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Innovative strategies to counteract Healthcare-Associated infections (HAIs): Antimicrobial properties of engineered materials for prosthetic use

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INTRODUCTION

Healthcare-Associated infections (HAIs) are those infections that patients get while or soon after receiving any health care activity and which were not present or incubating at the time of the treatment. Formerly called “hospital” or “nosocomial” infections because hospital was and is still the most common environment at risk to get this kind of infections. Nowadays, the wider denomination of HAIs includes infections acquired due to any type of assistance (hospital admission, day-hospital and day-surgery activities, home healthcare assistance). Specifically, hospital-acquired infections, are those infections occurring in a patient during their stay in hospital or after discharge, which were not present or incubating at the time of admission, that first appear 48 hours or more after hospitalisation or within 30 days after having received health care. These infections represent one of the leading sources of morbidity and mortality globally and are the second most common cause of death. Just think that for every hundred hospitalised patients, at least seven in high-income countries and ten in low-incoming or emerging economies have at least one type of HAIs. They also pose a huge problem in terms of economic expenditure, as they affect the time of departure and therefore directly or indirectly also the costs. Applying adequate prevention and control strategies for these infections by health professionals can, of course, reduce their incidence. The huge problem of HAIs is further exacerbated by the big challenge of antimicrobial resistance (AMR), a concern that has become increasingly common worldwide. These two serious conditions go together because very often the microorganisms

responsible for HAIs are at the same time antimicrobial-resistant, and since few new anti-microbials have been developed and placed on the market in recent times, the challenge of even more effective prevention activities becomes a crucial point. Nevertheless, an effective approach to containing HAIs must act on several fronts, which can include classic activities such as hand hygiene, a hygienic hospital environment, screening and categorising patients into cohorts, public health surveillance, antibiotic stewardship, and following good practices and safety guidelines. However, some innovative strategies could play a very crucial role in reducing the burden of HAIs and, in this context, the use of innovative materials with intrinsic antimicrobial activity for the manufacturing of medical devices could represent a turning point in the prevention of these infections. The objective of my doctoral course was to test new materials, which can be used for implantable prostheses, with intrinsic antimicrobial activity. In order to reduce the risk of contamination thereof, aiming to reduce this type of infections.

HAIs EPIDEMIOLOGY

HAIs (Alrebish et al., 2022) represent one of the leading causes of morbidity and mortality globally and the second leading cause of death globally. (Haque et al., 2020). They are linked to long-term damage, longer hospitalisations, higher rates of antibiotic resistance, and additional financial burdens (Allegranzi et al., 2011). Their impact is so powerful that their ability to impact quality of life has been demonstrated in a similar way to psychological trauma and long-term impairment (Koch et al., 2015). Approximately 7% of patients in high-income countries and 10% in those with low incomes or emerging economies acquire at least one HAI during their hospitalization, and even 10% of these also face death (World Health Organisation, 2016; Khan et al., 2017; Haque et al., 2018).

The ECDC reports more than 3.5 million annual cases of HAIS in the European Union and the European Economic Area, causing about 90,000 deaths and 2.5 million DALYs (corresponding to 501 DALY per 100,000/population). Of these infections, even 71% are caused by antibiotic-resistant bacteria, but more than 50% of these could be prevented (https://www.ecdc.europa.eu/en/healthcare-

associated-infections). The prevalence rate of HAIs in Lower- and Middle-Income Counties (LMICs) has been between 5.7 and 19.1% (Klevens et al., 2007; World Health Organisation, 2016). Globally, however, more than 1.4 million patients are acquiring at least one HAI in both developed and emerging countries, causing a considerable financial burden at the individual, community, and public levels (World Health Organisation 2009). In Italy, several studies have found an incidence rate of HAIs of 5-10% and a mortality rate of up to 20-30% (Messineo et al., 2015; Mancini et al., 2016). In Sicily, approximately 677 deaths per year are attributable to HAIs among people aged 45 years or older (Barchitta et al., 2021).

According to the Global Alliance for Infections in Surgery, HAIs include urinary tract infections (CAUTI's), central line-associated bloodstream infections (CLABSI's), Ventilator-Associated pneumonias (VAPs), Hospital-Acquired Pneumonias not associated with mechanical ventilation (HAPs), *Clostridium difficile* infections, and surgical site infections (Global Alliance for Infections in Surgery, 2022). The wide spread of this type of infections and the resulting phenotypic multiplicity are due to the fact that hospital environments can host pathogenic or opportunistic microorganisms that can infect a multitude of susceptible subjects, triggering the infection. This occurs extremely easily in controlled environments with immunocompromised patients and in operating rooms where a variety of risk factors can be present, such as inefficient ventilation systems and health workers who do not adhere perfectly to the measures for control of infections, and that can cause the onset of an HAI in weakened and unhealthy patients (Caraggiano et al., 2014). Nevertheless, it is crucial to apply a variety of prevention techniques to avoid the occurrence of HAIs.

MICRORGANISMS RESPONSIBLE FOR HAIS

Any type of microorganism can be cause of HAIs even if bacteria are the most common and typical etiological cause. A certain group of HAIs are also caused by fungi (especially yeasts belonging to the genus *Candida* spp.). (Szabó et al., 2022). Bacteria are often associated with a phenotype of resistance, which arises due to an excessive and irresponsible use of antibiotics especially in the hospital

setting. AMR is a complex phenomenon that is expressed by different mechanisms of action (inhibition of cell wall synthesis, DNA replication, and protein syntheses) (Awad et al., 2012). These microorganisms are often belonging to a group called "ESCAPE" by the Centres for Disease Control and Prevention (CDC), which includes vancomycin-resistant *Enterococcus faecium* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), *Clostridioides difficile*, multidrug-resistant (MDR) *Acinetobacter baumannii*, MDR *Pseudomonas aeruginosa* and *Enterobacteriaceae* including MDR *Klebsiella pneumoniae* (Medina et al., 2016). These bacteria found in hospital environments are often multidrug-resistant (MDR) or pandrug-resistant (PDR) (Correa-Martinez et al., 2020; Bezhadi et al., 2021; Mitevska et al., 2021) and can colonise operating rooms, surfaces, and medical devices, causing fearful infections that are difficult to fight due to the lack of new available antibiotics Facciolà et al., 2019). VRE and MRSA are the main Gram-positive bacteria of concern, while MDR *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae,* and *Enterobacter* spp. are the major resistant Gram-negative bacteria (Suetens et al., 2018; Levinson et al., 2020; Szab et al., 2022). For MRSA, which accounts for more than 50% of *S. aureus* strains isolated in hospitals in the US responsible for nosocomial infections (Levinson et al., 2020), the main transmission route is contaminated hands of healthcare workers. (Thompson et al., 1982). In the case of Gram-negative bacteria, however, a special concern is represented by the detection of species resistant to carbapenems, and belonging to both *Enterobacteriaceae* (e.g., *K. pneumoniae*, *E. coli*, *Enterobacters* spp*., Serratia* spp.) overall called Carbapenem-resistant Enterobacteriaceae (CRE) and non-fermenters (e.g., *P. aeruginosa* and *Acinetobacter baumannii*). Among all these strains, K. *pneumoniae* is by far the most commonly detected species (WHO 2017).

ROUTES OF TRANSMISSION

When dealing with HAI spread during health care, it is important to talk about pathways of transmission, i.e., the way in which a microorganism in a hospital environment spread and infects a susceptible host, represented by an already

defamed and immunodepressed patient. For HAIs, the most involved transmission modes include:

- Direct contact;
- Indirect contact;

Direct contact transmission involves the physical transfer of microorganisms through skin-to-skin contact. Patients can become infected by touching wounds or mucous membranes with hands colonised by microorganisms typical of the skin microbiota or contaminated by various infected body fluids (blood, urine, feces). Direct contact can also take place with healthcare workers (HCWs) and visitors. A patient may be visited up to 18 times per hour by HCWs or visitors. At least 27% of these visits involve physical contact (Infection Prevention and Control Practice Handbook, 2020). Therefore, there are plenty of opportunities for patients to be exposed to and infected by microorganisms innocuously carried by HCWs and visitors.

Indirect contact transmission involves the initial transfer of microorganisms from a host individual to an intermediate object or environmental matrix and, then, the further transfer to another individual. The classic example is the unwashed hands of the HCWs or the not often replaced gloves, which act as indirect contact transmission mediators (Hayden et al. 2008; Bache et al. 2013). Another example is represented by reusable medical devices that are not properly sanitised between patients (Kovaleva et al., 2013; Dirlam Langlay et al., 2013). As regards environmental matrices that can serve as a vehicle, we can consider air and surfaces within inpatient and operating rooms. In similar environments, especially when not properly aired, can occur a type of transmission by indirect contact such as that mediated by droplets, breathing drops emitted by sneezing, coughing, or talking, with a diameter of 30-50 μm, able to carry microorganisms from an infected individual and allow it to colonise other individuals or surfaces (Douedi S. & Douedi H., 2024). Larger droplets can travel only for very short distances (≤ 1 meter) before depositing and contaminating surfaces (Coia et al., 2013) or come into contact and potentially infect susceptible individuals. In any case, this type of transmission requires close contact between the infected host and other susceptible individuals. A similar type of transmission practicable in these environments is also

indirect transmission through the diffusion of small aerosols that can carry microorganisms. Aerosols are much smaller than droplets, so they can travel longer distances and stay in the air column for longer. Aerosol is produced by coughing, speaking, and breathing, as well as during clinical aerosol generation procedures such as suction, intubation, and chest physiotherapy (Tran et al., 2012). These aerosols can travel long distances and remain suspended in the air for prolonged periods of time, serving as the perfect vehicle for airborne diseases.

COMMON TYPES OF HAIs

The most common types of HAIs basically include:

- Urinary tract infections (CAUTIs);
- Central line-associated bloodstream infections (CLABSIs);
- Ventilator-associated pneumonia (VAPs);
- Hospital-acquired pneumonia not associated with mechanical ventilation (HAPs);
- *Clostridioides difficile* infections (CDIs);
- Surgical site infections (SSIs).

CAUTIs are urinary tract infections that occur in patients whose bladder has been catheterised within the last 48 hours. This type of HAIs is the most common, with about 1 million cases per year in the US, and represents the primary cause of secondary bloodstream infections (Werneburg 2022). The costs associated with preventable CAUTIs range from $115 million to $1.82 billion per year (Umscheid et al., 2011). Among urinary tract infections acquired in the hospital, about 75% are associated with a urinary catheter. It has been reported that in total, between 15 and 25% of hospitalised patients are forced to use urinary catheters during hospitalisation (Global Alliance for Infection Surgery, 2022). The most common microorganisms capable of causing complicated UTIs, wherein CAUTIs make up the majority of cases, are *Enterococcus spp*. (11%), *K. pneumoniae* (8%), *Candida spp*. (7%), *S. aureus* (3%), *Proteus mirabilis* (2%), *P. aeruginosa* (2%), and *Streptococcus group B* (2%) (Werneburg 2022). The most important risk factor for

the development of CAUTIs is prolonged use of the urinary catheter. The Global Alliance for Infections in Surgery (2022) has proposed a bundle for the prevention of catheter-associated urinary tract infections that includes:

- Insert the catheter only if it is absolutely necessary for the patient;
- Ensure maximum sterile barrier precautions upon insertion;
- Use standard precautions, including hand washing, during any manipulation of the catheter;
- Maintain an aseptic, continuously closed urinary drainage system;
- Remove the urinary catheter as soon as it is no longer needed.

