumannii* (AB1) | $1.3x10^3$ | $1.6x10^2$ | $5.4x10^3$ | $2.5x10^3$ |
| *A. baumannii* ATCC | $1.3x10^3$ | $3.1x10^2$ | $5.4x10^3$ | $1.25x10^3$ |
| **Yeast strains** | | | | |
| *C. albicans* (CA1) | $2.6x10^3$ | $1.0x10^4$ | $5.4x10^3$ | $1.0x10^4$ |
| *C. albicans* ATCC | $2.6x10^3$ | $1.0x10^4$ | $5.4x10^3$ | $1.0x10^4$ |

Concerning flow cytometric studies, FUN-1 staining demonstrated that cerium nitrate impairs *C. albicans* metabolism and ultimately can lead to cell membrane damage: the MLC concentration caused a critical decrease in the cellular metabolism, starting approximately after 1.5 h of exposure and increasing along the time; PI was able to stain approximately 50% of the cells treated with the MLC after 6 h of incubation and, after 24 h of treatment, almost 85% of cells exhibited membrane injury. MIC concentration was unable to induce membrane damage.

### Biofilm formation on polyurethane catheters

Cerium nitrate, at a concentration of $1.7x10^3$ mg/L, reduced the mean biofilm metabolic activity of *C. albicans* by 58.9% (±7.4) at 24 h and by 59.14% (±13.0) at 48 h. No relevant biofilm inhibition was found for bacterial strains with cerium nitrate at concentrations lower than MLC (Figure 4. A, B and C).

LMWC, at a concentration of 78 mg/L, reduced the biofilm metabolic activity of *S. epidermidis* by 80.46% (±0.0) (Figure 4. D). At a concentration of $5.0x10^3$ mg/L, LMWC reduced *C. albicans* biofilm metabolic activity by 87.5% (±0.0) at 24 h and by 90.06% (±0.0) at 48 h (Figure 4. F). However, inconsistent results were found with *S. aureus* and *A. baumannii* (Figure 4. D and E).

Hamamelitannin, at a concentration of 100 mg/L, decreased significantly the mean biofilm metabolic activity of *S. aureus* by 23.0% (±10.7%); of *S. epidermidis* by 42.2% (±3.78%) and of *A. baumannii* by 31.8% (±0.88) (Figure 4. G and H). Inconclusive results were found with *C. albicans* strains, at 24 and 48 h (Figure 4. I).

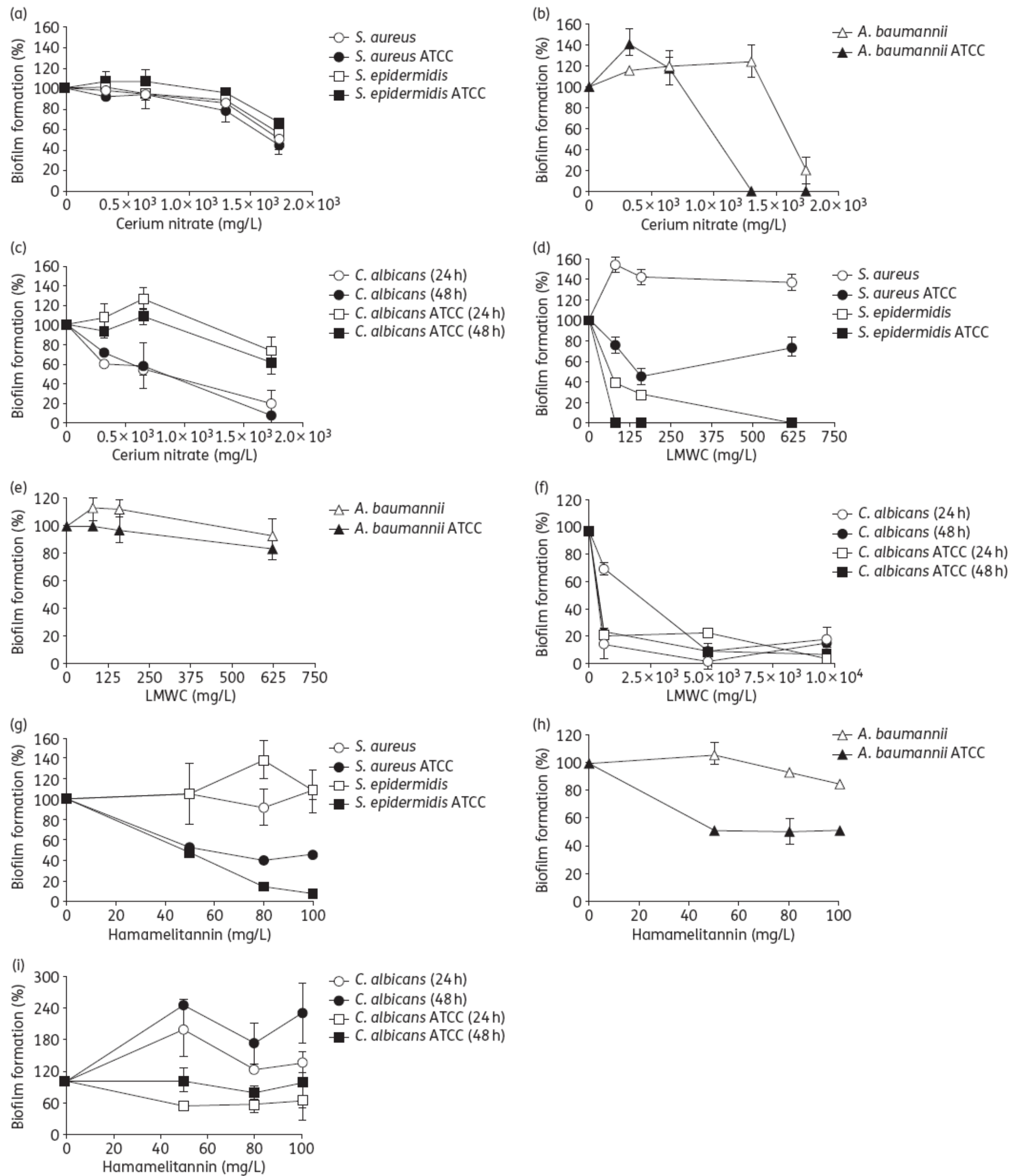


**Figure 4.** Effect of cerium nitrate (A, B and C), low molecular weight chitosan (D, E and F) and hamamelitannin (G, H and I) on biofilm formation (as percentage of the control) by Gram positive cocci, Gram negative bacteria and yeasts (at 24 and 48 h). Differences between control and antimicrobial groups were statistically significant ($P < 0.01$).

## Discussion

Indwelling medical devices are used for a wide array of purposes. Unfortunately, they are quite prone to colonization by biofilm producing microorganisms. New strategies aiming the blockade of such biofilms have been developed and although some are in use with variable success, there is still a wide field for further investigation.

A review conducted by Garner *et al.*, 2005, gathered information on the bacteriostatic effect of cerium nitrate against a panel of bacteria.[105] Our study not only adds to this effect but also documents unequivocally the microbicidal effect of cerium nitrate against bacteria and yeasts. This might be quite useful in medical applications because toxicity is rare with lanthanides since they do not penetrate the membranes of living mammalian cells.

Concerning microbial growth inhibition by cerium nitrate, our results indicate that MICs for *S. aureus* are similar to previous descriptions,[105] while we determined for the first time MICs for *S. epidermidis*, *A. baumannii* and *C. albicans*. Furthermore, the so far unclear cerium nitrate microbicidal effect was documented and MLCs were established for major colonizers of indwelling medical devices.

At lower concentrations, cerium nitrate was only effective against *C. albicans* biofilm, with no difference in its activity after 24 or 48 h of incubation.

No further experiments were then pursued in order to clarify the mechanism by which cerium nitrate inhibited in a different way biofilm formation by bacteria and yeasts.

The spectrum wideness of antimicrobial activity of chitosans depends on several factors, namely the molecular weight (MW).[108,109,111] However, inconclusive data regarding the actual efficacy of high MW, low MW and oligochitosans is available. Nevertheless, LMWCs seem to exhibit a strong antibacterial activity (which was described to be higher against Gram positive bacteria as their molecular weight increases and against Gram negative bacteria as their molecular weight decreases) and, also, antifungal activity.[130,131]

As far as antimicrobial activity is concerned, MICs and MLCs were determined for all tested microbial species. Although there is a tendency in literature to characterize chitosans as bacteriostatic and fungistatic, as stated by Goy *et al.*, 2009, our results suggest unequivocally that LMWC 107 kDa exhibits a microbicidal effect (Table 5).

At subinhibitory concentrations, a significant reduction of the biofilm metabolic activity was only found for *S. epidermidis* and *C. albicans*. This might somewhat decrease the medical interest of LMWC use in the prevention of indwelling device colonization, although these two species are very relevant and frequent CVC colonizers. A lower degree of acetylation might improve LMWC antimicrobial effectiveness, but no further studies on this topic were conducted.

Hamamelitannin produced no inhibition of microbial growth: no MICs or MLCs could be established at concentrations ranging between 10 to 100 mg/L, as expected.

Regarding the experiments on polyurethane catheter segments, inhibition of the biofilm metabolic activity was found at a concentration of 100 mg/L for all tested bacteria. The inhibition of the quorum sensing system of Gram positive bacteria, such as *S. aureus* and *S. epidermidis*, had already been documented by Kiran *et al.*, 2008. Interestingly, the biofilm inhibitory effect upon Gram negative bacteria such as *A. baumannii* was originally described by the present study. *A. baumannii* is a conspicuous pathogen usually related to MDR and invasive infections among critical care patients. It has been advocated that hamamelitannin might exhibit a better effect *in vivo* than *in vitro*.[117]

At present, medical device colonization is a global threat that may contribute to antimicrobial resistance. The microbicidal effect of cerium nitrate and LMWC upon usual CVC colonizers was clearly demonstrated. Moreover, at lower concentrations, both were found to be fungistatic. Hamamelitannin inhibited the biofilm metabolic activity of all tested bacteria: for *A. baumannii*, this effect was an original description.

# Task 3

## *In vivo* efficacy of treated catheters

## Materials and methods

### Chemicals

Cerium nitrate [cerium(III) nitrate hexahydrate, Sigma-Aldrich, Schnelldorf, Germany], chitosan [low-molecular-weight chitosan 107 kDa, 75-85% deacylated, Sigma-Aldrich, St. Quentin Fallavier, France; stock solution of 4% (w/v) prepared in 1% acetic acid, pH 4.0] and hamamelitannin [hamamelofuranose 2′,5-digallate, 2-C-(Hydroxymethyl)-D-ribofuranose 2′,5-digallate, Sigma-Aldrich, St. Quentin Fallavier, France] were used in the experiments.

### Microbial strains and inoculum preparation

Four type strains from the American Type Culture Collection were used: *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 155, *Acinetobacter baumannii* ATCC 19606 and *Candida albicans* ATCC 90028.

Bacterial strains were kept frozen in Luria-Bertani broth (LB; Difco Laboratories, Detroit, MI, USA) supplemented with 20% glycerol and yeast strains in yeast potato dextrose broth (YPD; Difco Laboratories) supplemented with 40% glycerol, at -70ºC until testing. For each experiment, the microorganisms were previously subcultured twice on LB agar at 37ºC during 24 h for bacteria or Sabouraud agar (Difco Laboratories) at 35ºC during 24 h for yeasts, in order to assess the viability and purity of the culture.

For the inoculum preparation, the microbial strains were grown overnight in LB (bacteria) or Sabouraud broth (yeast) at 37ºC and 180 rpm; cells were harvested by centrifugation (10000 g, 10 min), washed with PBS, counted in a Newbauer chamber and the concentration was standardized to $1x10^7$ cells/mL for bacteria and $1x10^6$ cells/mL for yeasts, in PBS.

### Polyurethane catheter implantation procedure

A mouse subcutaneous foreign body infection model as described by Rupp *et al.*[132] was used, with slight modifications. Eight-week-old pathogen-free female BALB/c mice (Charles River) weighting 20 g were used. Animals were housed in accordance with the Federation of European Laboratory Animals of science Associations (FELASA) criteria. Animal experiments were approved by the Animal Ethical Committee of Faculty of Medicine, University of Porto.

Prior to catheter implantation, mice were anaesthetized by intraperitoneal injection (1 mg/kg) of a mixture of xylazine (20 mg/mL) and ketamine (100 mg/mL) in a ratio of 1:2 (vol/vol) and the lower back of each animal was shaved with a hair clipper and disinfected with 0.5% chlorhexidine in 70% alcohol. A 2 mm incision was made longitudinally and the subcutis was dissected. Using an aseptic technique, six 1-cm segment polyurethane intravenous catheters (BD Vialon™ 16G 1.7x45 mm, Becton Dickinson and Company, Canada) were implanted into the subcutaneous space (Figure 6. A). Before implantation, catheters were soaked for 24 h with cerium nitrate, hamamelitannin (Figure 5) or LMWC at concentrations corresponding to the respective (previously determined in task 2) minimal inhibitory concentration (MIC), ½ MIC and ¼ MIC regarding each species. Catheters incubated in plain PBS were used as positive control. Five animals were used for each experimental condition. Thereafter, 300 μL of each standardized microbial suspension or of PBS (control) were injected into the pockets. The incision was closed with a monofilament suture and disinfected with 0.5% chlorhexidine in 70% alcohol (Figure 6. B).

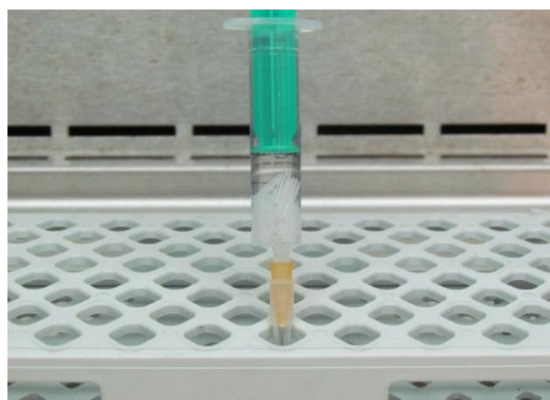

**Figure 5.** Catheter segments soaked with hamamelitannin before the implantation procedure.

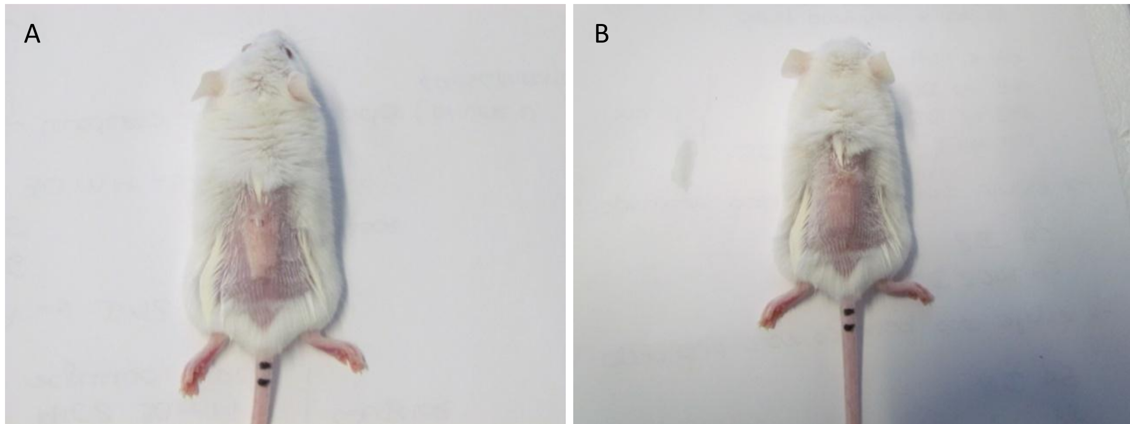


**Figure 6.** Subcutaneous pocket with catheter segments after implantation in the mice (A) and after inoculation and closure of the pocket (B).

### Biofilm formation assessment

At day 7, the animals were sacrificed, the catheters were aseptically removed (Figure 7. A and B) and biofilm was quantified spectrophotometrically by two different methodologies: XTT assay, measuring the biofilm metabolic activity at 492 nm, and crystal violet (CV) assay, measuring the biofilm total biomass at 590 nm.[133] In addition, catheter segments were submitted to scanning electron microscopy (SEM) imaging.

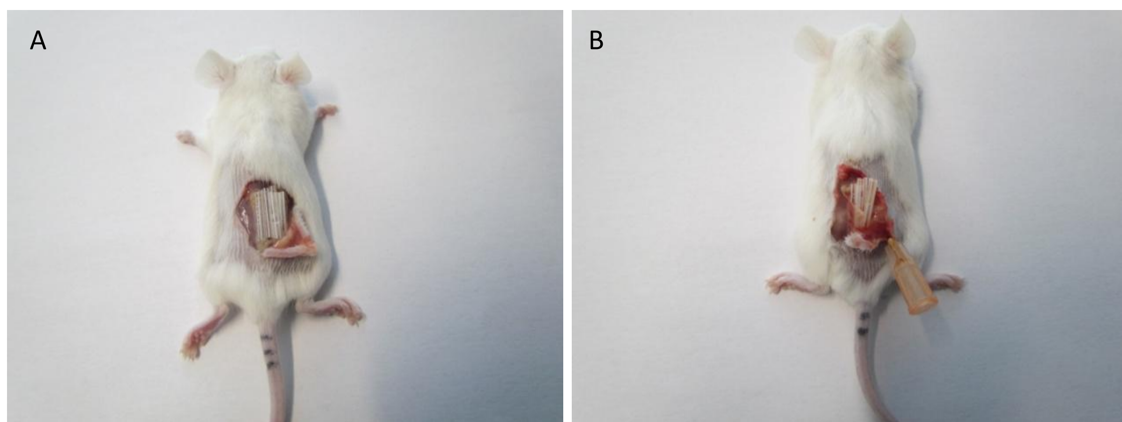


**Figure 7.** Mice at day 7 of subcutaneous pocket infection with *A. baumannii*: catheter segments treated with hamamelitannin (A) and without treatment (B).

*Scanning electron microscopy*

Catheters were washed in PBS, sectioned lengthwise and fixed overnight (4% formaldehyde and 1% glutaraldehyde v/v in PBS). The samples were rinsed in PBS and air dried in desiccators.[134] Samples were coated with gold/palladium (40%/60%) and observed in a scanning electron microscope (JEOL JSM 6301F/Oxford INCA Energy 350) in high vacuum mode at 15 kV, at CEMUP (Materials Centre of the University of Porto).

### Data analysis

Biofilm metabolic activity and total biomass formed in the presence of cerium nitrate, LMWC and hamamelitannin were expressed as the percentage in relation to the control. Values were divided by 1000 and arcsine square root transformed to achieve normal distribution and homoscedasticity. For each compound, each concentration and each microorganism, biofilm formation was compared by one-way ANOVA, followed by a Dunnett's test to identify significant effects; $P$ value $< 0.05$ was considered significant.

## Results

Cerium nitrate, at a concentration of $6.5 \times 10^2$ mg/L, reduced significantly the biofilm metabolic activity of *S. epidermidis* by 48.3% (±12.4) and its total biomass by 37.0% (±8.9) (Figure 8. A). For *C. albicans*, cerium nitrate at $1.3 \times 10^3$ mg/L inhibited the metabolic activity by 66.4% (±7.7) and the biomass by 28.0% (±4.3) (Figure 8. B).

LMWC, at 80 mg/L, decreased significantly the biofilm metabolic activity of *S. epidermidis* by 57.6% (±14.3) and its total biomass by 41.3% (±5.6) (Figure 8. C). Concerning *C. albicans*, $2.5 \times 10^3$ mg/L resulted in inhibition of the metabolic activity by 43.5% (±16.6) and of the total biomass by 23.2% (±13.7) (Figure 8. D).

Hamamelitannin reduced significantly all bacterial biofilms at subinhibitory concentrations: at 50 mg/L, *S. aureus* biofilm metabolic activity decreased by 59.3% (±19.1) and its total biomass by 48.4% (±8.0) (Figure 8. E); at the same concentration, *S. epidermidis* biofilm metabolic activity was reduced by 36.3% (±12.4) and its total

biomass by 34.6% (±6.9) (Figure 8. F); at 30 mg/L, hamamelitannin inhibited *A. baumannii* biofilm metabolic activity by 36.7% (±3.4) and its total biomass by 31.6% (±9.2) (Figure 8. G).

At other subinhibitory concentrations, no significant biofilm inhibition was found for the tested microbial strains (data not shown).

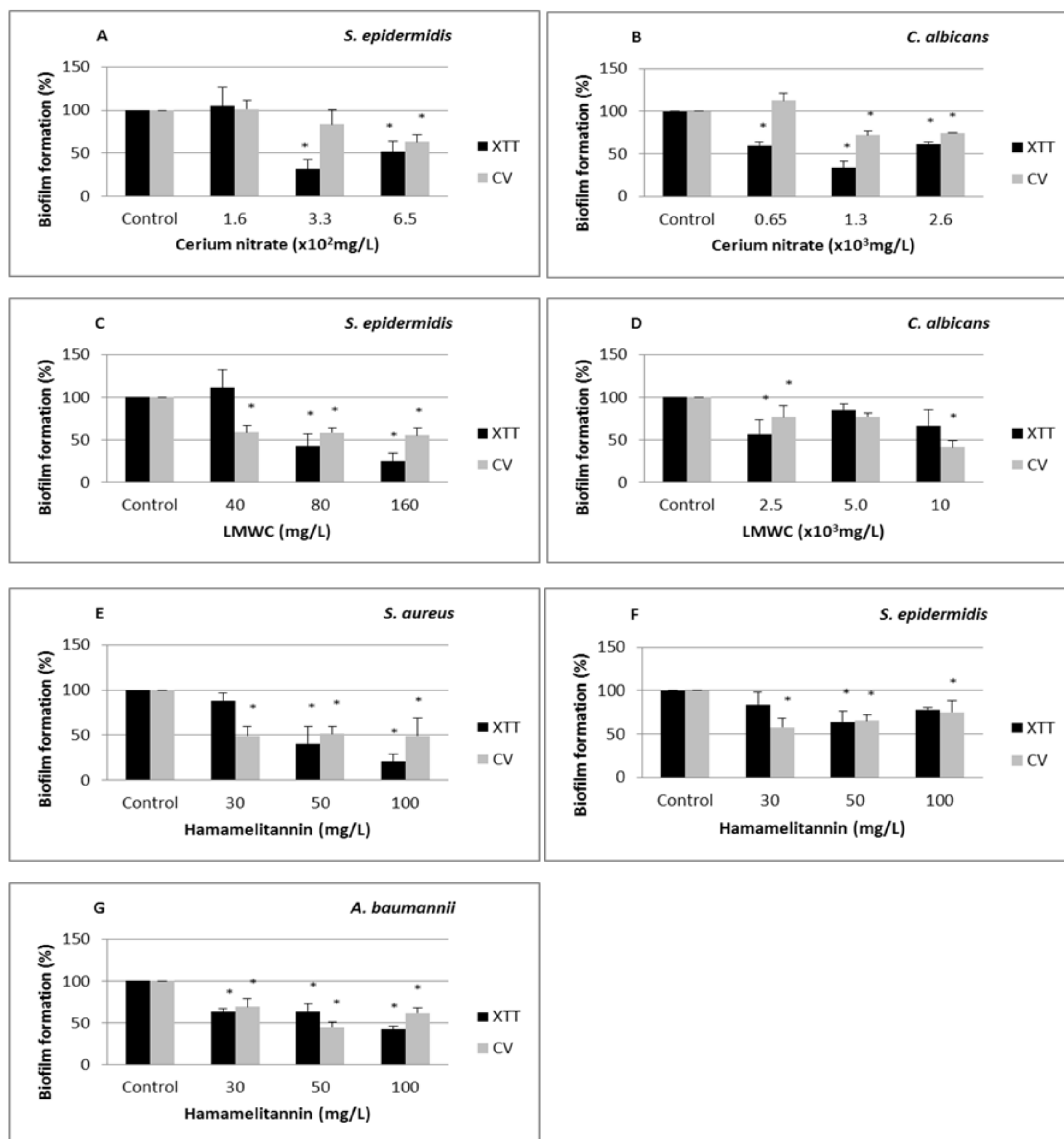


**Figure 8.** Effect of cerium nitrate (A and B), low molecular weight chitosan (LMWC) (C and D) and hamamelitannin (E, F and G) upon biofilm formation by *S. epidermidis* (A, C and F), *S. aureus* (E), *A. baumannii* (G) and *C. albicans* (B and D). XTT assay was used to determine the biofilm metabolic activity and CV assay to measure the total biomass. (*) *P* value <0.05.

Scanning electron microscopic examination was used to determine the architectural differences between biofilms. Untreated biofilms (Figure 9. A, B, I, J, M, N, Q and R) comprised a more dense network of microbial cells and exopolymeric matrix than treated biofilms (Figure 9. C to H, K, L, O, P and S to V).

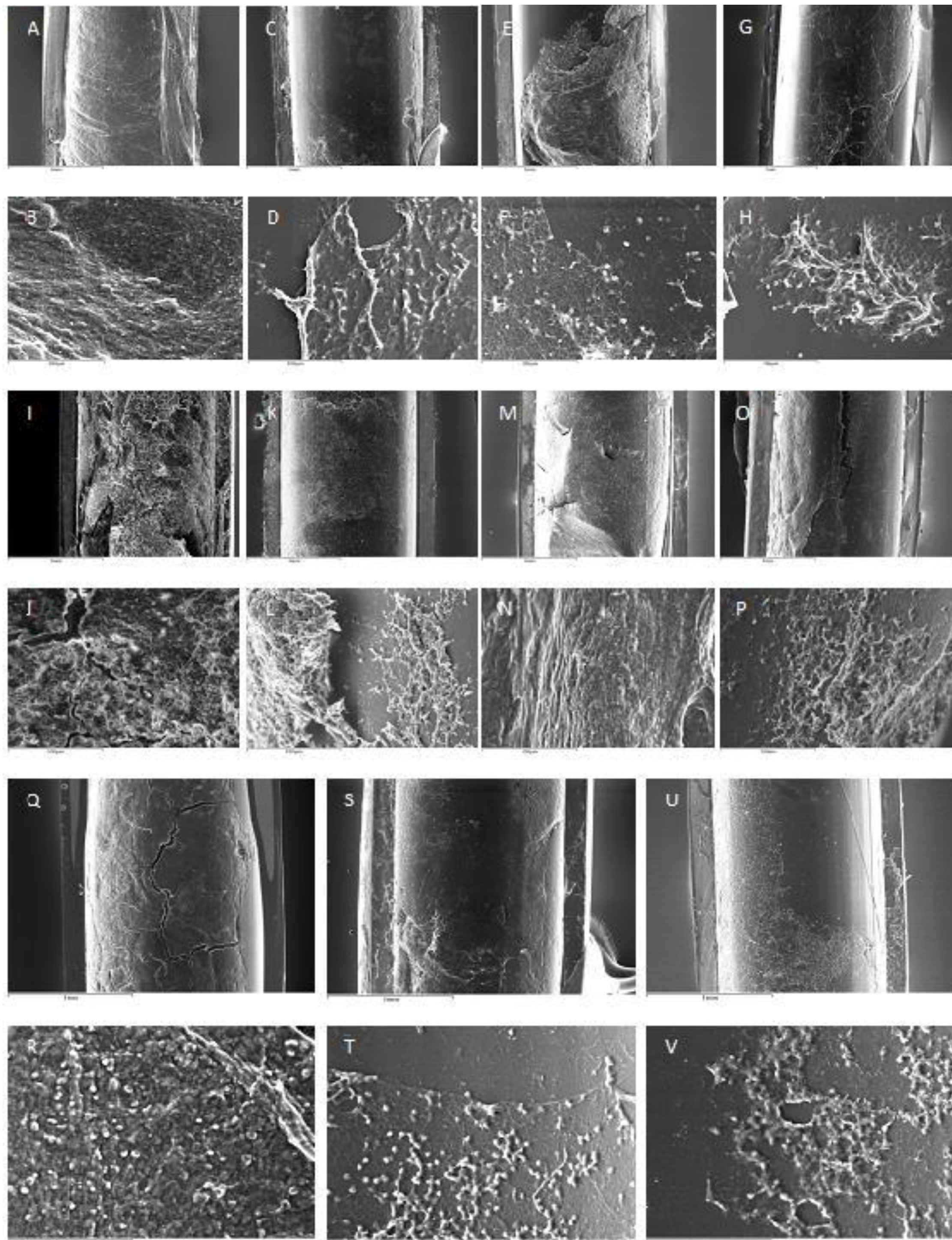


**Figure 9.** Scanning electron microscopic (SEM) examination of untreated biofilms of *S. epidermidis* (A and B), *S. aureus* (I and J), *A. baumannii* (M and N) and *C. albicans* (Q and R). SEM examination of *S. epidermidis* biofilms treated with cerium nitrate at $6.5x10^2$ mg/L (C and D), LMWC at 80 mg/L (E and F)

and hamamelitannin at 50 mg/L (G and H); *S. aureus* biofilms treated with hamamelitannin at 50 mg/L (K and L); *A. baumannii* biofilms treated with hamamelitannin at 30 mg/L (O and P); *C. albicans* biofilms treated with cerium nitrate at $1.3x10^3$ mg/L (S and T) and LMWC at $2.5x10^3$ mg/L (U and V).

## Discussion

In the long quest aiming to prevent medical device-related infections in a more effective way, many different strategies have been proposed. Until now, such objective remains to be fulfilled. Cerium nitrate, LMWC and hamamelitannin are biocompatible and relatively inexpensive compounds that could be used to coat CVCs and other medical indwelling devices. Differences in their mechanisms of action might explain the pattern of antimicrobial activity: whereas cerium nitrate and LMWC may disrupt the cell membrane,[105,111] hamamelitannin seems to inhibit bacterial cell communication.[117] The microbicidal effect of cerium nitrate and LMWC upon usual CVC colonizers was previously shown *in vitro*, as was the antibiofilm activity of hamamelitannin on polyurethane catheter segments. However, *in vivo* experiments with subinhibitory concentrations of those compounds remained to be made, since results obtained under *in vitro* conditions do not account for the intricate interaction between the host cells, immune system and biofilm community.

Garner *et al.*, studying the action of cerium nitrate on the treatment of severe burns, reported about its limited antimicrobial properties.[105] However, minimal lethal concentrations (MLCs) and MICs have been published more recently both for bacteria and yeasts.[135] *In vivo*, at subinhibitory concentrations, cerium nitrate exhibited an antibiofilm effect only against *C. albicans*. Such selective finding should not preclude its biomedical use since it is used routinely in medical burn wound care as an effective antiseptic, at much higher concentrations and with no known toxicity. In addition, *Candida* organisms are important pathogens regarding CRBSIs.

The microbicidal activity of LMWC upon usual CVC colonizers has already been documented *in vitro*. More recently, using an *in vivo* CVC model, Martinez *et al.* demonstrated the efficacy of chitosan against *Candida* species biofilms.[136] Our study not only supports these findings concerning *C. albicans*, at subinhibitory concentrations of chitosan, but further documents the antibiofilm efficacy against *S. epidermidis*, a

much more common CVC colonizer.[69] Given the known biocompatibility of chitosan, promising results could be achieved in the prevention of CRBSIs by a wider range of microbial colonizers if higher concentrations were used for treating the surface of CVCs.

At subinhibitory concentrations, the highest antibiofilm efficacy was obtained with hamamelitannin: a reduction in the metabolic activity and total biomass was documented for *S. aureus*, *S. epidermidis* and *A. baumannii*. This is the first report of *in vivo* biofilm inhibition for *A. baumannii*, confirming our previous *in vitro* description. Moreover, the antibiofilm efficacy was achieved at lower concentrations than expected considering our *in vitro* results regarding all tested bacteria, a fact that may be attributable to differences in environmental conditions. Concerning staphylococci, Kiran *et al.* attributed such discrepancy to the specific *in vivo* quorum-sensing inhibitor effect of hamamelitannin.[117] For pre-soaked grafts, the same authors reported no signs of staphylococcal infection *in vivo* with hamamelitannin concentrations of 30 mg/L, but the present study found a statistically significant reduction of biofilm formation by no more than ~50%, even at 100 mg/L. Nonetheless, hamamelitannin may be an excellent compound to coat medical devices given its bacterial range of biofilm inhibition.

# Conclusions

**Aetiology of CVC colonization (paper I)**

- The aetiology of CVC isolates recovered from national ICU patients was similar to European data except for *C. glabrata*, which ranked second among yeasts.
- Antimicrobial resistance was higher than expected, particularly regarding *S. epidermidis*, *S. aureus*, *E. faecium*, *P. aeruginosa* and *A. baumannii*. Conversely, Enterobacteriaceae were highly susceptible to cephalosporins. No antifungal resistance was found among the tested yeast strains.

- In order to ensure the efficacy of new therapeutic protocols considering the local patterns of antimicrobial susceptibility, continued surveillance of antimicrobial resistance is of paramount importance.

- The promotion of the essential elements of CVC care in this hospital is expected to result in a reduction of CRBSIs.

***In vitro* microbial biofilm formation on chemically treated catheters (papers II, IV-VIII)**

- Despite the controversy that could be found in previous published studies, cerium nitrate and LMWC displayed an effective microbicidal effect upon *S. epidermidis*, *S. aureus*, *A. baumannii* and *C. albicans*.
- In the case of *C. albicans*, such effect of cerium nitrate is related to cell membrane damage.