A CLABSI is a primary infection of the bloodstream in patients to whom a central venous catheter was applied more than 48 hours before the onset of the infection, confirmed by the detection of a pathogen from a blood culture. An estimated 250,000 bloodstream infections occur annually and their management involves very high costs, which correspond to about $46,000 per case (Haddadin et al. 2022). The microorganisms most involved in CLABSIs are:

- Gram-positive bacteria (coagulase-negative staphylococci, 34.1%; *enterococci*, 16%; and *Staphylococcus aureus*, 9.9%);
- Gram-negative bacteria (*Klebsiella*, spp. 5.8%; *Enterobacter* spp., 3.9%; *Pseudomonas* spp, 3.1%; *E. coli*, 2.7%; *Acinetobacter*, 2.2%), *Candida* species (11.8%), and others (10.5%) (Atilla et al., 2017; Wright et al., 2018).

The use of these venous catheters is essential for the administration of fluids, blood products, medicines, nutritional solutions, and haemodynamic monitoring, but being the main source of complications in hospitalised patients should be used only if really necessary. The site of insertion of the catheter affects the risk of catheter-related infections; femoral catheters are associated with a higher risk of infection and deep vein thrombosis compared to internal jugular or succlavial catheters. The Global Alliance for Infections in Surgery (2022) has proposed a bundle for the prevention of CLABSI that includes:

- Insert the central-line catheter only if it is absolutely necessary for the patient;
- Ensure maximum sterile barrier precautions upon insertion;
- Select the optimal site (avoid femoral vein in adults);
- Clean hands and respect infection prevention and control principles;
- Perform a daily review of central line necessity and remove promptly unnecessary lines.

A VAP is a lung infection that develops in patients who undergo mechanical ventilation for more than 48 hours. It is the second most common hospital-acquired infection among paediatrics and neonatal intensive care unit (ICU) patients (Kohbodi et al., 2023). This type of pneumonia is typically caused by causative agents of bacterial origin, and recently polymicrobial infections are on the rise. The most frequently causing organisms are *S. aureus* (28.4%), *P. aeruginosa* (25.2%), and other Gram-negative organisms (26.6%). (Iosifidis et al., 2018; Jain et al., 2023). The Global Alliance for Infections in Surgery (2022) has proposed a bundle for the prevention of ventilator-associated pneumonia infections that includes:

- Clean hands and respect infection control and prevention principles;
- Perform oral care and aspire to subglottic secretions;
- Control and maintain an optimal cuff pressure;
- Avoid elective changes of ventilator circuits and endotracheal tubes;
- Reduce the duration of mechanical ventilation when it is possible.

A HAP is also a nosocomial pneumonia that occurs after 48 or more days after hospitalisation and that is not present at the time of hospital admission but, unlike VAP, it is not caused by mechanical ventilation. Common pathogens of HAPs, which are similar to those of VAPs, include *P. aeruginosa*, *E. coli*, *K. pneumoniae*, *Enterobacter* spp., *Acinetobacter* spp. as Gram negative, and *S. aureus* and *Streptococcus* spp. as Gram positive. The etiological agent can therefore be varied and is fundamentally influenced by endogenous vectors of the patient and hospital contaminations (Kalil et al., 2016). HAPs affect 5 to 10 patients per 1,000 hospitalisations and they are considered the most common cause of acquired hospital infection in Europe and the US (Shebl & Gulick, 2023). The Global

Alliance for Infections in Surgery (2022) has proposed a bundle for the prevention of hospital-acquired pneumonia infections that includes:

- Clean hands and respect infection prevention and control principles;
- Perform oral care for the patients;
- Prevent aspiration;
- Change bed position;

CDIs are primary health-care-related infections caused by *C. difficile*, a Gram-positive and sporogenic bacterium that can colonise the gut of healthy people without causing infections and whose spores can survive on hospital surfaces and on the soil for prolonged periods. Contaminated surfaces and medical equipment in healthcare facilities can become reservoirs for spores of *C. difficile*, which must be destroyed through appropriate cleaning protocols (Carling et al., 2023). The most important risk factors for the onset of CDIs are the use of broad-spectrum antibiotics, followed by use of proton pump inhibitors, advanced age, immunosuppression, previous CDIs, , prolonged hospitalisations, inadequate control of infections, and unoptimal antibiotic management in healthcare (Mada & Alam, 2024). However, the best strategies for containing this kind of HAI are the prudent use of antibiotics, hand hygiene practices, environmental cleanliness, early diagnosis, and proper case management (Carling et al., 2023). The economic impact of this disease has been estimated to be $5 billion a year for the American health system (Dubberke & Olsen, 2012; Lessa et al., 2015). The Global Alliance for Infections in Surgery (2022) has proposed a bundle for the prevention of CDIs that includes:

- Enhance antimicrobial stewardship programs;
- Detect all cases and activate surveillance;
- Clean hands and use protective equipment;
- Clean and disinfect the environment;
- Educate staff and patients or visitors.

An SSI is an infection that occurs after surgery in the part of the body where the surgery took place. These infections may involve skin, subcutaneous tissues, organs, or implantable prostheses (Global Alliance for Infections in Surgery, 2022). The CDC has classified SSIs into:

- infections of superficial incisions that affect the skin and subcutaneous tissue.
- infections of deep incisions that affect the fascial and muscle layers.
- organ or space infections, which involves any part of the anatomy other than the incision that is opened or manipulated during the surgical procedure, for example, the joint or peritoneum. (National Collaborating Centre for Women's and Children's Health, 2008).

The risk factors that increased the chance of contracting this type of HAI are:

- Operations in parts of the body with a high concentration of microbial flora (such as the gut);
- Extended operations that increase tissue exposure time;
- Comorbidity (diabetes, malnutrition, cancer).

The bacterial general most commonly associated with these HAIs are *Staphylococcus* spp., *Streptococcus* spp., and *Pseudomonas* spp.

The Global Alliance for Infections in Surgery (2022) has proposed a bundle for the prevention of surgical site infections that includes:

- Have the patients take preoperative bathing/showering;
- Prescribe appropriate surgical antibiotic prophylaxis;
- Do not remove hair or remove hair immediately before surgery by clippers;
- Use a correct surgical hand scrub/preparation;
- Use a correct skin antiseptic preparation;
- Perform a correct post-surgery management of the wound (that has been demonstrated the most critical phase for the acquisition of a SSI)

In most instances HAIs are preventable, although the prevention and control of HAIs is highly complicated, and a multi-dimensional approach and strategies are required to address this significant public health concern. In fact, only through the integration of more preventive measures can the maximum reduction in the occurrence of HAIs be achieved. Among these approaches we have:

• Hand hygiene;

• Maintaining a safe, clean, hygienic hospital environment;

• Screening and categorising patients into cohorts;

• Public health strategies (surveillance, antibiotic stewardship, Following patient safety guidelines)

• Innovative strategies through the use of innovative materials for medical devices.

HAND HYGIENE

Hand hygiene (HH) is the simplest and most effective strategy to minimize the spread of infections in the hospital environment, as it is also effective against

antibiotic-resistant microorganisms, typical of HAIs. As early as the middle of the 19th century, researchers in Europe and the US realized that mainly HCWs could carry pathogenic and commensal microorganisms from one patient to another, causing infections (Haque et al., 2020). The hands of HCWs, in fact, are often colonised by MRSA and CRE, which can remain *in situ* vital for up to a maximum of 150 hours. In addition, the normal turnover of epithelial cells allows the elimination of 10,000,000 cells per day, which can transport microorganisms contaminating surfaces, objects, and the environment in the immediate proximity of the susceptible host (Global Alliance for Infection in Surgery, 2022). In addition, it is possible, through a lack of hand hygiene, even a cross-contamination between patients, simply by touching the patient during routine examinations, replacement of catheters, administration of drugs, blood samples, etc. To limit these risks, any healthcare professional must take care of the hygiene of his hands and must be able to perform it correctly and at the right time, so with a hydroalcohol solution if they are not visibly dirty or with water and soap otherwise. Generally, however, it is preferable to wash with water and soap. It is important to stress that the use of gloves cannot in any way replace the need to wash hands. According to the WHO, to limit the spread of pathogenic microorganisms in the hospital environment, hand hygiene must always be carried out:

- Before patient contact;
- Before aseptic task;
- After bodily fluid exposure;
- After patient contact and after contact with patient surroundings.

In literature, in fact, it has been widely reported that the strict practice of HH reduces nosocomial infections between 40% and 70% (Gawande, 2004; Kampf et al., 2009). Nevertheless, globally and in many hospital departments, regular HH by health professionals often does not exceed 40%, seriously endangering the health of patients (Schiffers et al., 2015; Geberemariyam et al., 2018; Longembe et al., 2020). Non-compliance with the HH guidelines is therefore a global phenomenon, which somehow contributes to the increased incidence of HAIs. The Joint Commission Journal on Quality and Patient Safety reports 24 reasons why HCWs do not perform strict HH (Chassin et al., 2015). The biggest problem following these studies is the lack of training of healthcare personnel on the need for strict

HH, which often leads to inadequate practices in ensuring and promoting HH as a key priority and a lack of understanding of how this is essential to high staff and patient safety. For example, some health professionals felt that wearing sterile gloves meant that HH was not necessary or that the requirements of HH set by the hospital management were too extreme. Following that, the Joint Commission proposed five essential plans for improving HH, using the acronym 'HANDS': H = "Habit," A = "Active feedback," N = "No One Excused," D = "Data-driven," S = "Systems" (Beckers; 2014). In this way, it aims to generate good hygiene habits in HCWs so that they wash their hands and maintain health hygiene as an automatic behaviour "upon entering or leaving a patient care area, as well as before and after patient care." According to Alsheari et al. (2018), a range of strategic methods is needed to raise HCWs' compliance regarding HH to an adequate level, but implementing all these might not be possible. Various interventions are suggested, such as the establishment of corporate educational programs, monitoring and feedback, ensuring logistical support, and improving access to HH agents (Alsheari et al., 2018).