- At subinhibitory concentrations:

    - Catheters treated with cerium nitrate were effective against *C. albicans* biofilm formation;

    - Catheters treated with LMWC inhibited *S. epidermidis* and *C. albicans* biofilm formation;

    - Catheters treated with hamamelitannin inhibited biofilm formation by all the tested bacteria.

### *In vivo* efficacy of chemically treated catheters (paper III)

- The *in vivo* antibiofilm efficacy of catheters treated with cerium nitrate, LMWC and hamamelitannin was found to be similar to that documented *in vitro*, notably regarding *A. baumannii*.
- The mouse foreign body infection model with subcutaneous implantation of catheter segments was documented to be a useful model for the study of indwelling catheter infection, both by quantitative and qualitative methods such as electron microscopy imaging studies.

# Future perspectives

A more durable coating strategy for cerium nitrate, chitosan and hamamelitannin and its further *in vivo* testing using the CVC animal model is the next step in the path to develop a more cost-effective and biocompatible indwelling catheter, that could reduce CRBSIs more effectively than the present available options.

In addition, studies are being conducted to clarify the mechanism of biofilm inhibition and the endovascular biocompatibility of these inhibitors, which may play promising roles in future biomedical applications.

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# Summary

Patients admitted at Intensive Care Units (ICUs) often require central venous catheters (CVCs) for multiple purposes. However, infectious complications may follow, ranging from simple catheter colonization to sepsis. Worldwide, the incidence rate of catheter-related bloodstream infections (CRBSIs) is high and its socio-economic impact is considerable. *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Acinetobacter baumannii* and *Candida albicans* are among microbial agents most frequently isolated from CRBSIs. Whichever might be the route of contamination, these organisms usually form biofilm on CVCs that help to promote microbial resistance. Several strategies have been proposed to control biofilm formation, but the success in reducing the infection rate was achieved with variable success and toxicity. Concerning other alternatives, cerium nitrate, chitosan and hamamelitannin are biocompatible and inexpensive compounds with known antimicrobial activity (although not fully elucidated) that could help to reduce CRBSIs.

The three main objectives of this investigation involved: (1) the determination of the aetiology of CVC colonization among intensive care patients at a national university hospital; (2) the study of *in vitro* biofilm formation by organisms commonly associated to CVC colonization and CRBSI, at the surface of cerium nitrate, low molecular weight chitosan (LMWC, 107 kDa) and hamamelitannin treated catheters; and (3) the evaluation of the *in vivo* efficacy of such coated catheters in the prevention of biofilm formation.

Regarding the first objective, a retrospective study of a laboratorial database was performed. Positive results from the culture of CVCs removed from ICU patients and the antimicrobial susceptibility pattern of isolates were reviewed. As expected, the aetiology of CVC colonization was similar to international data: *S. epidermidis* was the most frequent isolate, followed by *S. aureus*, Enterobacteriaceae, *Candida* spp, *Pseudomonas aeruginosa*, enterococci and *A. baumannii*. However, higher antimicrobial resistance rates of *S. aureus* to methicillin, of *E. faecium* to vancomycin and of *P. aeruginosa* and *A. baumannii* to carbapenems were found. Conversely, Enterobacteriaceae displayed high susceptibility to cephalosporins, while no resistance to azole antifungals was detected among *Candida* isolates. Routine surveillance for antimicrobial resistance and the prospective evaluation of new therapeutic protocols based on the local patterns of susceptibility might help to pursue a more strict selection of antimicrobials in this hospital.

Concerning the second objective, the antibiofilm effect of cerium nitrate,

LMWC and hamamelitannin was tested with *S. epidermidis*, *S. aureus*, *A. baumannii* and *C. albicans* strains. *In vitro* biofilm formation was assessed on polyurethane catheter segments and the metabolic activity was quantified by colorimetry. Cerium nitrate and LMWC displayed an effective microbicidal effect upon all the tested strains. Moreover, at subinhibitory concentrations, catheters treated with cerium nitrate were effective against *C. albicans* biofilm formation; catheters treated with LMWC inhibited *S. epidermidis* and *C. albicans* biofilm formation; and catheters treated with hamamelitannin inhibited the biofilm formation by all the tested bacteria. The *in vitro* activity against *A. baumannii* biofilm was an original description.

Since the results obtained were so promising, the pursuit of the third objective was addressed using all the compounds. The *in vivo* antibiofilm effect of cerium nitrate, LMWC and hamamelitannin was tested at subinhibitory concentrations, using a mouse foreign body infection model with catheter segments. Biofilm formation was assessed both with a crystal violet assay, a tetrazolium reduction assay and with scanning electron microscopy imaging. Results were found to be similar to those previously documented *in vitro*.

In summary, it was demonstrated that cerium nitrate, LMWC and hamamelitannin could be effective alternatives for the development of a more cost-effective and biocompatible indwelling catheter, reducing CRBSIs more effectively than the present available options.

# Resumo

Os doentes admitidos em Unidades de Cuidados Intensivos (UCI) são frequentemente submetidos a cateterização venosa central (CVC). Consequentemente, podem ocorrer complicações infecciosas, que variam de simples colonização do cateter a sépsis. Globalmente, a incidência de sépsis relacionada com cateter é elevada e o seu impacto sócio-económico é considerável. *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Acinetobacter baumannii* e *Candida albicans* são muitas vezes isolados nessas situações. Qualquer que seja a via de contaminação, estes agentes infecciosos tendem a formar um biofilme nos cateteres venosos centrais, o qual promove a resistência aos antimicrobianos. Várias estratégias foram propostas para controlar a formação de biofilme, apesar da redução da taxa de infecção ter sido conseguida com sucesso e toxicidade variáveis. Relativamente a outras alternativas, o nitrato de cério, os quitosanos e o hamamelitanino são compostos biocompatíveis, pouco dispendiosos, com actividade antimicrobiana conhecida (embora mal esclarecida) que poderiam ser úteis na redução da sépsis relacionada com CVC.

Os três objectivos principais deste estudo envolveram: (1) a determinação da etiologia da colonização de CVCs em doentes de UCIs de um hospital universitário nacional; (2) a investigação *in vitro* da adesão de microorganismos formadores de biofilme a cateteres tratados com nitrato de cério, quitosano de baixo peso molecular (QBPM, 107 kDa) e hamamelitanino; e (3) a avaliação *in vivo* da eficácia desses cateteres na prevenção da formação de biofilme.

Relativamente ao primeiro objectivo, procedeu-se a um estudo retrospectivo a partir de uma base de dados laboratorial. Os resultados positivos do exame cultural dos CVCs removidos dos doentes internados em UCIs e o padrão de susceptibilidade antimicrobiana das estirpes isoladas foram revistos. Conforme esperado, a etiologia da colonização dos CVCs foi semelhante à descrita internacionalmente: *S. epidermidis* foi o isolado mais frequente, seguindo-se *S. aureus*, Enterobacteriaceae, *Candida* spp, *Pseudomonas aeruginosa*, enterococos e *A. baumannii*. Todavia, encontraram-se taxas de resistência mais elevadas do *S. aureus* à meticilina, do *E. faecium* à vancomicina e da *P. aeruginosa* e *A. baumannii* a carbapenemas. Pelo contrário, observou-se uma elevada susceptibilidade de Enterobacteriaceae a cefalosporinas e nenhuma resistência de *Candida* a azoles. A vigilância rotineira da resistência antimicrobiana e a avaliação prospectiva de novos protocolos terapêuticos baseados nos padrões locais de susceptibilidade poderiam ser úteis para uma utilização mais rigorosa dos antimicrobianos neste hospital.

No que diz respeito ao segundo objectivo, o efeito antibiofilme do nitrato de cério, QBPM e hamamelitanino foi avaliado em estirpes de *S. epidermidis*, *S. aureus*, *A. baumannii* e *C. albicans*. A formação de biofilme foi avaliada em segmentos de cateteres de poliuretano e a actividade metabólica foi quantificada por colorimetria. O nitrato de cério e o QBPM demonstraram um efeito microbicida sobre todas as estirpes testadas. Adicionalmente, em concentrações subinibitórias, os cateteres tratados com nitrato de cério demonstraram eficácia sobre a formação de biofilme por *C. albicans*; os cateteres tratados com QBPM inibiram o biofilme de *S. epidermidis* e *C. albicans*; e os cateteres tratados com hamamelitanino inibiram a formação de biofilme por todas as bactérias testadas, tendo, pela primeira vez, sido demonstrada a eficácia do hamamelitanino contra a formação de biofilme por *A. baumannii*.

Finalmente, atendendo aos resultados promissores obtidos até então, foram utilizados segmentos de cateteres tratados com os mesmos compostos *in vivo*. O efeito antibiofilme do nitrato de cério, QBPM e hamamelitanino foi avaliado em concentrações subinibitórias num modelo animal de infecção de corpo estranho. A formação de biofilme foi avaliada por dois ensaios distintos de colorimetria e por microscopia electrónica de varrimento. Os resultados foram sobreponíveis aos observados *in vitro*.

Em resumo, ficou demonstrado que o nitrato de cério, o QBPM e o hamamelitanino podem representar excelentes alternativas para o desenvolvimento de CVCs biocompatíveis e com maior eficácia na redução da taxa de infecção relacionada com a sua utilização, comparativamente às opções disponíveis no mercado.

# Papers

# Paper I

## Colonization of central venous catheters in intensive care patients:

## A 1-year survey in a Portuguese university hospital

The authors thank Dr. Carolyn Gould, Dr. Arjun Srinivasan, and Mary Andrus for their invaluable contributions to the revision of the NHSN UTI criteria.
Disclaimer: The views expressed in this letter are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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## Colonization of central venous catheters in intensive care patients: A 1-year survey in a Portuguese university hospital

*To the Editor:*

Patients admitted at intensive care units (ICUs) often require central venous catheterization. Catheter colonization may lead to bacteremia and, eventually, end up as a catheter-related bloodstream infection.[1] To review the etiology and antimicrobial susceptibility pattern of central venous catheter (CVC) isolates from ICU patients, a retrospective study of the clinical database of Hospital of S. João, in Porto, was performed during a 12-month period. All positive results from the culture of distal tips of CVCs removed from ICU patients and antimicrobial susceptibility pattern of isolates were reviewed.

From 1482 cultures of CVC distal tips performed during the study period, 647 positive results (43.7%) were found. As expected,[2] coagulase-negative staphylococci (54.7%), mainly *Staphylococcus epidermidis* (40.2%), were the most common isolates; *S aureus* (12.8%), Enterobacteriaceae (8.6%), *Candida* spp (7.0%), *Pseudomonas aeruginosa* (6.2%), enterococci (4.8%) and *Acinetobacter baumannii* (2.8%) followed in the ranking. Of major concern was the resistance rate of *S aureus* to methicillin (80%), of *Enterococcus faecium* to vancomycin (85.7%), of *P aeruginosa* to meropenem (34.8%) and imipenem (65.2%) and of *A baumannii* to both carbapenems (94.4%).[3-7] Interestingly, no resistance to azole antifungals (fluconazole, itraconazole, voriconazole, posaconazole) was detected among the low number of tested *Candida* isolates (n = 35), in accordance with previous findings.[8] This fact might result from the limited exposure to antifungal agents in this hospital because no antifungal prophylactic treatment is routinely prescribed to such patients.

Globally, the etiology of CVC colonization in ICU patients was similar to international available data. However, higher levels of antimicrobial resistance were found for *S aureus*, *E faecium*, *P aeruginosa* and *A baumannii* isolates. Routine surveillance for resistance patterns and the prospective evaluation of new therapeutic protocols might be highly advisable in such setting.

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# Paper II

## Cerium, chitosan and hamamelitannin as novel biofilm inhibitors?

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Journal of Antimicrobial Chemotherapy

# Cerium, chitosan and hamamelitannin as novel biofilm inhibitors?

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**Objectives:** The colonization of indwelling medical devices and subsequent biofilm formation represents a global challenge since it promotes the persistence of infection and contributes to antimicrobial resistance. The aim of this study was to determine the antimicrobial activity of cerium, chitosan and hamamelitannin against usual microbial colonizers and to assess their efficacy regarding biofilm formation on polyurethane (PUR)-like catheters.

**Methods:** The antimicrobial and anti-biofilm effect of cerium nitrate, low molecular weight chitosan (LMWC) and hamamelitannin was tested against *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Acinetobacter baumannii* and *Candida albicans* strains. Biofilm formation was assessed with PUR-like catheter segments and the metabolic activity was quantified by colorimetry with a tetrazolium reduction assay.

**Results:** Cerium nitrate and LMWC inhibited the microbial growth of all microbial strains tested; hamamelitannin showed no inhibition. Regarding biofilm formation on PUR-like catheters, with subinhibitory concentrations: cerium nitrate significantly inhibited the metabolic activity of *C. albicans*; LMWC reduced the metabolic activity of *S. epidermidis* and *C. albicans*; and hamamelitannin decreased the metabolic activity of all tested bacteria, but not of yeasts.

**Conclusions:** The microbicidal activity of cerium nitrate and LMWC was clearly demonstrated in this study, as was their fungistatic effect at lower concentrations. Hamamelitannin significantly reduced biofilm metabolic activity of all tested bacteria. These microbial inhibitors may play a promising role regarding different biomedical applications.

**Keywords:** catheter-related bloodstream infections, central venous catheters, *S. epidermidis*, *S. aureus*, *A. baumannii*, *C. albicans*, cerium nitrate, low molecular weight chitosan, hamamelitannin

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## Introduction

The colonization of medical indwelling devices through microbial adhesion and subsequent biofilm formation may precede bacteraemia and sepsis in critically ill patients. Catheter-related bloodstream infections (CRBSIs) have been shown to result in longer hospital stays, increased costs and mortality.[1] *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Acinetobacter baumannii* and *Candida albicans* are among the most frequently isolated microorganisms from central venous catheters (CVCs).[2] Preventive methods have been suggested to reduce the incidence of CRBSIs, such as the coating of catheters with antibiotics or antiseptics.[3] Nevertheless, more effective and less toxic alternatives would be highly desirable.

Cerium is a rare earth element belonging to the lanthanide group. Cerium nitrate has been in use for a long time in the management of burns. Its direct antimicrobial properties remain controversial,[4] but the uptake of cerium into the cytoplasm with inhibition of cellular respiration, inhibition of glucose metabolism and disruption of the cell membrane have been the postulated mechanisms.

Chitosans are polyaminosaccharides obtained by deacetylation of naturally occurring chitin, with biocompatibility. Chitosans are being investigated for their broad spectrum of antimicrobial activity, which has been explained by (i) cell wall leakage by ionic surface interaction or by teichoic acid binding and extraction of membrane lipids, (ii) mRNA and protein synthesis

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inhibition and (iii) suppression of microbial growth through external barrier formation and metal chelation.[5]

Hamamelitannin is a naturally occurring polyphenol extracted from the bark of *Hamamelis virginiana* that belongs to the family of tannins. It is the ester of D-hamamelose (2-hydroxymethyl-D-ribose) with two molecules of gallic acid. Hamamelitannin seems to prevent graft-associated infections caused by the staphylococci tested so far (including methicillin-resistant *S. aureus* and *S. epidermidis*) by inhibiting the quorum sensing system of such bacteria, thereby reducing their virulence.[6]

The aim of this study was to determine the antimicrobial activity of cerium nitrate, low molecular weight chitosan (LMWC) and hamamelitannin against microbial strains usually involved in CRBSIs and to test their efficacy regarding biofilm formation on polyurethane (PUR)-like catheters.

## Materials and methods

### Chemicals

Cerium nitrate [cerium(III) nitrate hexahydrate; Sigma-Aldrich], LMWC (107 kDa, 75%–85% deacylated; Sigma-Aldrich; stock solution of $4.0\times10^4$ mg/L prepared in 1% acetic acid, pH 4.0) and hamamelitannin [hamamelofuranose 2′,5-digallate, 2-C-(hydroxymethyl)-D-ribofuranose 2′,5-digallate; Sigma-Aldrich] were used in the experiments.

### Microbial strains

A type strain and a clinical isolate of each of four microbial species were used. The type strains from the ATCC were *S. aureus* ATCC 29213, *S. epidermidis* ATCC 155, *A. baumannii* ATCC 19606 and *C. albicans* ATCC 90028. The clinical strains of *S. aureus* (SA1), *S. epidermidis* (SE1), *A. baumannii* (AB1) and *C. albicans* (CA1) had been previously isolated from cultures of CVCs removed from critical care patients. All clinical isolates had been identified by the Vitek system (bioMérieux, Vercieux, France).