Another systematic review concluded that electronic and video surveillance systems could be very effective in improving HH practice and prevention (Sringley et al., 2013) and thus controlling the spread of HAIs. The fault of such methods is that they are costly, so they may not be applicable to many hospitals, especially in low- and middle-income countries (LMIC). In 2005, the WHO and World Alliance for Patient Safety started a movement, the First Global Patient Security Challenge—"Clean"Care is Safer Care," (WHO 2009) aimed at improving HH in the healthcare system. This campaign, known as WHO-5, encourages a multimodal plan comprising five different elements: "system change, training and education, observation and feedback, reminders in the hospital, and a hospital safety climate" (Luangasanatip et al. 2015). Luangasanatip et al. (2015) have demonstrated how the use of the WHO-5 approach improved adherence to HH guidance among HCWs and highlighted that a strategy to improve HCWs' compliance with handwashing could be to clearly define desired goals, reward health professionals who achieve the goals with financial incentives, and stimulate the accountability of health professionals, regardless of their position. (Luangasanatip et al. 2015). Unfortunately, interventions to improve HH practice based on "knowledge,

awareness, control of action, and facilitation are not enough to change" HH practices (Huis et al., 2012). Although HH plays a fundamental role in preventing the spread of pathogens at the hospital level, it is unfortunately necessary to study and implement ever new strategies to allow adequate adherence of hospital staff to these norms of behaviour in order to increase the effectiveness of this practice.

ENVIRONMENTAL HYGIENE

Of course, another essential task for the prevention and control of HAIs is represented by environmental hygiene (Moffa et al., 2017). In fact, hospital surfaces can be contaminated by patients or HCWs with both microorganisms belonging to their own microbiota and with potentially antimicrobial- resistant pathogenic microorganisms (Leistner et al., 2023). That is why the daily cleaning of the frequently touched surfaces represents a gold standard of procedures aimed at the transmission of pathogens to vulnerable patients (Lax & Gilbert, 2015). In fact, hospital surfaces can serve as reservoirs and sources of transmission for microorganisms such as *C. difficile*, MRSA and VRE (Chemaly et al., 2014; Rupp et al., 2017; Kenters et al., 2018). Particularly at risk to be contaminated are surfaces and objects placed in close contact to patient as bed bars and sheets, bedside table and all is placed around the patient. Moreover, the so-called high-touched surfaces, as door and window handles, bathroom taps, light switches etc. can represent important reservoir. Other devices that have been highly studied as potential sources of pathogens causing HAIs are both tools used for the daily care of patients

(thermometers, blood pressure monitors, phonendoscopes etc.) and all the equipment represented by keyboards of machines for monitoring vital parameters (especially of patients in ICU), keyboards and mouse of personal computers, telephone handsets etc. For all these devices, daily sanitation and disinfection are crucial. The products used for hospital cleaning can contain approximately 275 different components and can be available in various formulations (spray, liquids, concentrated powders, gases) (The United States Environmental Protection Agency, 2014). There are currently three major strategies used for the manual maintenance cleaning of surfaces: soap-based (Bogusz et al., 2013; Stewart et al., 2014, 2018) and probiotic-based cleaning (Klasset et al., 2022). However, it is important that the cleaning done is not superficial, as negligence of this type could have a negative effect, dispersing the microorganisms on a larger surface and increasing the possibility that they may contaminate other objects. The cleaning must therefore be carried out in a standardised and careful manner, with means that ensure an adequate level of cleaning. For non-spore bacteria and viruses with envelopes, usually the removal of dirt from a surface is sufficient to inactivate them, as they lose an element that served as their protection against drying. It is different when we are dealing with microorganisms. Then different cleaning methods will be used, depending on the need for a total absence of microorganisms or not in a given environment. For example, disinfection can be performed if you want to eliminate all the vital microorganisms, but this practice does not allow the elimination of any bacterial spores present on inanimate objects. If a sporicidal effect is sought, sterilisation processes will be used. For example, ethylene oxide in gas can be used in case you want a sterilising effect, while hydrogen peroxide at 7.5%, isopropyl alcohols, and quaternary amino salts for a disinfectant action (Haque et al., 2020). In any case, there is no ideal disinfectant, and it is appropriate to choose the best compromise (between antimicrobial activity and the toxicity of the product) depending on the situation. In any case, this preventive measure also plays a crucial role in the transmission chain of HAIs.

Public health surveillance obviously plays a key role in the management and monitoring of HAIs. It consists of a continuous collection, analysis, interpretation and dissemination of data to be used to trace an exhaustive epidemiological picture about the spread of HAIs (incidence, prevalence) in a particular health setting to implement specific strategies to reduce morbidity and mortality. Van Bunnik et al. (2015) have demonstrated how the recognition time of a HAI can be reduced by using active surveillance and through greater adherence of hospitals to this system. The effectiveness of a surveillance system has also been confirmed in Germany and India (Zuschneid et al., 2010; Agarwal et al., 2017). The biggest problem in the surveillance system is given solely by the financial and practical constraints because often a specifically dedicated and expert personnel is necessary, but implementation of these systems at the global level will allow for better management of HAIs over time, helping the action of all other containment strategies.

ACTIVE SURVEILLANCE CULTURES AND ISOLATIONS

The two previous approaches, as widely discussed, despite a prominent effectiveness often do not prove sufficient to curb the phenomenon of the spread of HAIs, (Li et al., 2017; Irek et al., 2018). Therefore, additional and complementary strategies aimed at minimizing and controlling HAIs can be the active surveillance cultures (ASCs) or universal or targeted microbiological screening cultures for patients admitted to a hospital (McGinigle et al., 2008), which can be followed by the isolation of patients colonised by high-risk pathogens from susceptible subjects (Tacconelli 2009). This method can be effective in curbing the spread of HAIs, but its implementation is not easy, both for ethical and logistical/management issues. Therefore, considering these issues, a cost-benefit assessment is appropriate before implementation. Several countries have succeeded in reducing infectious outbreaks of MRSA by using “search and destroy” strategies at the national level (Vos et al., 2005), which consist of:

- Isolation of positive patients;
- Assessment of high-risk cases;
- Screening of patients and staff;
- Assessment of HCWs on leave as potential carriers;
- Total decontamination;
- Blocking of new admissions in a ward where one or more infected persons are present;

Numerous studies have suggested that patient isolation, ASCs, and staff screening can reduce the transmission of MDR pathogens (Duffy et al., 2011; Chemaly et al., 2014). These practices can be very useful especially for those units where particularly vulnerable patients are admitted such as ICU, oncology hematology and geriatric units etc, and can be achieved through the research of MDR microorganisms in rectal or cutaneous swabs soon before the admission. This practice is already common in many countries and can be an important tool for reducing the hospital transmission between patients thanks to the isolation and cohorting of those patients resulted positive to the analysis.

ANTIMICROBIAL STEWARDSHIP

Given that microorganisms causing HAIs show higher resistance rates to antibiotics than those causing community-acquired infections, antimicrobial stewardship also plays an important role in fighting these infections. In fact, it is nothing more than a set of coordinated strategies to improve the use of antimicrobial drugs with a view to improving patient health, reducing the phenomenon of antibiotic resistance, and reducing health costs. (The Society for Healthcare Epidemiology of America, 2019). These strategies also allow the correct selection of the dosage and duration of the antimicrobial treatment, which translates into a clinical outcome or a perfect prevention of infection with minimal toxicity for the patient and a minimal impact on the development of resistance (the right antibiotic for the right time and dose). The seven fundamental elements of the AS are:

- Leadership commitment;
- Accountability;
- Drug expertise;
- Action;
- Tracking;
- Reporting;
- Education (CDC, 2015)

Through these core elements, the AS aims to:

- A conscious use of antibiotics (correct antibiotic with precise targets, at the right doses, in the right timetables);
- Stop the excessive use of antimicrobials in the hospital and in the community;
- Block the phenomenon of antibiotic resistance. (Haque et al., 2020).

These results can be achieved by preventing doctors from prescribing antibiotics without prior authorisation and by assessing the spectrum of resistance of the infectious agent in the laboratory in such a way that only antibiotics suitable for treatment are used. (Doron & Davidson, 2011). Using these strategies reduces the incidence of infectious diseases in hospitalised patients and increases satisfaction regarding the quality of care in both doctors and patients (Fraser et al., 1997; Solomon et al., 2001; Baur et al., 2017). The introduction of AS is associated with a decrease in the use of antibiotics and overall health costs, with a significant reduction in the incidence of certain microbes (Timbrook et al., 2016).

FOLLOWING PATIENT SAFETY GUIDELINES

The correct implementation of HAIs guidelines, checklists, and control policies is an essential part of the fight against these dreadful infections. Despite this

importance, they are often interpreted and implemented in different ways, influenced by the perception of the individual, departments, and organisations based on local influences and practices. (Collins 2008; Carthey et al., 2011; Treadwell et al., 2013). This often results in a low or incorrect adherence of doctors to the guidelines, which inevitably leads to the failure of HAIs counter-strategies. (McInnes et al., 2014). In fact, strategic failure can also be attributable to excessive softness or a lack of clear information or guidelines that are contrary to other guidelines or too complicated to implement. Gerber et al. (2014; Ament et al. (2015). As mentioned earlier, the control and prevention of HAIs is therefore achieved through a broad and integrated approach, with cooperation between health facilities, public health authorities, health insurance, quality management and patient safety organisations, educational institutions, the public, and the veterinary sector (Gastmeier 2010).

INNOVATIVE MATERIALS

The use of innovative materials to coat or design surfaces, medical devices, or personal protective equipment can be another strategy to counter HAIs. Designed materials can basically counteract microorganisms either through an antiadhesive effect or through a biocidal/biostatic effect (Sheridan et al., 2022). An antiadhesive effect is achieved when a reduction in adhesion strength is generated between the solid surface and bacteria, which can then be easily removed before producing biofilms, a goal that can be achieved with superhydrophobic surfaces, zwitterionic polymers, and tailoring of surface nanostructure (Yang et al., 2013; Zhang et al., 2013).

While the antimicrobial effect is achieved through the release or non-release of a biocide that can kill the microorganism. These materials, which counteract bacterial viability, can also achieve the goal through various strategies, such as the use of quaternary ammonium compounds (QACs), nanoparticles, hydrogels, polymers (Elena & Miri 2018; Ealia & Saravanakumar 2017; Liu et al., 2022; Tang et al., 2023).

QACs are composed of nitrogen (N+)-containing compounds in which the N atom is attached to four different groups by covalent bonds. Most QAC salts are predominantly composed of chloride or bromide salts, while iodide salts tend to exhibit reduced solubility, and are therefore more difficult to use (Jiao et al., 2017). They are cationic and antimicrobial surfactants lethal to a wide variety of organisms, including vegetative cells of gram-positive and gram-negative bacteria, fungi, parasites and enveloped viruses (Haldar et al., 2006; Kenawy et al., 2007; Oblak et al., 2013). QACs are used for contact- or release-based applications since quaternary groups can be covalently bound to the polymer chain or electrostatically to opposite ions of a polyanion. They are used in antimicrobial research because they are considered environmentally suitable (Druvari et al., 2021). The antimicrobial effect is given by the ability of these compounds to cause a disruption of the cell membrane (Wessels & Ingmer, 2013) and is influenced by the length of the N -alkyl chain (Caillier et al., 2009; He et al., 2013). QACs can be used to form antimicrobial polymers, the efficiency of which is influenced by the charge density of the polymer and the alkyl chain length of the cationic fractions (Timofeeva & Kleshcheva, 2011; Gao et al., 2017). Quaternary ammonium groups with ten or more carbon atoms are preferable because they can interact more strongly with the lipid bilayer of microbial membranes, thus resulting in higher bactericidal activity (Li et al., 2013; Li et al., 2018). However, excessive chain length may have disadvantages in QAC-containing polymers such as aggregation, which would decrease the antimicrobial effect or haemolytic activity, which would affect biocompatibility; as well as the ratio between cationic and hydrophobic components; in fact, excessive hydrophobicity would cause high toxicity and poor solubility, while low hydrophobicity would lower antibacterial activity and cause hemoglutination (Druvari et al., 2021). The QACs that have shown the best performance in various studies are dodecyltrimethylammonium bromide (DTAB) and N , N -dimethyldodecylamine (DDA) (Smith & Lambert, 2008; Sharma et al., 2017; Ryšánek et al., 2019; Zhu et al., 2020).

NANOPARTICLES

Nanoparticles (NPs) are nano-objects that are entirely nanometric in size (Joudeh & Linke 2022). NPs can have different shapes (spherical, cylindrical and conical), sizes (as long as they remain between 1 and 100 nm in diameter) and can be composed of one or more layers (Ealia & Saravanakumar 2017). They can be synthesised using multiple techniques, which are classified into bottom-up or top-down methods (Ealia & Saravanakumar 2017). Bottom-up methods consist of starting from the atom to the synthesis of NPs (the sol-gel process, spinning, chemical vapour deposition and biosynthesis), while the top-down method is based on the reduction of a bulk material to NPs through mechanical milling, nanolithography, laser ablation, sputtering and thermal decomposition. Based on their constituents, they can be classified into metal-based nanoparticles (which include gold nanoparticles, iron nanoparticles, silver nanoparticles), metal oxide-based nanoparticles (copper oxide nanoparticles, titanium oxide nanoparticles iron oxide nanoparticles, zinc oxide nanoparticles, magnesium oxide nanoparticles, cerium oxide nanoparticles), carbon-based nanoparticles (graphene, fullerenes and carbon nanotubes), organic nanoparticles (chitosan) (Joudeh & Linke 2022) and ceramic/semiconductor nanoparticles (carbonates nanoparticles, phosphates nanoparticles, metalloids nanoparticles, CdS nanoparticles and ZnS nanoparticles). Their antibacterial activity is expressed in different ways that depend fundamentally on the nature of the particles themselves. In general, the bactericidal effect can be expressed through: Mechanical membrane damage (Nisar et al., 2019), release of harmful ions (Yin et al., 2012; Slavin et al., 2017), generation of reactive oxygen species (Manke et al., 2013; Ameh et al., 2022), alteration of protein expression (Roguska et al., 2015; Su et al., 2015).

HYDROGELS

Hydrogels are a class of highly hydrophilic polymers with a three-dimensional porous structure, which are formed by physical or chemical cross-linking of

polymer chains (Zhang & Khademhosseini, 2017; Liu et al., 2018), and which are widely used as active carriers for antimicrobial materials due to their similarity to human tissue structure (Cao et al., 2013). They possess soft texture, high water retention capacity, excellent biocompatibility (Suneetha et al., 2022), and lend themselves to being loaded with antibacterial agents that are effectively released, thus greatly improving performance and biocompatibility (Hu et al., 2017; Mensah et al., 2023). The different antibacterial agents that can be incorporated into the antibacterial hydrogels are: antibiotics; antibacterial polymers; nanoparticles, peptides, plant extracts (Tang et al., 2023). Hydrogels, based on antibacterial therapeutic strategies, can be summarised in the following categories:

- Antibiotic-loaded hydrogels;
- Antibiotic-free loaded hydrogels;
- Stimulus-sensitive smart antibacterial hydrogels;
- Light-mediated antibacterial hydrogels.

The latest frontiers of scientific innovation have led to the development of intelligent antimicrobial hydrogels that can be applied to wounds, i.e. new functional polymeric materials that are able to respond to external stimuli, which have antibacterial effects but also promote healing of drug release on demand (Chen et al., 2023). These smart hydrogels can undergo physical and chemical changes through reaction to external stimuli and have been applied in the pharmaceutical and medical fields, particularly for antimicrobial applications, allowing for slower and more controlled antibacterial release (Qiu, 2001; Wang et al., 2018). According to their sensitivity to the external environment, intelligent hydrogels can be classified into thermosensitive hydrogels, lightsensitive hydrogels, pH-sensitive hydrogels, enzyme-responsive hydrogels, and salt-sensitive hydrogels (Chene et al., 2023). These hydrogels can be used, for example, to fight wound infection (Pérez-Rafael et al., 2021; Tavakolizadeh et al., 2021; Crivello et al., 2023 et al.) or they can be part of a more complex structure. For example, Ribeiro et al, (2024) synthesised a three-layer system: the outer layer consisting of a fibrous film of polycaprolactone (PCL), which acts as a barrier to prevent microorganisms and impurities from reaching the wound; the middle layer consisting of a sodium alginate (SA) hydrogel loaded with ampicillin (Amp) to fight infection; and the inner layer consisting of a fibrous film of PCL and polyethylene glycol (PEG) to

facilitate cell recognition and prevent adhesion to the wound. The system demonstrated excellent absorption capacity of exudates (≈70 %), a controlled release of Amp for up to 24 hours, a great antimicrobial effect against *S. aureus* and *E. coli* and good biocompatibility on the human HaCaT cell line.

ANTIMICROBIAL POLYMERS

Even polymers as such can be used to make an antimicrobial coating on medical devices, the most commonly used being cationic polymers, i.e., environmentally friendly antibacterial agents that are not prone to causing drug resistance. They have attracted attention in the field of antimicrobial polymers due to their high effectiveness in inhibiting the growth of drug-resistant microbes. Their bactericidal effect is expressed by an initial electrostatic attraction with the bacterial cell membrane, followed by hydrophobic insertion into the lipid tails, and subsequent membrane lysis (Qiu et al., 2020). This is possible because they usually contain positively charged functional groups such as amine and guanidine in the molecules, whereas bacterial membranes are negatively charged. These polymers will only have a detrimental effect on the bacterial membrane when the cationicity reaches a certain amount. Since Gram-negative bacteria have an additional outer membrane that forms a barrier for large hydrophobic molecules, most cationic polymers have a lower potency against Gram-negative bacteria than against Gram-positive bacteria (Silhavy et al., 2010; Cox and Wright, 2013). However, cationic polymers still seem to have an effect on the LPS of Gram-negatives. There are also cationic polymers that act by inhibiting cell wall synthesis, as they target lipid II, an essential precursor for peptidoglycan synthesis (Malin and de Leeuw, 2019). Qiu et al. (2020) point out that the most promising antibacterial peptides for coating medical devices are:

- Polypyridine derivatives (PPy), which are heterocyclic cationic polymers containing quaternary ammonium groups.
- Poly ionic liquids, which are polyelectrolytes consisting of both anionic and cationic groups on the repeating units of the main polymer chains. These

possess positive residues that can interact with the bacterial cell membrane via electrostatic interaction.

- Guanidine-functionalised polymers that are able to destroy the biological structure of bacteria by extracting calcium ions from the bacterial outer Gram - membrane and disrupting the cytoplasmic membrane. This is because calcium ions are cross-linking agents for LPS in the outer membrane and their absence will increase its permeability.
- Conjugated oligomers (COEs) that are composed of hydrophobic and cationic building blocks in the backbone. These hydrophobic components will insert and break the bacterial cell membrane.
- Dendritic Polyethylene imines (PEI) contain both the hydrophobic polyethylene main chain and the imine group at the end. They have a high positive charge density and usually the lower molecular weight ones have a poorer bactericidal function, but the larger molecular weight ones are more toxic to animal cells. So it is important to find the correct balance.
- Antimicrobial peptides (AMPs) are basic polypeptides polymerised from 20~60 amino acid residues, widely studied by researchers for their broad-spectrum antibacterial activity. They can be isolated from bacteria or animal tissue, or they can be artificially synthesised. Amphiphilic AMPs possess positively charged groups that allow them to bind the bacterial cell membrane, while their hydrophobic mass inserts into the bacterial phospholipid bilayer inducing lysis of the bacterial cell membrane.

POSSIBLE PRACTICAL BIOMEDICAL APPLICATIONS OF INNOVATE MATERIALS IN REDUCING THE OCCURRENCE OF HAIs

With regard to personal protective equipment, for instance, Bhattacharjee et al. highlighted the antimicrobial effect of cotton/silk tissues containing rGO and Ag/CuNP fibres against *E. coli*, *P. aeruginosa*, and *S. aureus*. The fabric is also highly biocompatible and therefore can be an excellent method to produce protective clothing such as gowns for healthcare personnel. The same can be said of protective masks, which in one study were coated with colloidal AgNPs and

showed antibacterial activity against S. aureus and E. coli (Hiragond et al., 2018). Lovato et al. (2023) also collected in a review a whole series of studies demonstrating the possibility of manufacturing Medical Gloves with intrinsic antibacterial activity by incorporating compounds with antimicrobial activity such as L-ascorbic acid, Xanthones, Gardine solution, poly(hexamethylene biguanide) hydrochloride, chitosan, chlorhexidine and quaternary ammonium salts. These materials can also be used to ensure a more effective hygiene of the hospital environment. Baumler et al. (2021) report how antimicrobial coatings with nanoparticles of silver, zinc, copper and titanium, but also with chitosan, peptides, and compounds (like QACs) on hospital surfaces, especially on high-contact surfaces, can compensate for poor hand hygiene. A recent study shows how the ventilation system can also be treated with copper nanoparticles to prevent the spread of pathogenic microorganisms through the air (Mekapothula et al., 2024). Obviously, these antimicrobial materials can also be used directly on the patient, but only if they have proven biocompatibility and low toxicity. For example, coatings on urinary catheters with silver-phenolated lignin nanoparticles and poly(carboxybetaine) zwitterions and silver/gold nanoparticles with dopamine-modified gelatin have shown antimicrobial and antibiofilm activity against both Gram-positive and Gram-negative bacteria and high biocompatibility in both in vitro and in vivo tests (Puertas-Segura 2024a; 2024b), making them excellent candidates for human application to fight CAUTIs. Even coatings applied to venous catheters have shown promising results. For example, Lorente et al. (2014a; 2014b; 2015) have demonstrated that the use of chlorhexidine-silver sulfadiazine impregnated catheters is associated with a decrease in CLABSIs. Also for the prevention of VAPs, it would be possible to use coated endotracheal tubes, as illustrated by Chen et al. (2022), who tell us that various coatings have been studied over time with promising results that included organic polymers, bacteriophages, sphingosines, silver nanoparticles, ZnO, Nanosilica, Methylene Blue (MB), Titanium Dioxide (TiO2) and antimicrobial peptides. SSIs, on the other hand, can be prevented by applying hydrogel to the wound, which is manufactured to promote healing and at the same time exert antibacterial activity (Jia et al., 2023). Wang et al. (2017) succeeded in synthesising a novel quaternized cellulose-based hydrogel, which could be used in the biomedical field due to its high strength, self-healing, and antibacterial properties against E. coli. However, there are other examples of