### Antimicrobial activity

The MICs of chemicals were determined according to the CLSI microdilution reference protocol M07-A8[7] for bacteria and protocol M27-A3[8] for yeasts. The tested concentrations ranged from $5.4\times10^3$ to $3.3\times10^2$ mg/L for cerium nitrate, $1.0\times10^4$ to $1.6\times10^2$ mg/L for LMWC and 100 to 10 mg/L for hamamelitannin. The MIC endpoint was defined as the lowest drug concentration that completely inhibited the growth of microorganisms in microdilution wells.

To determine the minimal lethal concentration (MLC), 20 μL of each microdilution well was plated in Luria-Bertani (LB) agar (for bacteria) or Sabouraud agar (for yeasts). The plates were incubated at 37°C for 24 h (bacterial strains) or 35°C for 48 h (yeast strains) with subsequent enumeration of cfu. The MLC was defined as the lowest drug concentration that killed at least 99.9% of the final inoculum.

### Biofilm formation on PUR-like catheters

The strains were grown overnight and cells were harvested by centrifugation, washed with PBS and standardized to $1\times10^7$ cells/mL in LB broth for bacteria and $1\times10^6$ cells/mL in RPMI (Sigma-Aldrich) for yeasts. The anti-biofilm effect of the different test compounds was evaluated in the presence of four concentrations of cerium nitrate ($1.7\times10^3$, $1.3\times10^3$, $6.5\times10^2$ and $3.3\times10^2$ mg/L) and three of hamamelitannin (100, 80 and 50 mg/L). Regarding LMWC, concentrations tested were 625, 160 and 78 mg/L for bacteria and $1.0\times10^4$, $5.0\times10^3$ and 625 mg/L for yeasts. One single fragment of PUR-like intravenous catheters (BD Vialon™ 16G, 1.7×45 mm), with 1 cm length, was placed in each well of 12-well microplates, containing 1 mL of the standardized microbial suspensions.

After incubation (24 h for bacteria and 24 and 48 h for yeasts), at 37°C, catheter fragments were removed, gently washed with PBS and placed in new microplates to assess the biofilm metabolic activity with XTT, as previously described.[9] All the assays were performed in triplicate.

### Data analysis

Biofilm metabolic activity in the presence of cerium nitrate, LMWC and hamamelitannin was expressed as the percentage in relation to the control. Values were divided by 1000 and arcsine square root transformed to achieve a normal distribution and homoscedasticity. For each compound, each concentration and each microorganism, biofilm formation was compared by one-way ANOVA, followed by a Dunnett's test to identify significant effects; a $P$ value $<0.05$ was considered significant.

## Results

### Antimicrobial activity

Cerium nitrate MICs ranged between $3.3\times10^2$ and $1.3\times10^3$ mg/L for bacterial strains. For yeasts, concentrations up to $2.6\times10^3$ mg/L were needed. Globally, MLCs corresponded to ≥2× the MICs for all microbial species (Table 1).

For LMWC, MICs ranged between $1.6\times10^2$ and $3.1\times10^2$ mg/L for all bacteria. A higher concentration ($1.0\times10^4$ mg/L) was needed to inhibit *C. albicans* growth. The MLCs were nearly ≥2× the MICs for all bacterial species. The MLC and MIC were the same for *C. albicans* (Table 1).

Hamamelitannin resulted in no inhibition of microbial growth, at concentrations ranging from 10 to 100 mg/L.

### Biofilm formation on PUR-like catheters

Cerium nitrate, at a concentration of $1.7\times10^3$ mg/L, reduced the mean biofilm metabolic activity of *C. albicans* (24 h) by 58.9% (±7.4%) and of *C. albicans* (48 h) by 59.14% (±13.0%). No

**Table 1.** MICs and MLCs of cerium nitrate and LMWC

| | MIC | | MLC | |
|---|---|---|---|---|
| | cerium nitrate (mg/L) | LMWC (mg/L) | cerium nitrate (mg/L) | LMWC (mg/L) |
| Bacterial strains | | | | |
| *S. aureus* (SA1) | $3.3\times10^2$ | $1.6\times10^2$ | $5.4\times10^3$ | $3.1\times10^2$ |
| *S. aureus* ATCC | $3.3\times10^2$ | $1.6\times10^2$ | $2.6\times10^3$ | $1.25\times10^3$ |
| *S. epidermidis* (SE1) | $1.3\times10^3$ | $1.6\times10^2$ | $2.6\times10^3$ | $3.1\times10^2$ |
| *S. epidermidis* ATCC | $6.5\times10^2$ | $1.6\times10^2$ | $2.6\times10^3$ | $6.3\times10^2$ |
| *A. baumannii* (AB1) | $1.3\times10^3$ | $1.6\times10^2$ | $5.4\times10^3$ | $2.5\times10^3$ |
| *A. baumannii* ATCC | $1.3\times10^3$ | $3.1\times10^2$ | $5.4\times10^3$ | $1.25\times10^3$ |
| Yeast strains | | | | |
| *C. albicans* (CA1) | $2.6\times10^3$ | $1.0\times10^4$ | $5.4\times10^3$ | $1.0\times10^4$ |
| *C. albicans* ATCC | $2.6\times10^3$ | $1.0\times10^4$ | $5.4\times10^3$ | $1.0\times10^4$ |



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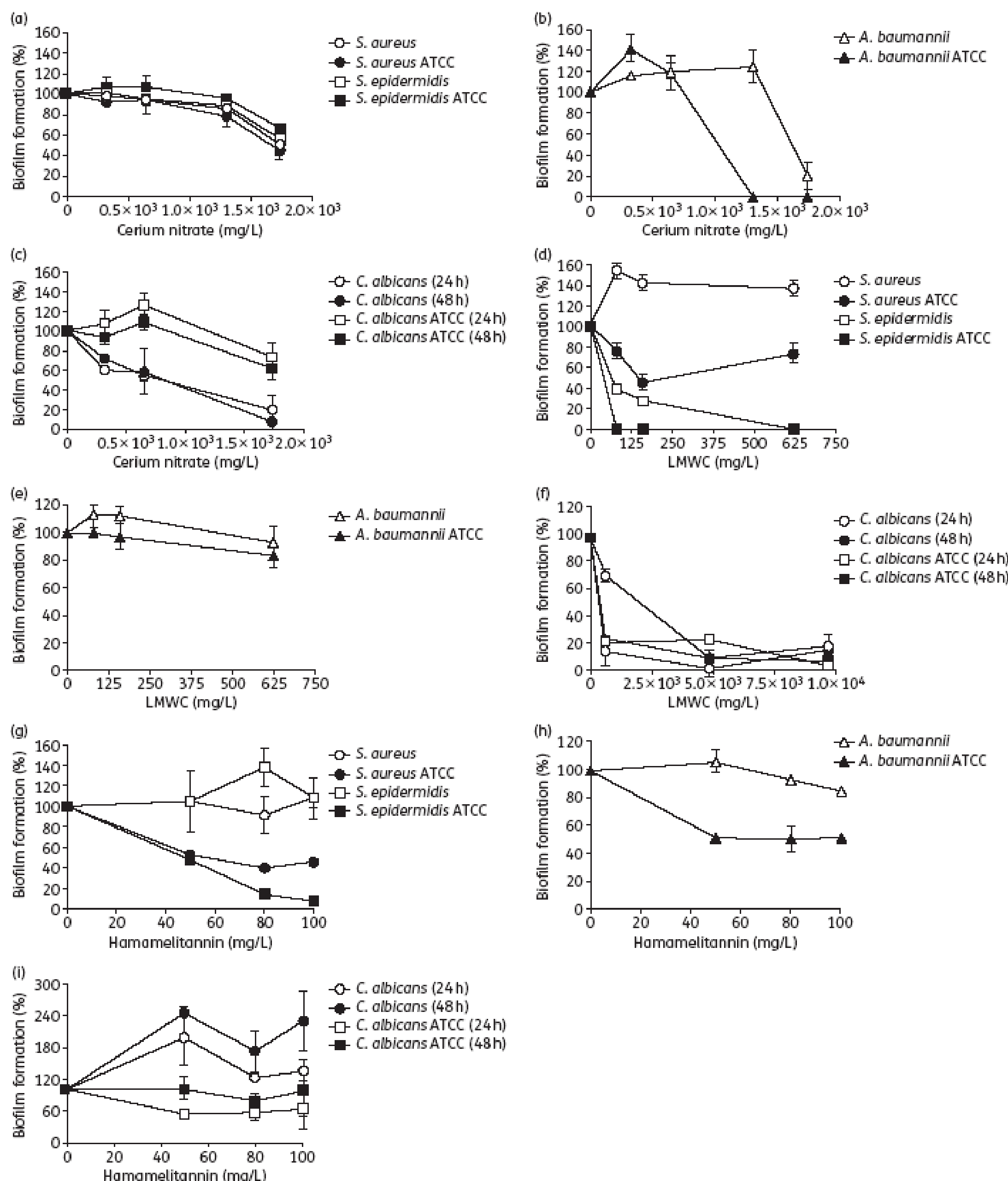


**Figure 1.** Effect of cerium nitrate (a, b and c), LMWC (d, e and f) and hamamelitannin (g, h and i) on biofilm formation (as a percentage of the control) by Gram-positive cocci, Gram-negative bacteria and yeasts (at 24 and 48 h). Differences between control and antimicrobial groups were statistically significant ($P<0.01$).



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relevant biofilm inhibition was found for bacterial strains with cerium nitrate at concentrations lower than the MLC (Figure 1a, b and c).

LMWC, at a concentration of 78 mg/L, reduced the biofilm metabolic activity of *S. epidermidis* by 80.46% (±0.0%) (Figure 1d). At a concentration of $5.0 \times 10^3$ mg/L, LMWC reduced *C. albicans* biofilm metabolic activity at 24 h by 87.5% (±0.0%) and at 48 h by 90.06% (±0.0%) (Figure 1f). However, inconsistent results were found with *A. baumannii* and *S. aureus* (Figure 1d and e).

Hamamelitannin, at a concentration of 100 mg/L, decreased significantly the mean biofilm metabolic activity of *S. aureus* by 23.0% (±10.7%), of *S. epidermidis* by 42.2% (±3.78%) and of *A. baumannii* by 31.8% (±0.88%) (Figure 1g and h). Inconclusive results were found with *C. albicans* strains at 24 and 48 h (Figure 1i).

## Discussion

A review conducted by Garner and Heppell.[4] gathered information on the bacteriostatic effect of cerium nitrate against a panel of bacteria. Our study not only adds to this effect, but also documents unequivocally the microbicidal effect of cerium nitrate against bacteria and yeasts. This might be quite useful in medical applications because toxicity is rare with lanthanides.

Concerning cerium nitrate microbial growth inhibition, our results indicate MICs for *S. aureus* similar to previous descriptions,[4] while determining for the first time MICs for *S. epidermidis*, *A. baumannii* and *C. albicans*. Furthermore, the so far unclear cerium nitrate microbicidal effect was documented and MLCs were established. At lower concentrations, cerium nitrate was only effective against *C. albicans* biofilm.

The broad spectrum of antimicrobial activity of chitosans depends on several factors, including the molecular weight.[10] However, inconclusive data regarding the actual efficacy of LMWC is available.

Although there is a tendency in the literature to characterize chitosans as bacteriostatic and fungistatic, as stated by Goy *et al.*[5] in 2009, our results suggest unequivocally that LMWC 107 kDa exhibits a microbicidal effect (Table 1). At subinhibitory concentrations, a significant reduction of the biofilm metabolic activity was only found for *S. epidermidis* and *C. albicans*.

Hamamelitannin produced no inhibition of microbial growth, as expected. However, regarding the experiments on PUR-like catheter segments, inhibition of the biofilm metabolic activity was found at a concentration of 100 mg/L for all tested bacteria. The inhibition of the quorum sensing system of Gram-positive bacteria, such as *S. aureus* and *S. epidermidis*, has already been documented by Kiran *et al.*[6] in 2008. The biofilm inhibitory effect upon a Gram-negative bacterium was originally described by our study. *A. baumannii* is a conspicuous pathogen usually related to multiresistance to antimicrobials in intensive care patients.

Nowadays, medical device colonization constitutes a global threat that may contribute to antimicrobial resistance. The microbicidal effect of cerium nitrate and LMWC upon usual CVC colonizers was clearly demonstrated. Moreover, at lower concentrations, both were found to be fungistatic. Hamamelitannin inhibited the biofilm metabolic activity of all tested bacteria: for *A. baumannii*, this effect was an original description. Further studies are being conducted in order to clarify the mechanism of biofilm inhibition and the endovascular biocompatibility of these inhibitors, which may play a promising role in future biomedical applications.

## Funding

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## Transparency declarations

None to declare.

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# Paper III

## *In vivo* antibiofilm effect of cerium, chitosan and hamamelitannin against usual agents of catheter-related bloodstream infections

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# *In vivo* antibiofilm effect of cerium, chitosan and hamamelitannin against usual agents of catheter-related bloodstream infections

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**Objectives:** Catheter-related bloodstream infections (CRBSIs) are common healthcare-associated infections associated with increased morbidity and medical costs. Antiseptic- and antibiotic-coated central venous catheters (CVCs) have been proposed to reduce the incidence of CRBSIs, with variable success. The aim of this study was to determine the *in vivo* antibiofilm activity of biocompatible and inexpensive compounds, such as cerium nitrate, chitosan and hamamelitannin, against usual agents of CRBSIs.

**Methods:** The antibiofilm effect of cerium nitrate, chitosan and hamamelitannin was tested against *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Acinetobacter baumannii* and *Candida albicans* in a mouse foreign body infection model, using polyurethane catheter segments. Biofilm formation was assessed with a crystal violet assay to quantify the total biomass, with a tetrazolium reduction assay to quantify the metabolic activity and with scanning electron microscopy.

**Results:** At subinhibitory concentrations, cerium nitrate significantly reduced biofilm formation by *C. albicans*, chitosan significantly decreased biofilm formation by *S. epidermidis* and *C. albicans*, and hamamelitannin significantly inhibited all bacterial biofilms.

**Discussion:** The *in vivo* antibiofilm effect of cerium nitrate against *C. albicans* and of chitosan against *C. albicans* and *S. epidermidis*, at subinhibitory concentrations, makes them promising alternatives to coat CVCs. Moreover, the microbicidal effect on a wider range of CVC colonizers was previously reported *in vitro* for both compounds, at higher concentrations. For all bacterial strains, the highest *in vivo* antibiofilm efficacy was achieved with hamamelitannin. For *A. baumannii*, this is the first report of *in vivo* inhibition.

**Keywords:** nosocomial infections, biofilm inhibition, CRBSIs

## Introduction

Catheter-related bloodstream infections (CRBSIs) are common healthcare-associated infections associated with increased morbidity, hospital stay and medical costs.[1] Antiseptic- and antibiotic-coated central venous catheters (CVCs) have been proposed to reduce the incidence of CRBSIs; catheters impregnated with chlorhexidine–silver sulfadiazine appear to be effective in reducing colonization and infection, but hypersensitivity reactions have been documented,[2] silver-impregnated catheters are not associated with a lower rate of colonization[3] and, despite catheters coated with minocycline and rifampicin significantly decreasing the incidence of CRBSIs, some concern may still exist regarding the development of antimicrobial resistance.[4] Therefore, additional strategies are being developed in order to reduce CVC colonization.

Among promising compounds, cerium nitrate, extensively used in the management of burn patients, has been shown to exhibit antimicrobial activity.[5] Chitosans are hydrophilic biopolymers obtained by N-deacetylation of chitin, with a proven antimicrobial effect.[6] Moreover, microbicidal activity was recently demonstrated for low molecular weight chitosan (LMWC).[5] Hamamelitannin is a polyphenol extracted from the bark of *Hamamelis virginiana*, and belongs to the family of tannins. In addition to inhibiting device-associated infections *in vivo* caused by Gram-positive bacteria,[7] *in vitro* activity against *Acinetobacter baumannii* has been documented.[5]

Overall, cerium nitrate, LMWC and hamamelitannin are biocompatible and relatively inexpensive compounds, displaying a wide range of antimicrobial activity. In order to determine their *in vivo* antibiofilm activity against the usual agents of CRBSIs, such as *Staphylococcus epidermidis*, *Staphylococcus aureus*,

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*A. baumannii* and *Candida albicans*, polyurethane catheter segments were used in a mouse foreign body infection model.

## Materials and methods

### Chemicals

Cerium nitrate [cerium(III) nitrate hexahydrate; Sigma–Aldrich, Schnelldorf, Germany], chitosan [LMWC 107 kDa, 75%–85% deacylated; Sigma–Aldrich, St Quentin Fallavier, France; stock solution of 4% (w/v) prepared in 1% acetic acid, pH 4.0] and hamamelitannin [hamamelofuranose 2′,5-digallate, 2-C-(hydroxymethyl)-D-ribofuranose 2′,5-digallate; Sigma–Aldrich] were used in the experiments.