hydrogels applicable for wound management that can express their antibacterial activity through Alpha-poly-l-lysine antibacterial peptides, positively charged ε-polylysine, Arnebia extract, nanoparticles, chitosan, and zwitterionic sulfopropylbetaine (Zou et al., 2018; Lei et al., 2021; Liu et al., 2021; Tavakolizadeh et al., 2021; Qu et al., 2022; Chen et al., 2023; Crivello et al., 2023). When the surgical cut is necessary for the implantation of prostheses, the latter can also become infected. Therefore, the synthesis of materials with intrinsic antimicrobial activity that can safely integrate into the body district in which they are implanted may be useful. For example, at the cardiac level, it is possible to use pyrolytic carbon coated with a film of AgNPs as an artificial heart valve, which showed antibacterial activity against MRSA, *Streptococcus pyogenes*, *E. coli*, *K. pneumoniae, P. aeruginosa*, and *P. vulgaris*. (Angelina et al., 2017). If, on the other hand, the prosthesis is for a bone problem, a collagen scaffold encapsulating AgNPs and bone morphogenetic protein 2 (BMP-2) can be used (Sun et al., 2015). This material exhibits high osteoconductivity and an antibacterial effect against MRSA. Dalavi et al. (2020), on the other hand, used particular microspheres, called COS-Ag-Alg-HA, containing AgNPs-coated chitooligosaccharide (COS), alginate (Alg) and hydroxyapatite (HA), as bone graft substitutes to aid in the removal/prevention of infections in the orthopaedic field caused by *S. aureus.*

EXPERIMENTAL PART

AIM OF THE THESIS

The aim of this doctoral thesis was to test two engineered materials, that can be used for the manufacturing of implantable prostheses, and, specifically, to evaluate their antibacterial and antibiofilm activity using different approaches. This in order to be able to hypothesize their possible future use in healthcare aimed at reducing infections related to the implantation of medical devices. Specifically, the efficacy of a hydrogel of Chitosan-Polyhedral Oligomeric Silsesquioxanes (chitosan-POSS) and that of titanium discs, the surface of which has been functionalised with quaternary ammonium salts (QAS), will be discussed.

CHITOSAN/POSS HYBRID HYDROGEL INTRODUCTION

The choice to synthetise a chitosan-POSS hydrogel stems from the desire to find innovative materials that can overcome some limitations of conventional bone grafts, with the aim of stimulating bone regeneration while also combating potential infections related to the prosthetic implants. These infections are often caused by Gram-positive bacteria (i.e., *E. faecalis*, *S. aureus*, including methicillin-resistant strains (MRSA), *S. epidermidis,* and other coagulase-negative staphylococci (CoNS)) and Gram-negative bacteria (for example, *K. pneumoniae*, *P. aeruginosa*, *A. baumannii*, *E. coli*, and *P. mirabilis*), which are biofilm producers (Veerachamy et al., 2014; Wolcott 2021). In this context, natural polymers capable of encapsulating cells and inducing the natural formation of bone tissue (Koons et al., 2020; Nallusamy & Das 2021), exhibiting also antibacterial and antibiofilm activity, which can serve as a countermeasure against implant-associated infections (Hamidi et al., 2023), may be the best choice. Therefore, these natural polymers serve as optimal constituents for the construction of bone scaffolds (Bose et al., 2020; Reddy et al., 2021). Among the various biocompatible biopolymers, chitosan (CS) has been selected. CS is a linear deacetylated polysaccharide that is abundantly found in the exoskeletons of crustaceans, non-toxic and recognized as safe by the United States Food and Drug Administration (FDA) (Yan et al., 2021). This has been widely studied in various fields of science, and its potential applications in

tissue engineering are constantly growing (Fatullayeva et al., 2022). CS has the ability to rapidly coagulate blood, promote healing, and produce a hypoallergenic response due to its biocompatibility and biodegradability, and it also exhibits good antibacterial activity (Rodríguez-Vázquez et al., 2015; Yilmaz Atay, 2020; Ferreres et al., 2023). The antimicrobial effect of CS is attributed to its positive surface charges, which allow it to interact with the negatively charged bacterial wall, leading to alterations in transmembrane transport and cellular homeostasis, as well as potentially binding to bacterial DNA, resulting in the inhibition of DNA replication and cell death (Yilmaz Atay, 2020). The 3D shapes that CS can take on are numerous after gelation and the use of crosslinking agents such as genipin or glutaraldehyde, making it truly versatile (Pistone et al., 2020). The limitation in the application of CS-based hydrogel lies in its low mechanical strength, which leads to rapid hydrolysis and release of CS. For this reason, the synthesised hydrogel has been reinforced with POSS, a class of nanostructured silica-based compounds that have been widely studied for the development of materials capable of inducing bone regeneration and serving as drug delivery agents (Oseni et al., 2015). These inorganic-organic hybrid molecules with a cage structure, whose repeating unit has the formula $RSiO_{3/2}$ and dimensions ranging from 1-3 nm, can be suitably functionalized and covalently bonded to different polymers to enhance their mechanical, rheological, and biological properties (Legnani et al., 2020; Tamburaci & Tihminlioglu, 2020).

Furthermore, it has been demonstrated that CS-POSS scaffolds increase alkaline phosphatase activity, osteocalcin secretion, and cell biomineralization, further highlighting that these composites are promising bioactive agents for bone tissue engineering (Tsai et al., 2020). Considering the interesting properties of CS-POSS hybrids for tissue engineering applications, the aim of this study was to synthesise a hybrid CS-POSS hydrogel by utilising the presence of an unsaturated ester group in a heptaisobutyl POSS structure to covalently bond it to CS. The Michael addition of the NH_2 group of CS to the double bond present in the structure of POSS was carried out using amounts of POSS equal to 50% relative to CS. The subsequent reaction with genipin, a natural compound with low toxicity (Visco et al., 2010) used as a crosslinking agent, , allowed for the formation of the corresponding hydrogels, for which chemical, physical, morphological, rheological, antibacterial, and biocompatibility characteristics have been studied. To demonstrate how the

composite material CS-POSS maintains adequate antimicrobial activity in addition to a greater stability, the potential bactericidal/bacteriostatic effect of CS-POSS hydrogels was assessed and compared with the one obtained with CS, on hospital strains isolated from cases of HAIs at the University Hospital "G. Martino" of Messina (Italy). This is aimed to verify the intrinsic antimicrobial and anti-biofilm properties of formulations for bone tissue engineering to use for the production of medical devices in orthopaedics, while biocompatibility has been tested on a cellular model of human foetal osteoblastic cells (hFOB 1.19 ATCC-CRL-11372™).

TITANIUM DISCS FUNCTIONALISED INTRODUCTION

The choice of titanium and a modification of its surface as an approach for the construction of an innovative material in the fight against HAIs (which can occur following a prosthetic implant surgery) is justified by the fact that, due to its biocompatibility and mechanical properties, it is already widely used in orthopaedic, dental, and cardiovascular implants (Kaur & Singh, 2019; Luo et al., 2020). Additionally, modifying the surface of implantable medical devices often helps to reduce the risk of infection and improve the device's longevity (Ahmadabadi et al., 2020). In fact, bacteria often manage to adhere to the surface of these prosthetic devices, creating biofilms and leading to the failure of the implant (Wang & Tang, 2019). In detail, the implanted devices could be contaminated before implantation by bacteria from droplets, air, or the hands of healthcare operators, or immediately at the moment of their introduction. After their introduction into the human body, these can be coated with serum proteins, aqueous humour, mucosal secretions, host microbiota, and extracellular fluids, which make the surface of the materials highly susceptible to microbial adhesion and infection (Katsikogianniet al., 2004; Josyula et al., 2021). Pathogens anchored on the surface of titanium through various surface interactions, such as van der Waals forces, hydrogen bonds, hydrophobic interactions and ion interactions, are able to multiply and colonise the surface, thus leading to an infection of the device (Celesti et al., 2022). Therefore, thinking about the development of functionalised titanium surfaces in order to confer or enhance antibacterial and antibiofilm properties is

essential to prevent and reduce infection rates (Huo et al., 2024). The most effective modification to enhance the antibacterial properties of titanium surfaces is chemical (Janson et al., 2019), which can use antimicrobial peptides, inorganic agents (such as metal ions, nanoparticles, non-metal compounds based on the presence of iodine and fluorine), polymers, and antibiotics (Akshaya et al., 2022). These agents can be covalently bonded or physically adsorbed onto the surface of titanium, providing a sustained release of compounds with antimicrobial activity (Multiple & Species, 2017; Celesti et al., 2022). Based on these considerations, it has been decided to develop titanium devices with intrinsic antibacterial activity by covalently bonding QAS to the surface. The choice fell on QAS because they possess a positively charged nitrogen atom, which allows them to disrupt the cell walls of microorganisms, leading to bacterial death (Dmochowska et al., 2016; Sayed et al., 2023) and preventing biofilm formation (Nada et al., 2022; Negut et al., 2022). Furthermore, QAS are particularly stable, non-volatile, and compatible with titanium (Gadenne et al., 2013), and they exhibit a greater effect against Gram positive bacteria (Jennings et al., 2015), which are the major responsible of prosthetic joint infections (Basile et al., 2021). QAS were covalently bonded to titanium discs using bifunctional silane coupling agents, capable of reacting with the hydroxyl groups present on the surface of titanium and with the reactive group of the QAS molecules. Four different QAS were chosen, anchored to the surface of titanium discs using 3-aminopropyltriethoxysilane (APTES), which differed in the length of the carbon atom chain:

- Acryloyloxyethyltrimethylammonium bromide (AHTEAB);
- Acryloyloxinonyltriethylammonium bromide (ANTEAB);
- Acryloyloxyundecyltriethylammonium bromide (AUTEAB);
- Acryloyloxydecyltriethylammonium bromide (ADTEAB).

In this way, it was possible to analyse the influence of the QAS chain on the antibacterial and antibiofilm effect against both MRSA and MSSA (Gram-positive), one of the main culprits of prosthetic joint infections (Kherabi et al., 2022).