### Microbial strains and inoculum preparation

Four type strains from the ATCC were used: *S. aureus* ATCC 29213, *S. epidermidis* ATCC 155, *A. baumannii* ATCC 19606 and *C. albicans* ATCC 90028.

Until testing, bacterial strains were kept frozen at −70°C in Luria–Bertani (LB) broth (Difco Laboratories, Detroit, MI, USA) supplemented with 20% glycerol and yeast strains in yeast potato dextrose (YPD) broth (Difco Laboratories) supplemented with 40% glycerol. For each experiment, the microorganisms were previously subcultured twice on LB agar at 37°C for 24 h for bacteria or Sabouraud agar (Difco Laboratories) at 35°C for 24 h for yeasts to assess the viability and purity of the culture.

For the inoculum preparation, the microbial strains were grown overnight in LB broth (bacteria) or Sabouraud broth (yeast) at 37°C and 180 rpm; cells were harvested by centrifugation (10000 **g**, 10 min), washed with PBS, counted in a Newbauer chamber and the concentration was standardized to $1\times10^7$ cells/mL for bacteria and $1\times10^6$ cells/mL for yeasts, in PBS.

### Polyurethane catheter implantation procedure

A mouse subcutaneous foreign body infection model as described by Rupp *et al.*[8] was used, with slight modifications. Eight-week-old pathogen-free female BALB/c mice (Charles River) weighing 20 g were used. Animals were housed in accordance with the Federation of European Laboratory Animal Science Associations (FELASA) criteria. Animal experiments were approved by the Animal Ethics Committee of the Faculty of Medicine, University of Porto.

Prior to catheter implantation, mice were anaesthetized by intraperitoneal injection (1 mg/kg) of a mixture of xylazine (20 mg/mL) and ketamine (100 mg/mL) in a ratio of 1:2 (v/v) and the lower back of each animal was shaved with a hair clipper and disinfected with 0.5% chlorhexidine in 70% alcohol. A 2 mm incision was made longitudinally and the subcutis was dissected. Using an aseptic technique, six 1 cm segment polyurethane intravenous catheters (BD Vialon™ 16G, 1.7×45 mm, Becton Dickinson and Company, Canada) were implanted into the subcutaneous space. Before implantation, catheters were soaked for 24 h with cerium nitrate, hamamelitannin or LMWC at concentrations corresponding to previously determined MIC, 0.5× MIC and 0.25× MIC for each species.[5] Catheters incubated in plain PBS were used as a positive control. Five animals were used for each experimental condition. Thereafter, 300 μL of each standardized microbial suspension or of PBS control was injected into the pockets. The incision was closed with a monofilament suture and disinfected with 0.5% chlorhexidine in 70% alcohol. At day 7, the animals were sacrificed, the catheters were aseptically removed and biofilm was quantified spectrophotometrically by two different methodologies: XTT assay, measuring the biofilm metabolic activity at 492 nm; and crystal violet (CV) assay, measuring the biofilm total biomass at 590 nm.[9]

### Scanning electron microscopy

Catheters were washed in PBS, sectioned lengthwise and fixed overnight (4% formaldehyde and 1% glutaraldehyde v/v in PBS). The samples were rinsed in PBS and air dried in desiccators.[10] Samples were coated with gold/palladium (40%/60%) and observed in CEMUP (Materials Centre of the University of Porto) using a scanning electron microscope (JEOL JSM 6301F/Oxford INCA Energy 350) in high-vacuum mode at 15 kV.

### Data analysis

Biofilm metabolic activity and total biomass formed in the presence of cerium nitrate, LMWC and hamamelitannin were expressed as the percentage in relation to the control. Values were divided by 1000 and arcsine square root transformed to achieve normal distribution and homoscedasticity. For each compound, each concentration and each microorganism, biofilm formation was compared by one-way ANOVA, followed by a Dunnett's test to identify significant effects; $P<0.05$ was considered significant.

## Results

Cerium nitrate, at a concentration of $6.5\times10^2$ mg/L, significantly reduced the biofilm metabolic activity of *S. epidermidis* by 48.3% (±12.4%) and its total biomass by 37.0% (±8.9%) (Figure 1a). For *C. albicans* and at $1.3\times10^3$ mg/L, cerium nitrate inhibited the metabolic activity by 66.4% (±7.7%) and the biomass by 28.0% (±4.3%) (Figure 1b).

LMWC, at 80 mg/L, significantly decreased the biofilm metabolic activity of *S. epidermidis* by 57.6% (±14.3%) and its total biomass by 41.3% (±5.6%) (Figure 1c). Concerning *C. albicans*, at $2.5\times10^3$ mg/L, inhibitions of the metabolic activity by 43.5% (±16.6) and of the total biomass by 23.2% (±13.7) (Figure 1d) were found.

Hamamelitannin significantly reduced all bacterial biofilms at subinhibitory concentrations: at 50 mg/L, *S. aureus* biofilm metabolic activity decreased by 59.3% (±19.1%) and its total biomass by 48.4% (±8.0%) (Figure 1e); at the same concentration, *S. epidermidis* biofilm metabolic activity was reduced by 36.3% (±12.4%) and its total biomass by 34.6% (±6.9%) (Figure 1f); and at 30 mg/L, hamamelitannin inhibited *A. baumannii* biofilm metabolic activity by 36.7% (±3.4%) and its total biomass by 31.6% (±9.2%) (Figure 1g).

At other subinhibitory concentrations, no significant biofilm inhibition was found for the tested microbial strains (data not shown).

In order to support the biofilm quantification by XTT and CV assays, scanning electron microscopic examination was used to determine the architectural differences between biofilms. Untreated biofilms (Figure 2a, b, i, j, m, n, q and r) comprised a more dense network of microbial cells and exopolymeric matrix than treated biofilms (Figure 2c–h, k, l, o, p and s–v).

## Discussion

In the long quest aiming to prevent medical device-related infections in a more effective way, many different strategies have been proposed. Until now, this objective remains to be achieved. Cerium nitrate, LMWC and hamamelitannin are biocompatible and relatively inexpensive compounds that could be used to coat CVCs and other medical devices. Differences in their



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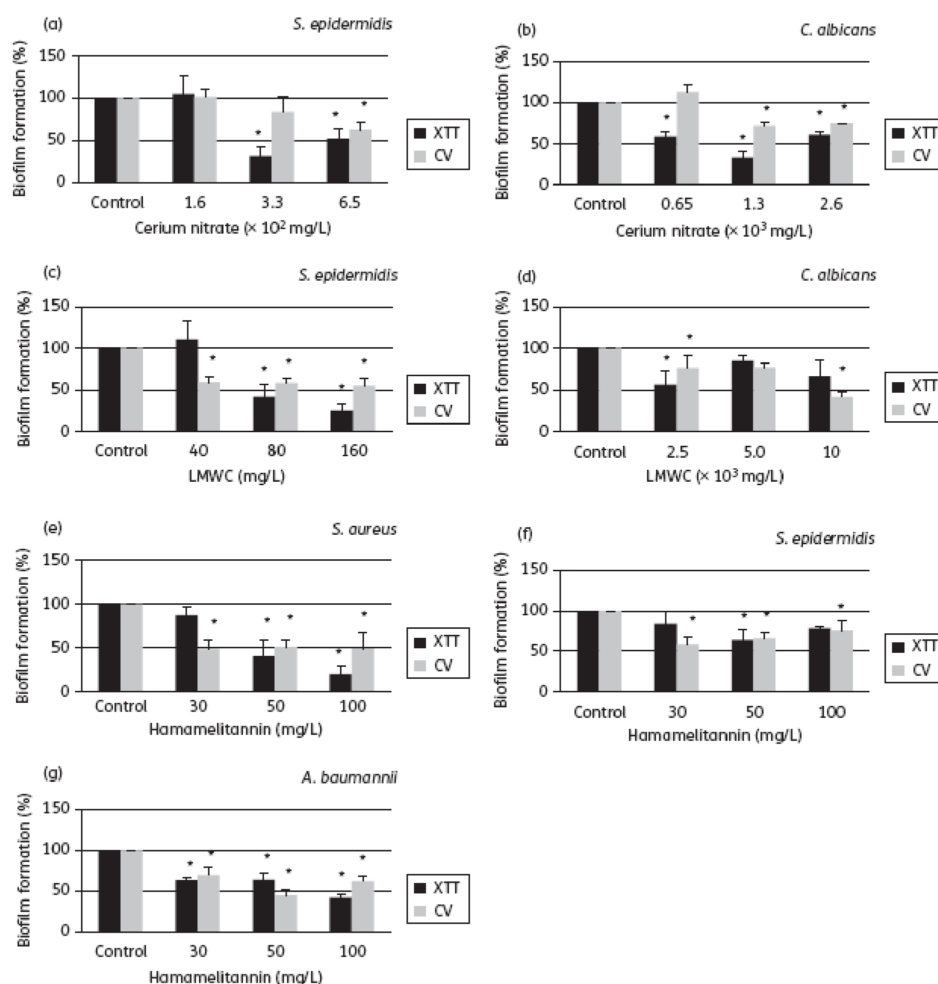


**Figure 1.** Effect of cerium nitrate (a and b), LMWC (c and d) and hamamelitannin (e, f and g) on biofilm formation by *S. epidermidis* (a, c and f), *S. aureus* (e), *A. baumannii* (g) and *C. albicans* (b and d). The XTT assay was used to determine the biofilm metabolic activity and the CV assay was used to measure the total biomass. *$P<0.05$.

mechanisms of action might explain the pattern of antimicrobial activity; whereas cerium nitrate and LMWC may disrupt the cell membrane,[6,11] hamamelitannin seems to inhibit bacterial cell communication.[7] The microbicidal effect of cerium nitrate and LMWC upon usual CVC colonizers was previously shown *in vitro*, as was the antibiofilm activity of hamamelitannin on polyurethane catheter segments.[5] However, *in vivo* experiments with subinhibitory concentrations of the compounds were still required, as the results obtained under *in vitro* conditions do not account for the interaction between the host cells, immune system and biofilm.

Garner and Heppell,[11] studying the action of cerium nitrate on the treatment of severe burns, reported that it has limited antimicrobial properties. However, minimal lethal concentrations (MLCs) and MICs have been published more recently for both bacteria and yeasts.[5] *In vivo*, at subinhibitory concentrations, cerium nitrate exhibited an antibiofilm effect only against *C. albicans*. Such a selective finding should not preclude its





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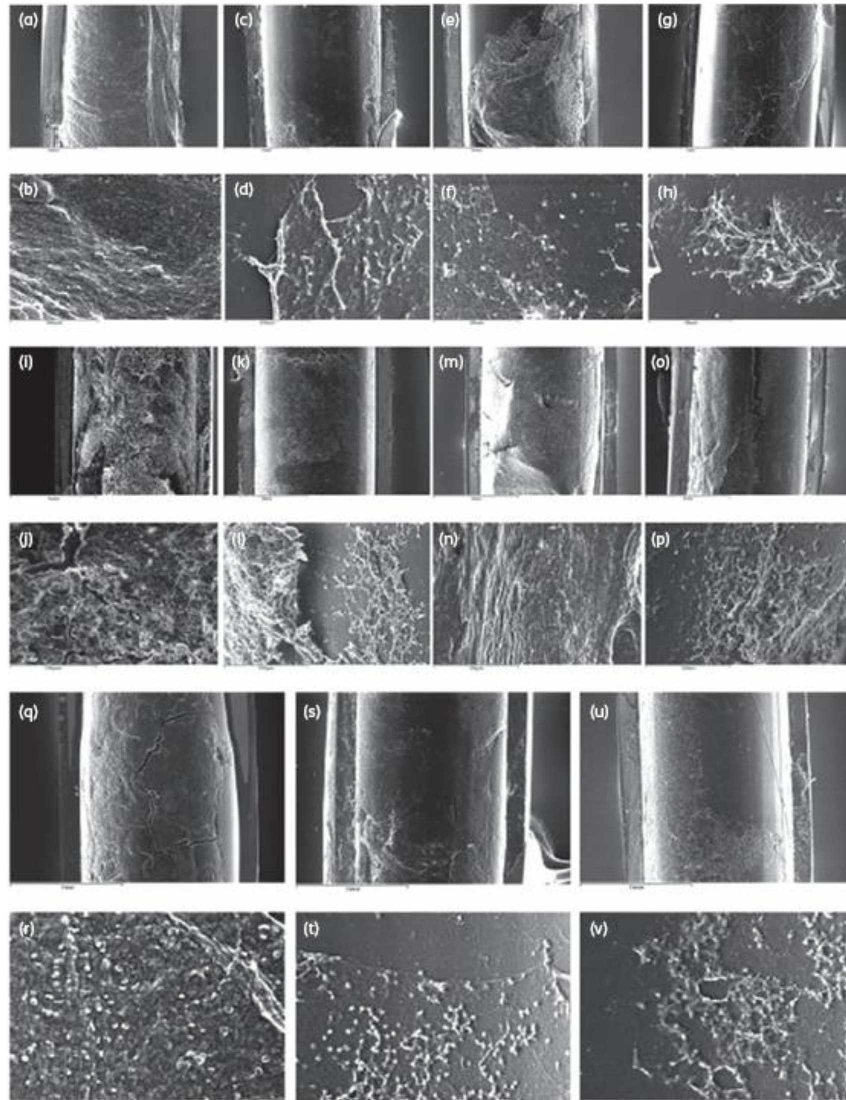


**Figure 2.** Scanning electron microscopic (SEM) examination of untreated biofilms of *S. epidermidis* (a and b), *S. aureus* (i and j), *A. baumannii* (m and n) and *C. albicans* (q and r). SEM examination of *S. epidermidis* biofilms treated with cerium nitrate at $6.5\times10^2$ mg/L (c and d), LMWC at 80 mg/L (e and f) and hamamelitannin at 50 mg/L (g and h), *S. aureus* biofilms treated with hamamelitannin at 50 mg/L (k and l), *A. baumannii* biofilms treated with hamamelitannin at 30 mg/L (o and p) and *C. albicans* biofilms treated with cerium nitrate at $1.3\times10^3$ mg/L (s and t) and LMWC at $2.5\times10^3$ mg/L (u and v).

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biomedical applicability since in medical burn wound care it is used routinely as an effective antiseptic, at much higher concentrations and with no known toxicity. In addition, *Candida* organisms are important pathogens causing CRBSIs.

The microbicidal activity of LMWC against usual CVC colonizers has already been documented *in vitro*.[5] Using an *in vivo* CVC model, Martinez *et al.*[12] demonstrated the efficacy of chitosan against *Candida* species biofilms. Our study not only supports these findings concerning *C. albicans*, at subinhibitory concentrations of chitosan, but further documents the antibiofilm efficacy against *S. epidermidis*, a more frequent CVC colonizer.[13] Given the known biocompatibility of chitosan, promising results could be achieved in the prevention of CRBSIs by a wider range of microbial colonizers if higher concentrations were used for treating the surface of CVCs.

At subinhibitory concentrations, the highest antibiofilm efficacy was obtained with hamamelitannin; a reduction in the metabolic activity and total biomass was documented for *S. aureus*, *S. epidermidis* and *A. baumannii*. This is the first report of *in vivo* biofilm inhibition for *A. baumannii*. Moreover, the antibiofilm efficacy was achieved at lower concentrations than expected considering the previous *in vitro* results for all the tested bacteria, a fact that may be attributable to differences in environmental conditions. In the case of staphylococci, Kiran *et al.*[7] attributed such variation to the specific quorum-sensing inhibitor effect of hamamelitannin. For pre-soaked grafts, the same authors reported no signs of staphylococcal infection *in vivo* with hamamelitannin concentrations of 30 mg/L, but the present study found a statistically significant reduction in biofilm formation by no more than ~50%, even at 100 mg/L. Nonetheless, hamamelitannin may be an excellent compound to coat medical devices given its bacterial range of biofilm inhibition.

A more durable coating strategy for cerium nitrate, chitosan and hamamelitannin and their further testing with a CVC model *in vivo* is the next step in the path to develop a more cost-effective and biocompatible catheter that can prevent CRBSIs more effectively than the current alternatives.

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## Transparency declarations

None to declare.

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# Paper IV

## Antibiofilm effect of cerium nitrate against *Candida albicans*

# Antibiofilm effect of cerium nitrate against *Candida albicans*

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## Abstract

*Candida albicans* is a frequent agent of catheter-related bloodstream infections, which increase the time of hospitalization and related medical costs. In order to find new strategies to reduce the colonization of *C. albicans* on indwelling medical devices, this research aimed to determine the antibiofilm effect of cerium nitrate.

XTT and crystal violet assays were used to quantify biofilms challenged with cerium nitrate. Two strains of *Candida albicans* were used, grown on polystyrene surfaces.