MATERIALS AND METHODS

SYNTHESIS AND PURIFICATION OF CS-POSS POWDERS

CS-POSS hybrid powder has been synthesized through Michael type addition reaction (Sashiwa et al., 2003). Specifically, CS powder (200 mg) (medium molecular weight and deacetylation degree of 75–85%, Sigma Aldrich, St. Louis, MO, USA) was melt in a solution of 2% aqueous acetic acid (10mL) for 30 min at 45°C and then mixed with acryloxypropylheptaisobutyl-POSS (MA0701, C34H72O14Si8, MW: 929.61 g/mol, Hybrid Plastics, Hattiesburg, MS, USA), 200 mg (1 equiv, 0.21 mmol). This mixture was subjected to magnetic stirring, at reflux for 12 h at 50°C. The obtained CS-POSS was treated with a saturated solution of NaHCO3 to reach a neutral pH and then purified through dialysis bags (MW: 12,000 Da) for two days. The purified sample was lyophilized by freeze-drying at -80°C for 72 h and then used for the subsequent characterizations. The conjugation of POSS with the polymer was confirmed by FTIR spectroscopy and TGA analysis performed under inert atmosphere. The percentage of free amino groups in both CS and CS-POSS hybrid powder was evaluated by UV–vis absorption spectra, after reaction with ninhydrin measuring the absorbance of the solutions at 570 nm (Curotto & Aros, 1993) and were found to be of 82% and 45% for CS and CS-POSS hybrid, respectively.

SYNTHESIS OF CS AND CS-POSS HYBIRD HYDROGELS

For the synthesis of the two distinct types of hydrogels, CS powder (200 mg) or CS-POSS (200 mg) were dissolved in a 2% aqueous acetic acid solution for 30 min, at 45°C. Cross-linking agent genipin 0,1 mmol (20 mg) (purity > 98%, Carbosynth, St. Gallen, Switzerland) was slowly added to the mixture until the formation of a 3D gel, as it allows the link of two amino groups between the neighbouring chains of the CS polymer. After being cleaned with deionised water, the hydrogels that were created were kept at 15°C in a hermetic covered pan with a consistent relative

humidity. The water content was evaluated by drying the hydrogels in a becker for 24 h at 37°C at a vacuum drying pressure of 65 mbar until constant weight and was found to be equal to 94 wt% for CS and 78 wt% for CS-POSS hydrogels, respectively. Then, for both CS and CS-POSS hydrogels, rheological characterizations, that is the frequency response of G0 and the complex viscosity η*, monitored 30 min after the start of crosslinking, were performed. The complex viscosity and the elastic modulus were evaluated as a function of frequency, in the range of 0.01–200 rad/s. From these analyses emerged that the G0 value of CS (at 0.1 rad/s) was of 118,183 Pa, with a viscosity value of 1,197,866 Pa*s, while for the CS-POSS sample, these values were, respectively, reduced to 3684 Pa and 50,183 Pa*s. These results are in agreement with the data obtained from the evaluation of the free amino groups by UV-vis since the decrease of these groups leads to a progressive reduction of the reticulation degree with a consequent decrease in stiffness and in structural complexity. Synthesis, purification and characterisation of hydrogels were conducted in collaboration with professor Daniela Iannazzo and professor Consuelo Celesti, at the engineering department of the University of Messina.

BIOCOMPATIBILITY OF CS AND CS-POSS HYBRID HYDROGELS

To evaluate the biocompatibility of the synthesized CS- and CS-POSS-based hydrogels, were used, as in vitro model, the hFOB cells. Specifically, the cells were cultured in 1:1 mixture of Ham's F12 and DMEM media without phenol red and with the addition of 2.5mML-glutamine, 0.3 mg mL^{-1} G418, and 10% of foetal bovine serum at 34°C in 5% CO_2/95% air humidified atmosphere. CS- and CS-POSS (100 µL) were gelled at room temperature in 6-well cell culture plates in order to obtain hydrogels. In each well, the area covered by the hydrogels was calculated using ImageJ software (imagej.nih.gov/ij/index.html) in order to use only wells with a sample area equal to 35% (±5) of the entire well area. In each well, a cell suspension ($5x10^4$ cells/mL) was added, cells cultured in wells with their specific medium and without the gelled samples were used as negative controls. Cells were grown on the formed hydrogels for 48 h at 34°C. By employing

fluorimetric analysis (exc 493–em 636 nm) with the DNA intercalating probe propidium iodide (PI) (Sigma) at a concentration of 20 µg/mL, for 15 min at 20°C, the biocompatibility of the investigated materials was assessed. The analyses were performed at least in triplicate both on the cells present in the medium, to detect the detached cells (death cells) and, after cell permeabilization by methanol, on the adherent cells to assess the viable cells. The data were presented as the mean standard deviation (SD) and were analysed by one-way analysis of variance (ANOVA) to assess possible significant differences, which were accepted at $P < 0.05$ (GraphPad Prism 8). Additionally, to evaluate morphological modifications due to the effects of the hydrogels, confocal microscope observations were performed by a laser scanning confocal microscopy (CLSM) equipped with a 401.0 NA immersion objective and a TCS SP2 instrument (Leica Microsystem Heidelberg, Mannaheim, Germany). Specifically, the cell-permeable metachromatic fluorophore acrydine orange (AO) was used, because it allows to visualise both the cytoplasmic compartment where, due to the presence of the acid compartment, the organelles emitting red fluorescence can be seen, and the nuclear one emitting green fluorescence due to the bond between AO in monomeric form and DNA double helix.

ISOLATION AND CHARACTERISATION OF BACTERIAL STRAINS FROM CLINICAL ISOLATES

Bacterial strains used in this study were isolated from clinical samples of patients affected by HAIs hospitalized at the University Hospital “G. Martino” of Messina, Italy. Specifically, a total of 44 bacterial strains were used, of which 38 Gram-positive [methicillin-susceptible *S. aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), methicillin-susceptible *S. epidermidis* (MSSE), methicillin-resistant *S. epidermidis* (MRSE), vancomycin-susceptible *E. faecium* (VSE), and vancomycin-resistant *E. faecium* (VRE)], and 6 Gram-negative (non-MDR and MDR *P. aeruginosa* (PSEAER)) bacteria. The bacterial detection was performed using common growth media specific for the different strains. Specifically, Mannitol Salt Agar (MSA) was used for the detection of staphylococci, Bile Esculine Agar (BEA)

for enterococci and MacConkey Agar (MCA) for PSEAR. All the plates were incubated in aerobic conditions at 37°C for 24 h. After the detection and the subsequent isolation, the microbial identification and the antimicrobial susceptibility were performed by the automatized system VITEK® 2 COMPACT (bioMérieux Clinical Diagnostics). After that, strains were stored at -20°C in Luria-Bertani (LB) Miller formulation Broth with 15% glycerol. For their use, strains were unfrozen and plated on the same agar plates used for their detection. The plates were, then, incubated at 37°C for 24 h. From these subcultures on solid plates, a suspension in Mueller-Hinton Broth (MHB) (BD DIFCO™, Franklin Lakes, NJ, USA) with an optical density (OD) of 0.5 was obtained (595nm).

ANTIMICROBIAL ACTIVITY OF CS AND CS-POSS HYBRID HYDROGELS

The antibacterial activity of CS and CS-POSS was tested both in liquid and in solid phase. The first one was performed by dissolving the materials in the bacterial suspension. In particular, the treatment in liquid phase was performed by adding 200 µL of CS or CS-POSS (4 mg mL^{-1}) to 1 mL of bacterial suspension with an OD of 0.5 (595 nm) on MHB. The suspension was incubated for 24 h at 37°C under shaking to ensure a good dispersion of the materials. After 24 h, the OD of each treated and untreated suspension was measured. The activity in solid phase was performed by creating a button of CS and CS-POSS hydrogels with a dimension of about 2 cm^2 (800 µg mL^{-1}) on the middle of a Petri plate with Muller-Hinton (MH) agar medium and left to solidify. After that, 10 µL of bacterial suspensions with an OD of 0.5 (595 nm) were plated on the same plates. Then, the plates were incubated at 37°C for 24 h. After the incubation, the growth in plates without and with hydrogels' buttons was evaluated and the ODs of the suspensions derived from the two plate sections were measured. All OD measures were performed in triplicate and the average values were reported with standard deviations.

MICROBIAL MEMBRANE PERMEABILITY ASSAY USING PROPIDIUM IODIDE

The evaluation of the membrane permeability was carried out using the fluorochrome PI, according to the literature (Li et al., 2023). Specifically, bacterial suspensions were incubated for 24 h at 37°C in MHB, then collected, washed, and resuspended in a buffer solution containing 5 mM glucose and 5 mM HEPES at pH 7.2 to obtain a solution with an OD value of ~0.25 (600 nm). Then, 150 µL of this bacterial suspension were put into wells of a 96-well plate together with 10 µL of PI solution (50 µM) and firstly incubated for 10'. Following this first short incubation, florescence was measured for the next 8' with a time interval of 2' using a microplate reader (535 nm excitation, 617 nm emission, Tecan, Switzerland). After this, 30 µL of CS or CS-POSS (800 µg mL^{-1}) were added, and the florescence intensity was monitored after 10', 20', and 30'. The assay was performed in triplicate, and the SD average values were reported.

EVALUATION OF BACTERIAL CELL-SURFACE

Specifically, the solvents ethyl acetate (as electron donor) and chloroform (as electron acceptor) were used to evaluate any change of charges on cellular surface. Briefly, bacterial cells were grown overnight in MHB at 37°C without and with CS and CS-POSS (800 µg mL^{-1}), then collected by centrifugation (6,000 rpm for 10 min), washed twice with phosphate buffered saline (PBS), and resuspended in a volume of PBS to obtain a bacterial suspension with an OD between 0.5 and 0.7 (400 nm) (A0). Aliquots (3 mL) of each treated and untreated bacterial suspensions were added to a tube containing 0.4 mL of ethyl acetate, a strong basic solvent, and chloroform, an acidic solvent which exhibits negligible basic character. After strong agitation by vortex, phases were allowed to separate for 10'at 30°C and the OD (400 nm) of the aqueous phase was measured (A1). The percentage of affinity to each solvent was calculated as follows:

$$\% \text{ Affinity} = A0 - A1/A0 \times 100$$

CONFOCAL MICROSCOPIC OBSERVATION OF BIOFILM

The capacity of CS and CS-POSS to inhibit the production of bacterial biofilm was investigated by CLSM. As described by Spanò et al. (2016), aliquots of overnight bacterial cultures in MHB (adjusted to OD600 = 0.5) were distributed into chamber slides (Nunc Inc., Naperville, IL, USA), previously coated with CS and CS-POSS. After incubation at 37°C for 24 h, those bacteria that have not attached to the surface of the chamber slide were eliminated while the cells that adhered to the slide were washed with PBS, then heat fixed and finally stained with 20 μg mL^{-1} PI, an intercalating of nucleic acids that has the fluorescence excitation and emission maximum equal to 535 nm and 617 nm, respectively. The slides were incubated in the dark at 30° C for 5' to allow the fluorescent labelling of bacteria. The observations were performed by CLSM using a TCS SP2 microscope (Leica Microsystems Heidelberg, Mannheim, Germany), equipped with an Ar/Kr laser and coupled to a microscope (Leica DMIRB). *Staphylococcus* spp. were considered representative target bacteria.