Concerning the biofilm metabolic activity of *C. albicans*, cerium nitrate at $6.5 \times 10^2$mg/L inhibited significantly both strains. Referring to the total biomass, cerium nitrate at a considerably lower concentration ($0.8 \times 10^2$mg/L) inhibited both strains of *C. albicans.*

The fact that cerium nitrate inhibited more effectively the total biomass production than the metabolic activity of the yeast, at subinhibitory concentrations, may be explained by interference with extracellular matrix production or with intercellular communication. Whichever might be the case, cerium nitrate has a potent antibiofilm effect upon *C. albicans* and deserves further attention in strategies that could lower microbial colonization of indwelling medical devices.

**Key words**: Biofilm, cerium nitrate, *Candida albicans*.

## Introduction

Among bloodstream infections caused by *Candida* species, *C. albicans* is the most frequent isolate, being associated with a high mortality rate. As a whole, *C. albicans* stands on as the fourth leading cause of catheter-related bloodstream infections (CRBSIs) (Polderman *et al.*, 2002; Cobrado *et al.*, 2010), which increase the time of hospitalization and related medical costs (Orsi *et al.*, 2002; Tamura *et al.*, 2003).

Data from Mah and Toole, 2001, show that over 65% of hospital infections are originated from biofilm forming organisms. A major concern when biofilms develop is the emergence of microbial strains resistant to the host immune system and to antimicrobial therapy (Kiran *et al.*, 2008). A correlation between *Candida* spp. biofilm formation and resistance to antimicrobial agents was already documented (Pranab *et al.*, 2004). Despite antifungal therapy, the persistence of fungi was noticed.

Therefore, new agents that could inhibit biofilm formation would be highly desirable. Cerium is a rare earth element of the lanthanide group and cerium nitrate is currently used in the management of burn wounds, with a reduction in patient morbidity and mortality (Garner *et al.*, 2005). Cerium nitrate was reported to reduce the activity of microbial pathogens due to their action at different cellular targets (Dwight *et al.*, 1967).

The aim of this research was to determine the antibiofilm effect of cerium nitrate upon *Candida albicans*, a frequent microbial colonizer of indwelling medical devices like central venous catheters (CVCs).

## Materials and methods

### Microbial strains and chemicals

*C. albicans* ATCC 90028 (from the American Type Culture Collections) and a clinical isolate (from a CVC removed from a critical care patient admitted at Hospital S. João, Porto, Portugal) were used. The clinical isolate had been identified by Vitek® System (bioMérieux, Vercieux, France).

Yeast strains were kept frozen in yeast potato dextrose medium (YPD) (Difco Laboratories) supplemented with 40% glycerol, at -70ºC, until testing. For each experiment, the microorganisms were subcultured twice on Sabouraud agar, at 35ºC, for 24 h (Difco Laboratories) to assess the purity of the culture and its viability.

The chemical used in the experiments was cerium nitrate [cerium (III) nitrate hexahydrate, Sigma-Aldrich].

### Biofilm formation

#### *Inoculum preparation*

Yeast cells were grown for 18 h in Sabouraud broth at 35ºC, at 180 rpm. Afterwards, cells were washed in PBS, ressuspended in RPMI and adjusted to 0.5 McFarland density in order to achieve $10^6$ cells/mL.

#### *Biofilm growth with cerium nitrate*

The effect upon biofilm formation was tested with four subinhibitory concentrations of cerium nitrate ($6.5x10^2$ mg/L, $3.3x10^2$ mg/L, $1.7x10^2$ mg/L and $0.8x10^2$ mg/L), which were selected based on the results obtained from *C. albicans* growth experiments (minimal inhibitory concentration of $2.6x10^3$ mg/L, as described by Cobrado *et al*., 2012). Each concentration was added to 12-well polystyrene microtiter plates containing the inoculum in order to obtain a final volume of 1 mL. In order to obtain biofilm formation, *Candida* cells were incubated for 24 h at 37ºC.

### Biofilm quantification

#### *Metabolic activity of biofilm cells - XTT Assay*

A XTT reduction assay was used to determine the *in situ* biofilm metabolic activity. After biofilm formation, supernatants were removed from the plates and 1 mL of PBS buffer was added and gently mixed. Following PBS rejection, 1 mL of XTT [2,3-bis (2methoxy-4-nitro-5sulfophenyl)-2H-tetrazolium-5-carboxanilide] solution was added to each prewashed biofilm and control wells. Plates were further incubated for 5 h at 37ºC, in the dark, and finally the optical density (OD) was measured at 492 nm. The XTT solution was prepared in PBS (4 mg XTT in 10 mL prewarmed at 37ºC); this solution was supplemented with menadione prepared in acetone. All the assays were performed in triplicate.

#### *Biomass formation of biofilm cells - Crystal Violet Assay*

Biofilm fixation was performed with 1 ml 99% methanol (15 min), after which supernatants were removed and the plates air-dried. Afterwards, 1 ml of a crystal violet (CV) solution (0.02%; v/v) was added to each well; following 20 min, the excess of CV was removed by washing the plates twice with distilled water. Finally, bound CV was released by adding 1.5 ml of acetic acid (33%; v/v). The optical density (OD) was measured at 590 nm. All the assays were performed in triplicate.

### Data analysis

Biofilm metabolic activity and total biomass with cerium nitrate were expressed as the percentage in relation to the control. Values were divided by 1000 and arcsine square root transformed to achieve normal distribution and homoscedasticity (Zar, 1996). For each concentration and each strain, biofilm formation was compared by one-way ANOVA, followed by a Dunnett's test to identify significant effects. $P$ value< 0.05 was considered significant.

## Results

### Biofilm formation on polystyrene plates

Concerning the biofilm metabolic activity of *C. albicans*, cerium nitrate at $6.5x10^2$ mg/L inhibited significantly the clinical strain by 36% (±12) and the ATCC strain by 28% (±4) (Figure 1. A).

Referring to the total biomass of *C. albicans*, cerium nitrate at $0.8x10^2$ mg/L inhibited significantly the clinical strain by 81% (±11) and the ATCC strain by 50% (±4) (Figure 1. B). Doubling the concentration of cerium nitrate to $1.6x10^2$ mg/L, there was a reduction in the total biomass of the clinical strain by 87% (±11) and of the ATCC strain by 80% (±5) (Figure 1. B).

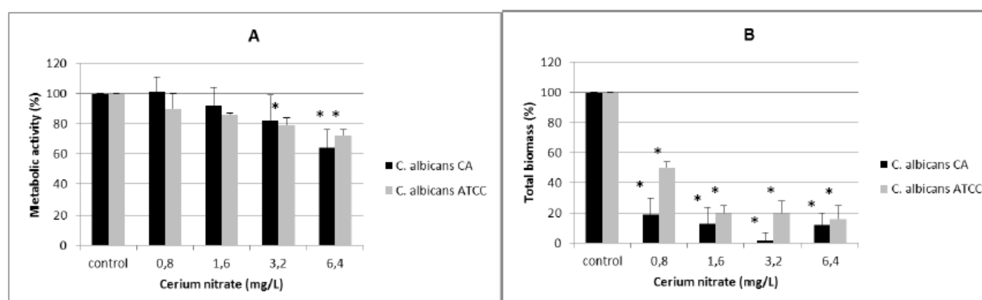


**Figure 1.** Effect of cerium nitrate on metabolic activity (A) and total biomass (B) (as a percentage of the control) by *Candida albicans* clinical strain (CA) and ATCC strain. (*) $P$ value <0.05.

## Discussion

The frequent use of indwelling devices in medical care makes patients more prone to develop biofilm-related infections, with a negative impact on the clinical outcome and hospital costs. The ability of nosocomial pathogens such as *C. albicans* to form biofilms is clinically relevant, since their formation impairs the efficacy of antimicrobial therapy (Donlan and Costerton, 2002). Therefore, it is crucial to find new modes of prevention and effective alternatives to antimicrobial treatment.

Cerium nitrate is thought to enter the cytoplasm of microorganisms and inhibit cellular respiration, glucose metabolism and, eventually, induce disruption of the cell membrane (Dwight *et al.*, 1967). According to the results of Garner *et al.*, 2005, cerium nitrate exhibits a bacteriostactic effect, but further testing demonstrated a microbicidal effect against a wide range of microbial pathogens (Cobrado *et al.*, 2012). Moreover, a potent antibiofilm activity against *C. albicans* grown on polyurethane catheter segments was documented with concentrations lower than MIC: cerium nitrate, at $1.7x10^3$ mg/L, inhibited biofilm formation by ~60%. The results were similar either at 24 h or 48 h of incubation.

The present study confirms the antibiofilm effect of cerium nitrate against *C. albicans* even at lower concentrations. Despite both XTT and CV assays were reproducible, as estimated by the low values of standard deviation, major differences in the quantification of the biofilm were observed. The XTT assay is based on the reduction of tetrazolium salts by mitochondrial dehydrogenases of yeasts and it is used for the quantification of metabolically active cells. The less expensive and time-consuming CV assay is commonly used for the quantification of biofilm biomass, staining the cells (living and dead) and the matrix produced (Peeters *et al.*, 2008). The fact that cerium nitrate inhibited more effectively the total biomass production than the metabolic activity of the yeast cells (Figure 1. A and B), at subinhibitory concentrations, may be explained by its interference with intercellular communication or with extracellular matrix production. Whichever might be the case, further studies are being held to clarify the mechanism of action and the endovascular biocompatibility of cerium nitrate, in order to support the clinical use of such promising compound to coat indwelling medical devices.

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There is no conflict of interest to declare.

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# Paper V

## Anti-Biofilm activity of Low molecular weight Chitosan Hydrogel against *Candida* species

# Anti-Biofilm activity of Low molecular weight Chitosan Hydrogel against *Candida* species

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**Abstract**

*Candida* invasive infections have increased in frequency during the last decades. Such infections are often associated to medical indwelling devices like central venous catheter. The recurrent nature and difficulties in its treatment infections are often related to biofilm formation. Herein, we report about the in vitro anti-biofilm activity of low molecular weight chitosan hydrogel (LMWCH), a natural biopolymer obtained from the N-deacylation of crustacean chitin, upon distinct clinical relevant *Candida* strains. Most importantly, LMWCH was able to significantly inhibit biofilm formation by *Candida albicans* and *Candida parapsilosis* in an in vivo catheter mouse model. LMWCH revealed to be a promising compound for treatment of candidosis or its prevention through medical device coating.

**Keywords**: *Candida*; Chitosan; Biofilm; Candidosis; Catheter-related bloodstream infections; central venous catheter.

## Introduction

Classical antifungal drugs not always represent a viable therapeutic approach to treat mucocutaneous and systemic candidosis since clinical resistance, namely to azoles, is emerging. Therapeutic failure occurs especially in the setting of fungal cells organized in biofilm, a microbial community encased within a polysaccharide-rich extracellular matrix (ECM). Biofilm formation has a clearly established role during systemic and mucosal *Candida* infections. It represents a reservoir of fungal cells with promoted antifungal resistance, also enhancing the microorganism ability to invade host tissues and also protecting the pathogen from the host immune system response [1-3] .

The continuous search for compounds displaying intrinsic antimicrobial properties is crucial for the prevention as well as for treatment of *Candida* infections. Chitosan is a natural polysaccharide biopolymer which exhibits notable biological properties, including non-toxicity, biodegradability and biocompatibility, making it a good candidate for biomedical applications [4-8]. Chitosan also displays antimicrobial activity against a wide range of microorganisms such as algae, fungi and bacteria [7, 9-14]. Furthermore, it was shown that treatment with chitosan can efficiently reduce or prevent bacterial biofilm formation, both in vitro and in vivo [15-20]. Despite its unequivocal antimicrobial effect upon planktonic cells, few studies were performed to clarify its anti-biofilm action against fungi, namely *Candida* spp. Therefore we evaluated the in vitro and in vivo anti-biofilm activity of LMWC upon distinct *Candida* spp with clinical relevance.

## Methods

### Strains

Six clinical isolates of each *Candida* species, corresponding to *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. guilliermondii*, were used in this study (Table 1). All strains had been isolated from patients admitted at Hospital S. João (Porto, Portugal), and identified using the Vitek 2 system (bioMérieux, Vercieux, France). *C. albicans*

ATCC 90028 strain belonging to American Type Culture Collection was also used. All the strains were kept frozen in yeast potato dextrose medium (YPD) (Difco Laboratories) supplemented with 40% glycerol at -70°C until testing. For each experiment, the microorganisms were subcultured twice on Sabouraud agar (Difco Laboratories), 35°C, 24h to assess the purity of the culture and its viability.

**Table 1**- *Candida* strains used in this study: distribution by species and provenance.

| Strain | Isolate | Site of isolation or Source |
|---|---|---|
| **O32** | *C.albicans* | Blood |
| **O63** | *C.albicans* | Blood |
| **OL028** | *C.albicans* | Catheter |
| **OL057** | *C.albicans* | Urine |
| **OL060** | *C.albicans* | Tracheal bronchial secretions |
| **OL075** | *C.albicans* | Catheter |
| **MC433** | *C.albicans* | Mucocutaneous |
| **MC437** | *C.albicans* | Mucocutaneous |
| **ATCC 90028** | *C.albicans* | American Type Culture Collection |
| **O21** | *C. parapsilosis* | Blood |
| **O39** | *C. parapsilosis* | Blood |
| **OL007** | *C. parapsilosis* | Tracheal bronchial secretions |
| **OL021** | *C. parapsilosis* | Catheter |
| **OL031** | *C. parapsilosis* | Urine |
| **OL056** | *C. parapsilosis* | Catheter |
| **MC428** | *C. parapsilosis* | Mucocutaneous |
| **MC429** | *C. parapsilosis* | Mucocutaneous |
| **OO6** | *C. tropicalis* | Blood |
| **O77** | *C. tropicalis* | Blood |
| **OL006** | *C. tropicalis* | Faeces |
| **OL017** | *C. tropicalis* | Urine |
| **OL038** | *C. tropicalis* | Urine |
| **OL053** | *C. tropicalis* | Urine |
| **MC374** | *C. tropicalis* | Mucocutaneous |
| **MC418** | *C. tropicalis* | Mucocutaneous |
| **OO1** | *C.glabrata* | Blood |
| **OO4** | *C.glabrata* | Blood |
| **O12** | *C.glabrata* | Blood |
| **OL044** | *C.glabrata* | Faeces |
| **OL090** | *C.glabrata* | Faeces |
| **OL071** | *C.glabrata* | Urine |
| **MC376** | *C.glabrata* | Mucocutaneous |
| **MC425** | *C.glabrata* | Mucocutaneous |
| **O14** | *C.krusei* | Blood |
| **O131** | *C.krusei* | Blood |
| **OL012** | *C.krusei* | Tracheal bronchial secretions |
| **OL091** | *C.krusei* | Tracheal bronchial secretions |
| **OL099** | *C.krusei* | Tracheal bronchial secretions |
| **OL103** | *C.krusei* | Catheter |
| **OL109** | *C.krusei* | Faeces |
| **OL101** | *C.krusei* | Tracheal bronchial secretions |
| **32** | *C. guilliermondii* | Blood |
| **33** | *C. guilliermondii* | Blood |
| **OL072** | *C. guilliermondii* | Tracheal bronchial secretions |
| **OL077** | *C. guilliermondii* | Cerebrospinal fluid |
| **MC23** | *C. guilliermondii* | Mucocutaneous |
| **MC27** | *C. guilliermondii* | Mucocutaneous |
| **MC37** | *C. guilliermondii* | Mucocutaneous |
| **MC38** | *C. guilliermondii* | Mucocutaneous |

**Chemicals**

Low molecular weight chitosan (LMW) (Sigma Aldrich), 50 KDa, 92% deacetylation degree was solubilized in lactic acid (2% v/v) solution to a final concentration of 4% (w/w). Subsequent dilutions of this stock solution were prepared in RPMI 1640 cell culture medium (Sigma-Aldrich).

**Adhesion and in vitro biofilm formation assays**

Yeast cells were grown overnight at 35°C, 180 rpm in Sabouraud broth (Difco Laboratories). After washing twice with phosphate-buffered saline (PBS), a suspension containing $1x10^6$ cells/ml, counted with a hemacytometer, was prepared in RPMI.

**Adhesion assay** The effect of LMWCH upon *Candida* adhesion to polystyrene was evaluated accordingly to a reduction assay, as previously described [1]. Briefly, 12-well polystyrene microplates uncoated or coated with LMWCH at the respective sub-inhibitory concentration (half the minimum concentration that inhibited 50% of microbial growth ($MIC_{50}$)) for each species, were used. Plates were incubated for 90 min with a cell suspension containing $1x10^6$ cells/ml of each strain, and then washed twice with PBS to remove non-adherent yeasts. Adherent cells were quantified using the semi-quantitative 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay[21].

The $MIC_{50}$ and $MIC_{90}$ for planktonic cells were determined accordingly to the CLSI M27-A3 protocol [22]. Lethal concentrations (MLC) were determined accordingly with Canton *et al* protocol [23] (Table 2).

**Table 2**- Susceptibility of distinct *Candida* species under planktonic and biofilm associated growth conditions to LMWCH, determined accordingly CLSI and XTT methods. **a)** The MIC end point for planktonic cells was based upon visual determination of the lowest drug concentration that caused a 50% or 90% decrease in growth relative to the growth in the chitosan-free control well. **b)** The sMIC end point for biofilms corresponds to the lowest chitosan concentration resulting in a 50% or 90% of RMA relative to the metabolic activity of the untreated growth control, measured by the XTT reduction assay. Results are representative of at least three independent experiments, performed in triplicate.

| | **Planktonically grown cells** | | **Biofilm at 24 h** | |
|---|---|---|---|---|
| | **$MIC_{50}$** | **$MIC_{90}$/ MLC** | **$SMIC_{50}$** | **$SMIC_{90}$** |
| *C. albicans* | 0.04 | 5 | 0.31 | >10 |
| *C. parapsilosis* | 0.005 | 0.02 | 0.02 | 2.5 |
| *C. glabrata* | 5 | 7.5 | 1.25 | >10 |
| *C. tropicalis* | 0.04 | 1.25 | 0.02 | >10 |
| *C. krusei* | 0.005 | 0.05 | 0.08 | >10 |
| *C. guilliermondii* | 0.0025 | 0.0025 | 0.08 | >10 |

**Biofilm Formation** Yeast suspensions were allowed to form biofilms for 24 hours [21] in the presence and absence of four LMWCH concentrations: ½ $MIC_{50}$, $MIC_{50}$, $MIC_{90}$value and 10 mg/ml. After 24h of incubation biofilms were washed gently with PBS to remove non adherent cells and afterwards quantified using two different methodologies: the semi-quantitative XTT reduction assay, which measures biofilm cellular metabolic activity [21] and the quantification of biofilm total biomass, using the crystal violet staining assay [24].

**Biofilm susceptibility to LMWCH** Biofilms of each *Candida* species were allowed to form in polystyrene microplates for 24 hours, and afterwards challenged with LMWCH in serial 1:2 diluted concentrations, ranging from 10 to 0.01mg/ml. The minimal LMWCH concentration which caused 50% or 90% reduction in metabolic activity (50% or 90% RMA) of biofilm compared with the respective control strain (incubated in the absence of drug) was defined as the $sMIC_{50}$ or $sMIC_{90}$ respectively (minimal inhibitory concentration of sessile cells) [21].

**In vivo biofilm formation assay**

A mouse subcutaneous foreign body system was used [25]. One *C. albicans* strain and one *C. parapsilosis* strain (both isolated from central venous catheter CVCs) were tested with this model. Eight-week-old specific pathogen-free female BALB/c mice (Charles River), weighting 20g were used. Animals were housed in an environmentally controlled room with 12-h light-dark cycle and were kept on a standard *ad libitum* diet. Animals were maintained in accordance with the Federation of European Laboratory Animals of Science Associations (FELASA) criteria. Animal studies were approved by the animal ethical committee of the Faculty of Medicine, University of Porto.

Mice were anesthetized and the lower back was shaved and disinfected with 0.5% chlorhexidine in 70% alcohol. Subsequently, six 1-cm segments of polyurethane intravenous catheters (BD Vialon™ 16G 1,7x45 mm) were implanted into the subcutaneous space. Before implantation, catheters were soaked for 24 hours with LMWCH at concentrations corresponding to the $MIC_{90}$ for each species (5mg/ml for *C. albicans* and 0.02mg/ml for *C. parapsilosis*). Catheters incubated in PBS were used as a positive control. Five animals were used for each experimental condition. Subsequently, $1x10^6$ yeast cells/ml in PBS or plain PBS (control), was injected into the pockets; the incision was closed and disinfected with 0.5% chlorhexidine in 70% alcohol. At day 7, animals were sacrificed; catheters were aseptically

removed and biofilm quantified spectrophotometrically following the XTT assay [21].

**Biofilm imaging by scanning electron microscopy (SEM)**

In order to visualize biofilms formed in the wells of 12-well polystyrene plates, the bottom of each well was extracted from the plate. To visualize biofilms formed at the surface of catheter segments, each segment was transected lengthwise. Samples were placed overnight in a fixative solution of 4%formaldehyde v/v, 1% glutaraldehyde v/v in PBS. After incubation, biofilms were washed with PBS and air dried in desiccators [1]. For analysis, samples were coated with gold/palladium (40%/60%) and observed in a scanning electron microscope (JEOL JSM6301F/OXFORD INCA ENERGY 350) in high vacuum mode at 15 kV.

**Statistical analysis**

Statistical analysis started with distribution normality assessment using the Kolmogorov-Smirnov test and histogram evaluation. Since all the variables' distribution was normal only parametric tests were used. Chitosan adhesion and biofilm inhibitory effect, at different concentrations, was evaluated with One-Way ANOVA (with the Bonferroni correction). The student's t test was used to evaluate the activity of chitosan treatment in pre-formed biofilms. Correlation analysis was assessed using the Pearson correlation test. Statistical significance was considered as a p value inferior to 0.05. All statistical analysis was conducted using the SPSS software (v. 20.0).

**Results**

**Adhesion and Biofilm formation in presence of LMWCH**

All strains adhered promptly to polystyrene plates. However, adherence was significantly reduced in the presence of LMWCH, excepting for *C. krusei* ($p$ 0.24) and *C. guilliermondii* ($p$ 0.39). The extend of reductions was 9.4% for *C. albicans* ($p$ 0.03), 53.4% for *C. glabrata* ($p$<0.001), 31.2% for *C. parapsilosis* ($p$ 0.001) and 14.5% for *C. tropicalis* ($p$ 0.002) (Fig.1A).

The significant decrease of yeast adhesion to polystyrene caused by LMWCH led to the investigation of its activity against biofilm development by *Candida* isolates, which was quantified both by total biofilm biomass and by using a semi quantitative XTT reduction assay for metabolic activity determination.

For all species, except *C. parapsilosis*, the presence of LMWCH at subinhibitory concentrations during biofilm development decreased significantly its metabolic activity (45 to 81%) (Fig. 1B). Conversely, the total biomass was not affected (Fig. 1B). Despite the reduction of the biofilm metabolic activity, no significant correlation was found between biofilm inhibition and reduced percentage of adhesion ($p$> 0.05).The $MIC_{50}$ concentration decreased significantly biofilm biomass of species like *C. glabrata* and *C. tropicalis*, being the reduction less prominent in the case of *C. guilliermondii*. The highest concentration tested (10 mg/ml) was the most effective, causing biomass reductions of all *Candida* species, up to 99% of biofilm reduction when compared with non-treated biofilms (Fig. 1B).

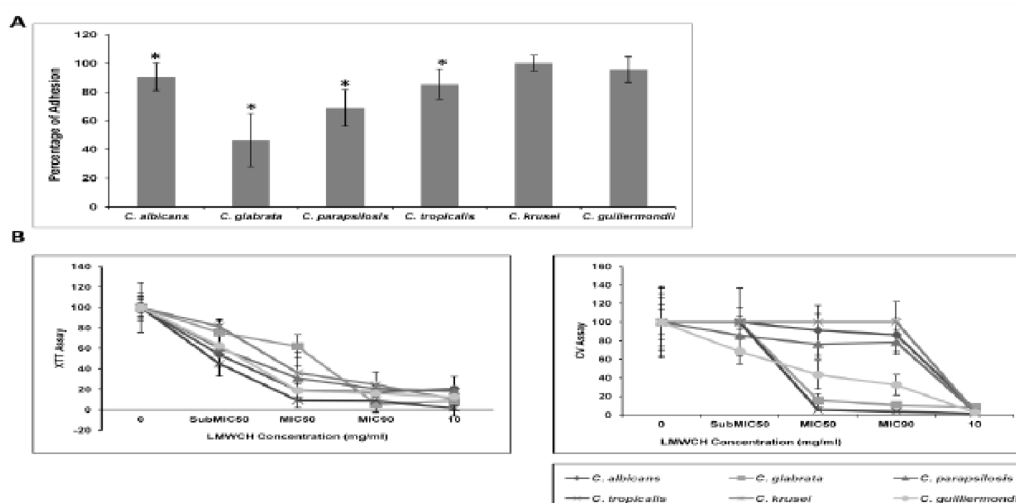

**Figure 1- Activity of LMWCH upon adhesion and biofilm formation. A)** Evaluation of *Candida* adhesion to polystyrene in the presence of LMWCH subinhibitory concentrations (*C. albicans* 0.02 mg/ml; *C. glabrata* 2.5 mg/ml; *C. parapsilosis* 0.0025 mg/ml; *C. tropicalis* 0.02 mg/ml; *C. krusei* 0.0025 mg/ml and *C. guilliermondii* 0.00125 mg/ml). **B)** Quantification of biofilm metabolic activity (left) and biomass formation (right) of *Candida* biofilm in the presence of LMWCH. The subinhibitory concentrations used for adhesion assays, $MIC_{50}$, MLC value and 10 mg/ml concentrations were tested for each species.

Adhesion and biofilm results were normalized to control (0 mg/ml), which was taken as 100%. Each result is representative of at least three independent experiments performed in triplicate. * $p < 0.05$ when compared with control (without treatment) evaluated with One-Way ANOVA (with the Bonferroni correction) and with error box graphics.

**Biofilm susceptibility to LMWCH**

Regarding the activity upon pre-formed biofilm, LMWCH exhibited a dose dependent reduction of biofilm metabolic activity. A significant direct correlation was found between LMWCH concentration and the metabolic activity/biomass for all *Candida* species ($p<0.05$).

In general, the $sMIC_{50s}$ were 2 to 5 dilutions above the planktonic $MIC_{50}$. Interestingly, such value decreased in the case of *C.glabrata* and *C. tropicalis* (two of most resistant chitosan strains) comparatively to planktonic cells (Table 2). Nevertheless, $sMIC_{50}$ values were below the $MIC_{90}$ for most of the species. With the exception of *C. glabrata* and *C. guilliermondii*, in all other species reductions ranging between 80 to 93% in RMA were obtained (Fig. 2A). Generally, biomass reduction ranging between 80 to 96% was achieved with 10 mg/ml of LMWCH, which was found to be the most effective concentration against all species (Fig. 2B).

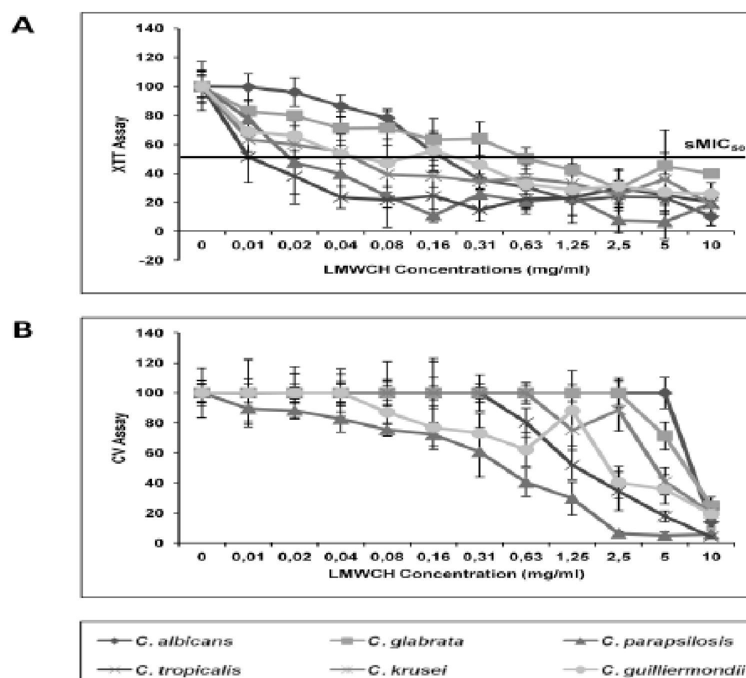


**Figure 2- Biofilm susceptibility to LMWCH.** Graphics show the effect of LMWCH upon pre-formed biofilms; 24 hours biofilms were treated with serial concentrations of LMWCH, ranging from 10 to 0.01mg/ml. After incubation with LMWCH for an additional 24 hours, the metabolic activity (A) and biomass (B) were evaluated. Results were normalized to control (0 mg/ml), which was taken as 100%. Each result is representative of at least three independent experiments performed in triplicate.

**Scanning electron microscopy**

Imaging of biofilms formed in polystyrene plates demonstrated that biofilm architecture varies not only among the different species but also slightly varies among strains of the same species (data not shown). Nevertheless LMWCH activity was generally consistent and constant within the same species.

Representative images of the four most medical relevant *Candida* species are depicted in Fig. 3. *C. albicans* untreated biofilms showed an heterogeneous architecture with a continuous layer of yeasts and hyphae interconnected (Fig. 3A). *C. parapsilosis* displayed micro colony/water channel morphology with yeast cells aggregates and filamentous forms, disposed in a discontinuous monolayer (Fig. 3C). *C. tropicalis* and *C. glabrata* exhibit the most compact biofilms (Fig. 3B, D). While *C. glabrata* biofilms are only composed by a multilayer of yeasts cells closely packed with each other, *C. tropicalis* exhibits filamentous forms interconnecting continuous blastospore layers.

*C. albicans*, *C.glabrata* and *C. tropicalis* biofilms grown in the presence of the $MIC_{90}$ are less compact but do not exhibit evident morphological alterations (Fig. 3E, F, H). Only in the case of *C. parapsilosis* was possible to observe the absence of filamentous forms found in the untreated control and smaller blastopores, most of which appear to be collapsed (Fig. 3G). The 10mg/ml LMWCH concentration unable mature biofilm formation in *C. albicans*, *C. glabrata* and *C. parapsilosis* strains (Fig. 3I-K). Apparently only a few yeasts were able to adhere to polystyrene and to each other, but were incapable to start the biofilm formation process. *C. tropicalis* seems to create a more organized structure but yeast cells and pseudohyphae are mostly collapsed (Fig. 3L).

Treatment of preformed biofilms with 10mg/ml of LMWCH resulted in biofilm disorganization, in all tested species (Fig. 3M-P). Curiously, in the case of *C. albicans* and *C. parapsilosis,* only yeast forms persisted whereas, in the case of *C. tropicalis,* mainly the hyphal structures remained. The concentration corresponding to the $SMIC_{50}$ led to a reduction of biofilm biomass, as well as morphological alterations, being the particulary evident in *C. parapsilosis* biofilm (Fig. 3O, W).

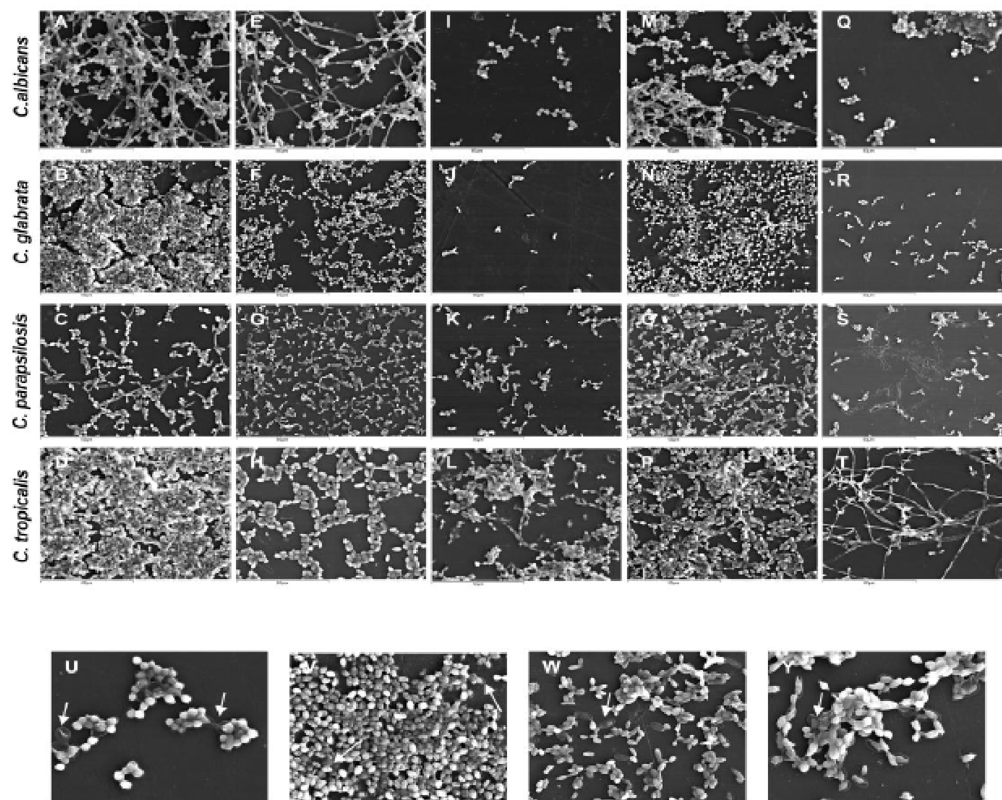

**Figure 3- Scanning electron microscopy images of *Candida* biofilms formed in polystyrene plates. A-D,** *Candida* biofilm without LMWCH treatment (control); **E-L,** *Candida* biofilms grown in the presence of $MIC_{90}$ (E to H) or 10 mg/ml (I to L) of LMWCH for 24 h; **M-T,** 24 h preformed *Candida* biofilms treated with LMWCH concentrations corresponding to the $sMIC_{50}$ of each species (M to P) or 10mg/ml of LMWCH (Q to T) for an additional 24 h period. **U-Y**, Higher magnification of biofilms treated with 10 mg/ml of LMWCH. White arrows show collapsed cells in the remaining biofilm of different species (U- *C. albicans*, V- *C. glabrata*, W- *C. parapsilosis*, Y- *C. tropicalis*).