FUNCTIONALIZED TITANIUM DISCS

SYNTHESIS OF AATEABS

For the synthesis of AATEABs was used a convenient, practical approach, without any need for chromatographic purification, suitable for large-scale and industrial application. The ammonium salts AHTEAB, ANTEAB, AUTEAB and ADTEAB were prepared with a two-steps synthetic approach, which consists of:

- First step: Esterification of ω-bromo-1-alkanols with acryloyl chloride (carried out in the presence of molecular sieves (3Å), to obtain ω-bromoalkyl acrylates.
- Second step: Quaternization with Et_3N. (Mancuso et al.,2017).

The AATEABs, thus obtained, were covalently linked to Titanium discs (25 mm in diameter and 0.5 mm thick), purchased from Merck Life Science, with chemical

and mechanical properties conforming to the ASTM-B-265/ASME-SB-265 standard. 3-Aminopropyltriethoxysilane, hydrogen peroxide, sulphuric acid and the solvents toluene (99.8%), ethanol (99.8%) and methanol (99.8%) were purchased from Merck Life Science and used without further purification for the realization of the binding between titanium discs and salts in a multi-step approach.

ACTIVATION OF TITANIUM-BASED SAMPLES

Titanium discs were ultrasonically cleaned in ethanol and deionized water (10 minutes for each one). The surfaces of titanium discs were, then, activated by soaking the discs for 48 hours in a piranha base solution (100 mL), i.e. a 3:1 mixture of ammonium hydroxide (NH_4OH) and hydrogen peroxide (H_2O_2). After this treatment, the samples were washed with ethanol and dried with nitrogen gas. In this way, Ti-OH alkaliactivated samples were obtained, which are essential to enable the next silanization step. Titanium surface activation by basic treatment was confirmed by field emission gun scanning electron microscopy (FEG-SEM), FT-IR analysis, and EDX mapping analysis.

SILANIZATION OF ACTIVATED TITANIUM DISCS

For the silanization of Ti-OH samples was used a solution (2.0 vol.%) of APTES in anhydrous toluene (Celesti et al., 2021). Ti-OH discs, placed in a glass flask equipped with a reflux condenser, were heated for 36 hours at 70° C in a nitrogen atmosphere. Then, the discs were washed with ethanol and dried with nitrogen gas. The silanization step of the TI-OH discs surfaces is crucial for immobilising the chosen bioactive molecules. In fact, this method exploits the spontaneous formation of strong siloxane bonds from the reaction between the alkoxy groups of the APTES and the hydroxyl groups of the titanium, while then the nucleophilic amino group is used for the further bonding with ammonium salts. To confirm silanization, FEG-SEM, FT-IR analysis and EDX mapping analysis were performed.

SYNTHESIS OF AATEABS-FUNCTIONALIZED TITANIUM DISCS

Ti-APTES samples were placed in a flask equipped with a reflux condenser and then treated with different solutions of the AATEABs (AHTEAB, ANTEAB, AUTEAB, or ADTEAB, 10 mg mL^{-1}), in methanol (25 mL). The discs were heated for 36 h at 80° C, then washed with ethanol and dried with nitrogen. With this final step, Ti-AATEABs discs were obtained. To confirm the successful functionalization reaction, FT-IR analysis was performed and surface roughness and hydrophobicity were evaluated. FEG-SEM analysis established that of the starting solution, approximately 2% of the salts covalently bonded to the surface of the titanium, corresponding to a concentration of 200 µg/mL. Also in this case, synthesis and characterisation of engineered materials were conducted in collaboration with professor Daniela Iannazzo and professor Consuelo Celesti, at the engineering department of the University of Messina.

EVALUATION OF THE ANTI-BACTERIAL AND ANTI-BIOFILM ACTIVITIES OF AATEABS

The antibacterial activity of AATEABs was assessed against MSSA (Newman D2C [NCTC 10833]) and MRSA (TCH1516 [USA300-HOU-MR]) using both a simple absorbance measurement (595 nm) and the resazurin assay in order to evaluate both the inhibition of bacterial growth and the inhibition of metabolic activity. Resazurin (Sigma-Aldrich, St. Louis, USA) is a weakly fluorescent, non-toxic, cell-permeable, and redox-sensitive blue-violet phenoxazine dye. Resazurin can be irreversibly reduced to pink resorufin (7-hydroxy-3H-phenoxazin-3-one) if bacterial cells are metabolically active. Specifically, 10 µL of a resazurin solution (150 µg mL^{-1}) were added to 100 µL of bacterial suspension, previously incubated for 24h at 37° C in stirring incubator with the AATEABs. The absorbance values were recorded after 3 hours (570 nm). Then, the MBC test was used to determine the lowest concentration at which an antimicrobial agent kills a particular microorganism. This is a test used to determine minimum concentration of antimicrobial agent that has bactericidal effects. To perform the test, 10 µL of bacterial suspensions treated for 24 h at 37 °C with the different salt concentrations

were taken and spotted on a Petri dish with Mueller Hinton Agar. After a further 24 h, photographs were taken to assess the bactericidal doses of the AATEABs.

ASSESSMENT OF THE ANTI-BIOFILM ACTIVITIES OF AATEABS

The antibiofilm activity was investigated using the same bacterial strains incubated in 96-well polystyrene microplates. Suitable aliquots (200 µL) of each overnight culture in MHB (OD600nm = 0.01) were poured with different salts concentrations in the microwells, and the microplates were incubated at 37° C for 48 h, without shaking. Non-adherent bacteria were removed by 3 washes with distilled water, sucking gently, and air dried. Biofilm was stained with 0.1% (*w*/*v*) crystal violet solution and incubated at room temperature for 20 min. To eliminate the excess stain, the plates were washed with distilled water (5 times) and air-dried (for 45 min). The stained biofilms were solubilized with absolute ethanol. Biofilm mass was spectrophotometrically determined (595 nm) by the level of the crystal violet present in the destaining solution, using a microtiter plate reader (Multiskan GO, Thermo Scientific, Waltham, MA, USA). Each data point was averaged from three replicated microwells, and the standard deviation (SD) was calculated. The antibiofilm effect of AATEABs was evaluated also by CLSM. Aliquots of overnight bacterial cultures in MHB (adjusted to OD600 = 0.01) were distributed into chamber slides, with different salts concentrations. After incubation at 37° C for 48 h, without shaking, supernatant bacteria were eliminated while the cells that adhered to the slide were washed with PBS, then heat fixed and finally stained with 20 µg mL^{-1} PI. The slides were incubated in the dark at 30°C for 5' to allow the fluorescent labelling of bacteria. The observations were performed by CLSM using a TCS SP2 microscope (Leica Microsystems Heidelberg, Mannheim, Germany), equipped with an Ar/Kr laser and coupled to a microscope (Leica DMIRB).

To evaluate antibacterial properties of functionalized titanium discs, ATCC strains of MSSA (Newman D2C [NCTC 10833]) and MRSA (TCH1516 [USA300-HOU-MR]) were used. Bacteria were firstly cultured in Brain-Heart Infusion (BHI) broth (Merck KgaA, Darmstadt, Germany) at 37° C for 24h in agitation. From primary cultures, a 0.5 McFarland solution of both the strains was obtained and a plate count method on Petri dishes including, in the middle, the titanium disc was performed. Specifically, on 35 mm Petri dishes, after inoculation of 10 µL of the 0.5 bacterial suspension on the surface of the disk, 1 mL of merged Plate Count Agar (PCA) (Merck KgaA, Darmstadt, Germany) was added in order to obtain an extremely thin layer of agar above the disk. After solidification, Petri dishes were incubated at 37° C for 24h. After the incubation period, colonies were imaged with a digital camera and quantified using the free program ImageJ (imagej.nih.gov/ij/index.html) (Sieuwerts et al., 2008).

ASSESSMENT OF THE ANTI-BIOFILM PROPERTIES OF FUNCTIONALIZED TITANIUM DISCS

For the anti-biofilm activity, not functionalized and functionalized discs were put in contact with a 0.01 McFarland bacterial suspension of both the strains and incubated at 37° C for 48h. Then, after being washed three times with sterile PBS in order to remove all the non-adherent bacteria, discs were sonicated (Brandon 3210 Ultrasonic Cleaner) for 20 minutes in 5 ml of sterile PBS to detach the eventually formed biofilm from the surface of the disc. From the collected suspension, ten-fold serial dilutions were obtained and plated on PCA for the count. Plates were, then, incubated at 37° C for 24h and colonies were quantified using the free program ImageJ. At the same time, from the collected suspensions, we set up slides to obtain confocal microscopy images of the bacterial biofilm. Specifically, bacteria were fixed by heat and stained with PI (20 µg mL^{-1}) (Sigma-Aldrich, Italy), a nucleic acids' intercalating and fluorescent agent. The slides were incubated in the dark at 30° C for 5 min to enable the fluorescent labelling of the bacteria. Observations were performed by CLSM using a TCS SP2 microscope (Leica Microsystems Heidelberg, Mannheim, Germany), equipped with Ar/Kr laser, and coupled to a microscope (Leica DMIRB). The fluorescence excitation maximum is

535 nm and the emission maximum is 617 nm. All the experiments were performed in triplicate.

STATISTICAL ANALYSIS

Statistical analyses, for both the Chitosan/POSS Hybrid Hydrogel and Functionalised Titanium discs experiments, were performed using Prism 4.0 software (GraphPad, San Diego, CA, USA). Stratified data were statistically analyzed using one-way ANOVA and *t*-tests. Significance was assessed at the $p < 0.05$ level.

RESULTS AND DISCUSSION

BIOCOMPATIBILITY OF CS AND CS-POSS HYBRID HYDROGELS

The cytotoxicity and biocompatibility of the CS and CS-POSS hydrogels were evaluated in vitro using hFOB cells. In both cases, the cytotoxicity was minimal and nearly equal to the control. Even, the CS hydrogel decreased cell death, demonstrating the substrate's higher level of biocompatibility when compared to the plastic materials used for cell cultures. The partial coating of the wells appears to create a microenvironment that is physiologically more appropriate for the used cell model. In the hybrid material, the presence of POSS resulted in a cell death rate overlaid on that of the control cells (11.1% vs. 9.8%) (Figure 1).

Utilising the AO probe, microscopic observations verified the outcomes as well. The morphological similarity between the cells growing in the presence of hydrogels and the control is evident from the CLSM pictures displayed in Figure 2. The results obtained show that both CS and CS-POSS hydrogels are absolutely biocompatible. This is basically due to the key elements that compose them. In fact, the chitosan is a non-toxic, non-immunogenic and biocompatible compound, which owes these characteristics to its similarities to glycosaminoglycans which are found in the extracellular matrix (Harugade et al., 2023). The same applies to POSS nanostructures, which are biocompatible and non-toxic as well as improve mechanical properties, and resist biodegradation, which is very important in tissue engineering (Ozimek & Pielichowski, 2022).