Images represent typical fields of view. Scale bars correspond to 60 µm at 1000x magnification for A trough T panels and to 30 µm at 2000x magnification for U trough Y panels.

**In vivo LMWCH biofilm inhibition**

To demonstrate the effectiveness of LMWCH, as a coating agent of medical indwelling devices, in impairing fungal adherence, biofilm formation and, ultimately invasive candidosis, a mouse subcutaneous foreign body system was used [26]. Since *C. albicans* and *C. parapsilosis* are frequently isolated from *Candida* infected CVCs, one strain of each species (previously isolated from CVCs) was tested with this model. Catheter segments soaked with LMWCH $MIC_{90}$ concentration resulted in a marked impairment of biofilm metabolic activity of *C. albicans* (91.4% ±8.9 of biofilm RMA) and of *C. parapsilosis* (95.7% ±3.3 of biofilm RMA). SEM images demonstrated that the catheter surface is generally free of biomass, except some residual spots (Fig. 4c, d, g, h). In addition, the structure of the biofilm formed in the presence of LMWCH is quite different from the control, grown in the absence of chitosan. In non-treated catheters, both *Candida* species formed a compact multilayer biofilm where hyphae, pseudohyphae and yeasts were present and mainly immersed in a dense matrix, covering the entire catheter surface (Fig. 4a, b, e, f). The sparse biofilm formed in chitosan treated catheters was a thin monolayer of yeast, hyphae or pseudohyphae embedded with a scarce amount of matrix (Fig. 4d, h).

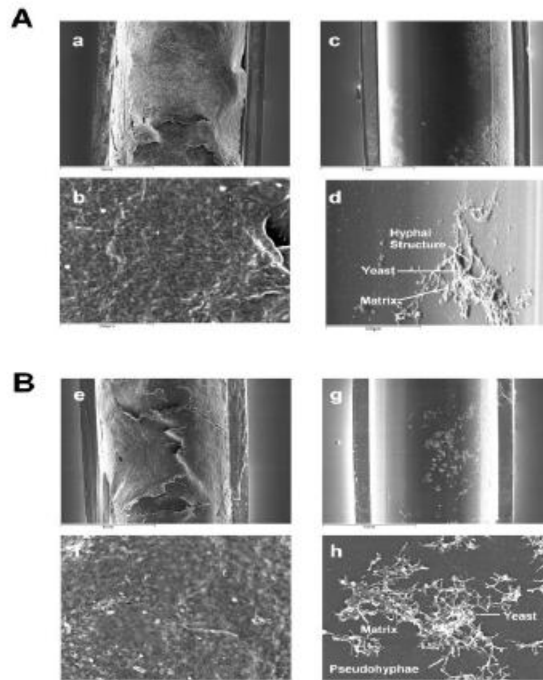


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**Figure 4- Architecture of in vivo *C. albicans* (A) and *C. parapsilosis* (B) biofilms.** Scanning electron microscopy images show the intraluminal surface of catheter segments after 7 days of in vivo implantation. Catheters treated with of LMWCH (c-d and g-h) or without treatment (a-b and e-f) were challenged with *C. albicans* and *C. parapsilosis*. At day 7, he surface of untreated catheters was completely covered with a dense multilayer biofilm while catheters soaked with LMWCH failed to form continuous and structured biofilms. Scale bars correspond to 1 mm at 50x magnification for catheter cross sections and 100 µm with 500x magnification for detailed biofilm structures.

**Discussion**

Chitosan is known to be fungicidal to *Candida* planktonic cells [7, 9, 11-13, 18]. In present study, we demonstrated its *Candida* anti-biofilm activity, by reducing considerably the biofilm metabolic activity (up to 90%) of pre-formed biofilms and further promoting extensive biomass disaggregation of mature biofilms. Interestingly, the concentrations needed to obtain a 50% reduction of cellular metabolism by the different species are closely correlated with the susceptibility of planktonic cells, suggesting an identical antimicrobial mechanism. It is plausible that the positively charged chitosan molecules interact with negatively charged yeast cell membranes inducing intracellular constituents' leakage, reducing cellular metabolism, ultimately resulting in cell death (which was already demonstrated by flow cytometry [13]). Interestingly, a recent study explains the heterogeneity of planktonic susceptibility between the different *Candida* species, is resulting from the ionic reaction between chitosan amino free groups and ionic charges at the cell surface [27]. The authors correlate the yeast surface charge to the chitosan sensitivity and demonstrate that *C. albicans* and *C. glabrata* are less susceptible due to the reduced prevalence of negative charges at its cell surface; following this rank are *C. tropicalis*, *C. parapsilosis* and *C. guilliermondii* [27]. Curiously, SEM images of biofilms treated with chitosan corroborate this theory: yeast cells displayed a wrinkled surface, far from its usual smooth regular surface, and even collapsed in a large extend. Another proposed mechanism involves the penetration of chitosan into yeast nuclei, it's binding to DNA and inhibition of RNA synthesis with obvious consequences in the cellular metabolism. All these phenomena could cause a serious physical stress upon biofilm structure leading to its disruption [7, 28]. However, the discrepancy found among distinct species suggests a non common mechanism, being dependent of physiological differences and variations in ECM components [29, 30]. Moreover, interactions between chitosan and the ECM components of the different species, could promote chitosan binding to the matrix, delaying its penetration. This assumption could explain the differences between $sMIC_{50}$s. Nevertheless, the concentration of 10mg/ml LMWCH efficiently disorganized biofilm.

Our study also showed that LMWCH reduced significantly adhesion of *C. albicans*, *C. glabrata, C. parapsilosis* and *C. tropicalis* to polystyrene and inhibited its biofilm formation. However, biofilm formation may not necessarily correlate with adhesion, as previously reported for *Cryptococcus neoformans* [14], demonstrating that adhesion is an important step although not a crucial event for biofilm formation.

The anti-biofilm activity of chitosan upon pre-formed *C. albicans* and *C. parapsilosis* biofilms, and its ability to prevent *C. albicans* biofilm formation had been previously mentioned in two elegant studies [20, 28]. However, no reference to the type of chitosan was made in one [28] while, in the other, a medium molecular weight chitosan was used [20]. Furthermore, data obtained resulted only from testing a single strain of each *C. albicans* and *C. parapsilosis*, and the range of tested chitosan concentrations was extremely narrow.

In our study, we performed for the first time a comprehensive evaluation of LMWCH effect against several *Candida* species.

Moreover, the efficacy of chitosan to impair *C. parapsilosis* biofilm formation in vivo was originally demonstrated. Although *C. albicans* is still the fungal species most frequently isolated from infected CVCs, *C. parapsilosis* has been increasingly associated with medical indwelling device infections. Recently, Cobrado *et al* [26] demonstrated that LMWC reduced in a extend of 50% *C. albicans* biofilm formation at a concentration of 2.5 mg/ml, using a similar animal model. Notably, we achieved an inhibition of 91.4% just by doubling the concentration. Our findings are in accordance with Martinez *et al* [28] results. Even though, in such study catheters were removed soon after 24 h of implantation while in the present study catheters were only removed 7 days later. Our results demonstrate that devices coated with LMWC exhibit a durable antifungal activity. Such fact might help to avoid frequent catheter removal in critical patients, a medical procedure

that is indicated but implies several complications to the patient. The 5mg/ml concentration is also non-toxic for human endothelial cells [28] making this compound a serious candidate for biomaterial applications, such as coating of medical indwelling devices.

The classical antifungals often fail to eradicate fungal biofilms, being echinocandins and amphotericin B lipid formulations the few efficient therapeutic options for sessile cells [31, 32]. We showed that LMWC significantly reduced both in vitro and in vivo *Candida* biofilm formation, being also able to disrupt a pre-formed biofilm, not only by reducing yeast metabolic activity but also by promoting ECM disaggregation. Considering its intrinsic antifungal, anti-biofilm activity and biocompatibility with human cells, LMWCH represents a promising compound for the management of mucocutaneous fungal infections, as well as an interesting approach to coat medical indwelling devices in order to prevent its colonization by *Candida* organisms.

**Funding**

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# Paper VI

## Cerium, Chitosans and Hamamelitannin:

## the Rise of New Microbial Inhibitors?

## Cerium, Chitosans and Hamamelitannin: the Rise of New Microbial Inhibitors?

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### Abstract

**K-124**

**Background** Indwelling medical devices are related to bacteremia, mainly in critically ill patients, with important morbidity, mortality and costs. *S. epidermidis*, *S. aureus*, *A. baumannii* and *C. albicans* are among the most frequently medical device-associated microoganisms isolated. Coating of devices with antibiotics or antiseptics has been tried with variable success. Chitosan, cerium and hamamelitannin may be related to the reduction of virulence of biofilm-associated microorganisms. The objective of this study was to evaluate the potential of these substances in the prevention of biofilm formation.

**Methods** To study biofilm inhibition, two strains (one from ATCC and another clinical) of each microbial species have been grown overnight, in 12 well plates, with rising concentrations of cerium ($1.9x10^{-4}$ to $1.5x10^{-3}$ M), chitosan 107 kDa ($3.9x10^{-3}$ to $6.25x10^{-3}$ %) and hamamelitannin (10 to 50 µg/ml). All of those agents were at sub-inhibitory concentrations. Biofilm was quantified by spectrophotometry, with XTT tetrazolium and crystal violet assays.

**Results** Cerium, at $1.5x10^{-3}$ M, inhibited the biofilm metabolic activity of both strains of *S. epidermidis*, *S. aureus* and *C. albicans* in 20 to 60%, comparatively to positive controls. Total biofilm mass was reduced in 20 to 90%. No such relevant inhibition was documented for *A. baumannii*. Chitosan 107 kDa, at $6.25x10^{-3}$ %, inhibited the biofilm metabolic activity of *S. epidermidis* and *S. aureus* in 10 to 20%. Total biofilm mass was reduced by 40 to 90%. Conflicting results have been observed regarding *A. baumannii* and *C. albicans* strains. Hamamelitannin had no significant effect in biofilm inhibition, even at the higher concentration tested.

**Conclusions** An important reduction in biofilm mass and metabolic activity of *S. epidermidis*, *S. aureus* and *C. albicans* has been observed for cerium, for the first time. Chitosan 107 kDa inhibited rather the gram positive bacteria tested. No consistent biofilm inhibition has been demonstrated for *A. baumannii*. Further knowledge on the applicability of cerium and chitosans on medical devices would be welcome.

# Paper VII

## Cerium Nitrate:

## Anti-*Candida* Activity Due to Cell Membrane Damage

## Cerium Nitrate: Anti-Candida Activity Due to Cell Membrane Damage

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P-037

## Abstract

**Objectives:** Cerium is a member of the lanthanide series or rare earth elements. Several medical applications have addressed cerium compounds namely antiemetic, antineoplastic, antiseptic and immunomodulatory properties. All these applications are still controversial, with the exception of its use as a topical agent for the treatment of burn wounds. In fact some studies present a reduction in mortality of 20-50% following treatment with cerium. This finding is mostly attributed to its immunomodulatory activity since studies addressing its antimicrobial effect produced contradictory results. Our aim was to clarify Cerium nitrate antimicrobial activity against *Candida* species by the classical methodologies and also by Flow Cytometry using FUN-1 (a metabolic marker) and Propidium iodide (PI) (a selective marker of dead cells with membrane damage).

**Methods:** Cerium nitrate, clinical isolates of *Candida* (n=36), corresponding to *C. albicans* (n=6), *C. parapsilosis* (n=6), *C. glabrata* (n=6), *C. tropicalis* (n=6), *C. Krusei* (n=6), and *C. guilhermondii* (n=6), were tested accordingly to the classical microdilution antifungal susceptibility protocol M27-A3. Serial concentrations of Cerium nitrate ranging to 250 a 0.125mM were used and the minimal inhibitory (MIC) and minimal lethal (MLC) concentrations were determined. MIC end point was defined as the lowest drug concentration that inhibited 50% the growth when compared to the control.

To investigate cerium nitrate antimicrobial activity by Flow Cytometry one *C. albicans* strain was incubated with different concentrations of cerium (125, 50, 8 and 0.125mM) for 30min, 90min, 3h, 6h and 24h at 35°C with agitation. At each time point, yeast cells were harvested by centrifugation, washed twice and ressuspended in a solution with FUN-1 0.5µM or PI 1µg/ml (Molecular probes). Following an additional incubation of 30min at 35°C yeast cells were analyzed in a flow cytometer (FACSCalibur BD Biosciences, Sydney) and 20000 events were collected. Cytometric readings were performed in the FL2 fluorescence channel (BP 585/42 nm) for FUN-1 staining and FL3 fluorescence channel (LP 670 nm) for PI. From each suspension, yeast cells were plated on Sabouraud dextrose agar and incubated for 24h for assessment of viable cells.

**Results:** Cerium MIC was 0.125 mM and the MLC was 8 mM for all *Candida* strains. Cytometric analysis showed that cerium nitrate produces not only a dose and time dependent decrease of the cell metabolic activity but also results in a definitive membrane injury that is a lethal effect.

**Conclusions:** Cerium nitrate exhibits antimicrobial activity against *Candida* species, being a promising agent for prevention or treatment of mucocutaneous candidosis.

# Paper VIII

## Cerium Nitrate:

## Anti-*Candida albicans* Activity in Planktonic Cells and Biofilms

# Cerium Nitrate: Anti-*Candida albicans* Activity in Planktonic Cells and Biofilms

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## Abstract

**Objectives:** Cerium is a member of the lanthanides or rare earth elements. Several medical applications have been addressed to cerium compounds, based upon its antiemetic, antineoplastic, antiseptic and immunomodulatory properties. All these applications are still controversial, with the exception of its topical use for the treatment of burn wounds. Nevertheless studies addressing its antimicrobial effect produced contradictory results. Our objective was to clarify its antimicrobial activity against *Candida albicans*. We also tested its ability to impair biofilm formation and its potential use in biofilm treatment.

**Methods:** Six clinical isolates of *C. albicans* and the type strain ATCC 90028, were tested accordingly to the protocol M27-A3. Serial concentrations of cerium nitrate (ranging from 1 to 0.244M) were used and the minimal inhibitory (MIC) and minimal lethal (MLC) concentrations determined. A cytometric kinetic study was also performed by incubating *C. albicans* ATCC90028 with each of 3 concentrations (0.03, 8 and 50 mM) during 30min, 90min, 3h, 6h and 24h. At each time point yeast cells were stained with FUN-1 0.5µM (a metabolic marker) or with Propidium iodide 1µg/ml (a membrane injury marker) and 20,000 events were analyzed in a flow cytometer (FACSCalibur BD Biosciences, Sydney). From each suspension, yeasts were plated on Sabouraud dextrose agar for viability assessment.

Biofilms growth was promoted in the presence of 3 cerium concentrations (0.03, 8 and 50 mM) for 24hours. The biofilm metabolic activity and total biomass were quantified colorimetrically with XTT and crystal violet assays, respectively. Biofilm susceptibility was also assessed (tested concentrations ranging from 0.5 mM to 1 M).

**Results:** Cerium MIC and MLC were 0.03 mM and 8 mM, respectively. Flow cytometry showed a considerable decrease in cellular metabolic activity, after 3h of incubation with 8 and 50mM, accompanied by a definitive membrane injury.

Cerium nitrate was able to impair efficiently biofilm formation at 8 mM. Treatment of pre-formed biofilms with cerium resulted in 90% reduction in the biofilm metabolic activity with 500 mM, and 92% of total biomass disaggregation with 16 mM.

**Conclusions:** Cerium nitrate exhibits not only antimicrobial but also anti-biofilm activity against *C. albicans,* being a promising agent for prevention or treatment of candidosis associated with medical indwelling devices.