ANTIBIOTIC SUSCEPTIBILITY OF THE TESTED STRAINS

Analyses performed with the VITEK® 2 COMPACT to test for antimicrobial susceptibility showed that obviously the resistant strains had a higher average percentage of overall resistance than the sensitive strains. Specifically, VRE resulted the most resistant strains among the resistant ones, while enterococci showed the highest average value of resistance overall (Table 1). These results are confirmed by literature data showing that VRE was one of the most important and

the fastest growing resistant pathogen in Europe in 2022 (+21%) (Meschiari et al., 2023).

ANTIBACTERIAL PROPERTIES OF CS AND CS-POSS

Treatment with CS and CS-POSS in MHB showed a significant decrease in bacterial growth, although in the presence of a certain variability among the strains. The presence of CS resulted in a drop of −55.26% and −60.63% for MSSA and MRSA, −69.56% ($p < 0.01$) and −53.60% for MSSE and MRSE, −62.75% and −36.36% for VSE and VRE, and −54.90% and −73.58% ($p < 0.0001$) in non-MDR and MDR PSAER. The decrease, more contained, was also highlighted by the CS-POSS treatment, and was equal to −34.21% and −29.86% for MSSA and MRSA, −65.21% ($p < 0.01$) and −42.74% for MSSE and MRSE, −47.06% and −32.73% for VRE and VSE, and finally, −49.02% and −66.04% ($p < 0.0001$) for non-MDR and MDR PSAER (Figure 3 A).

Exposure to the hydrogel in plate confirmed the antimicrobial activity of the tested materials and how POSS-conjugated chitosan still retains its antibacterial activity. Despite the substantial inter-strain heterogeneity, there was a significant decrease in bacterial growth for CS, with values consistently exceeding 40%. MRSA (−74.99%; $p < 0.0001$), MSSE (−78.32%; $p < 0.01$), MRSE (−62.96%; $p < 0.01$), non-MDR PSAER (−55.29%; $p < 0.05$), and MDR PSAER (−77.64%; $p < 0.0001$) showed the greatest declines. Exposure to CS-POSS hydrogel resulted in homogenous drops of −17.00 to −29.00% for all the tested strains, with the exception of the VSE, which decreased by −53.99%.There was no significant difference in antibacterial characteristics of tested materials between antibiotic sensitive (−62.13% for CS and −32.71% for CS-POSS) and resistant (−61.49% for CS and −25.37% for CS-POSS) strains (Figure 3B - C).

The results are very promising because a hydrogel containing only chitosan presents problems such as low mechanical strength, sudden drug release and rapid hydrolysis, which limit its use for bone tissue engineering applications (Alven et al., 2020; Islam et al., 2020; Pistone et al., 2020). Thus, through the addition of POSS, the stability of the hydrogel was enhanced while maintaining good

antibacterial activity, making it a perfect candidate for the construction of a bone scaffold with intrinsic antibacterial activity to combat HAIs following prosthetic implants. Although the results obtained seem to show a lower efficacy, in terms of antibacterial activity, of the CS-POSS hydrogel compared to CS alone, it must actually be considered that following the addition of POSS, the free amino groups (responsible for the antibacterial effect of CS) decrease from 82% present in the CS powder to 45% for the CS-POSS powder, respectively. Thus, for the same number of free amino groups, the antibacterial effect is superimposed with a more pronounced stability of the chitosan-POSS hydrogel.

ANTIMICROBIAL MECHANISM

The affinity for chloroform and ethyl acetate was investigated to assess the impact of CS and CS-POSS on bacterial surface charges. The tests revealed a reduced chloroform affinity that was marked for CS and nearly superimposable on the control for CS-POSS, indicating a change in surface charges only for staphylococci. As shown in figure 4, treatments with the materials under research resulted in a reduced affinity for chloroform in staphylococci compared to basal conditions. MSSA showed an affinity of 39.00% and 59.11% after CS and CS-POSS treatment, respectively. For MRSA, CS treatment lowered chloroform affinity to 34.15%, while CS-POSS treatment reduced it to 76.40%. The MSSE and MRSE chloroform affinity were lowered to 48.97% and 66.67%, respectively, after CS treatment, and 76.13% and 80.28% after CS-POSS treatment. The results showed that treatment with CS and CS-POSS resulted in a greater affinity for ethyl acetate, with values of 41.94% and 58.00% for VSE and 51.24% and 48.65% for VRE, respectively. There were no differences in surface bacterial strains between non-MDR-PSAER and MDR-PSAER compared to the control strain. The DNA-binding PI fluorochrome doesn’t happen in the presence of microorganisms with intact membranes, but produces significant florescence when the membrane is damaged, allowing it to intercalate with DNA. Figure 5 shows that CS and CS-POSS treatment increased the florescence of bacterial strains, implying that these materials' antimicrobial activity is due to bacterial wall/membrane damage. The results revealed that membrane damage occurred promptly in all examined strains, however membrane

permeability varied between microbial species, most likely due to differences in cell surface structures and compositions. Fluorescence levels rose considerably with exposure time in MSSA, MRSA, VSE, and VRE for both the materials ($p < 0.05$). In MSSE and MRSE, fluorescence increased significantly following CS treatment, whereas the strain treated with CS-POSS showed a lesser and non-significant rise. Concerning non-MDR- and MDR PSAER, there was a delay in the entry of the fluorochrome, which was evident only after 30′ of exposure, most likely due to the bacterium's characteristic composition, which includes a dense mucous layer that can make it difficult for exogenous substances to pass through. Even though there was no link with the exposure time, all of the tested strains showed a considerable increase in fluorescence at time 30′ compared to time 0. The results obtained confirm that the mechanism of action by which chitosan performs its antimicrobial activity in these types of hydrogels probably involves the interaction between its positively charged free amine groups and the negatively charged membranes of microbial cells. Indeed, this interaction alters the charges and permeability of the microbial cell (Guarnieri et al., 2022). In addition, CS can also act as a calcium chelator in the bacteria cell walls, exerting its antimicrobial effect by interacting with the stability of peptidoglycan and changing the osmotic balance of the membrane wall (Young & Kaus, 1983; Goy et al., 2009). The mechanism of action by which chitosan manifests its antimicrobial activity against Gram-positive and Gram-negative bacteria is slightly different, as are the external structures of the bacteria. In Gram-negative bacteria, chitosan performs its antibacterial action by interacting with anionic structures present on their surface, such as lipopolysaccharides and proteins (Nikaido & Vaara, 1985). In Gram-positive bacteria, on the other hand, the polymer interacts directly with their cell wall layer, which is made up of the negative charges of peptidoglycan and teichoic acids (Hosseinnejad, M. & Jafari, 2016). The time difference in the onset of membrane damage between the Gram-positive and -negative strains used for the experiments can probably be attributed to this, in addition to the fact that a medium molecular weight CS was used for the synthesis of the hydrogels, seeking a compromise and a broad spectrum action, as the higher molecular weight CS is more effective for Gram positive bacteria while the lower molecular weight CS is more effective for Gram negative bacteria (Eaton et al., 2008).

ANTIBIOFILM PROPORTIES OF CS AND CS-POSS HYDROGELS

The inhibitory properties of CS and CS-POSS against bacterial biofilm formation were investigated using *Staphylococcus* spp. as the target organisms. Compared to the basal state, where the biofilm was compact with well-clustered internal bacterial cells, the CLSM images demonstrated how CS-POSS and CS treatments were able to remove the dense biofilm structure in MSSA, MRSA, MSSE, and MRSE (Figure 6). The good biofilm prevention effect found for the two hydrogels is certainly useful for their future use in the biosanitary field, as the biofilm formed by pathogenic bacteria is considered as one of the major resistance mechanisms against the rationally used antibiotics (Khan et al., 2020). The biofilm is a structural polymeric architecture composed of extracellular polymeric substance (EPS), including lipids, polysaccharides, proteins, extracellular nucleic acids (eDNA), and ions, which act as a barrier against drying, action of host's immune system and the antibiotics used during the treatment (Iaconis et al., 2024). The antibiofilm effect exerted by the two hydrogels is again due to the positive charges of chitosan, which interact with the negatively-charged biofilm components such as EPS, proteins and DNA, resulting in an inhibitory effect on bacterial biofilm (Shrestha et al.,2012; Jiang et al., 2014).

FUNCTIONALIZED TITANIUM DISCS

ANTIBACTERIAL ACTIVITY OF AATEABS AND THEIR MICROBICIDE EFFECTS

To evaluate the antimicrobial properties of AATEABs on MSSA and MRSA, a simple absorbance measurement and the resazurin test were used. The absorbance measurement showed an antibacterial dose-dependent effect that, on both the sensitive and resistant strains, was correlated with the chain length of the salt anchored to the titanium disc (Figure 7). With regard to the susceptible strain, significant differences in antibacterial activity were found for ANTEAB (p<0.005), AUTEAB (p<0.001) and ADTEAB (p<0.0001) compared to AHTEAB. Significant differences were also found for the resistant strain between the longer-chain salts

and AHTEAB, albeit smaller (p<0.05). Also the resazurin test showing a dose-dependent effect for all the salts tested and for both the strains used as a model. No significant differences were found between the sensible and resistant strains in their metabolic activity. AHTEAB was the salt that lost mostly its effect to the concentration decreased, ANTEAB and AUTEAB were effective in the range 800-100 μg mL^{-1}, while the ADTEAB was the salt with the greatest inhibitory effect on metabolic activity, in fact it inhibits the metabolic activity in the range 800-25 μg mL^{-1} (Figure 8). Therefore, for MSSA significant differences in metabolic activity were found for AUTEAB (p<0,05) and ADTEAB (p<0,01) compared to AHTEAB, while for MRSA only ADTEAB shown significant correlation (p<0,005) (Figure 9).

The MBC test underlined that there was no bactericidal dose of AHTEAB for either of the tested strains while for ANTEAB and AUTEAB the lowest bactericidal dose was equals to 200 μg mL^{-1} for both MRSA and MSSA strains. Instead, for ADTEAB the bactericidal dose was 100 μg mL^{-1} for MRSA and 50 μg mL^{-1} for MSSA (Figure 8 -9).

The antibacterial effect exhibited by AATEABs results from the electrostatic interaction between the positive N-atom of QAS and the negatively charged teichoic acid present in the peptidoglycan of *S. aureus* (Odžak et al., 2023). As a result of the interaction, QAS can also integrate into the membrane, facilitating its perforation (Alkhalifa et al., 2019). In the experiments, the antimicrobial activity of the AATEABs has been shown to be dependent on the alkyl chain length of the salt. This finding has previously been documented in the literature and is due to the salt's higher cationic charge, which allows for greater interaction with the peptidoglycan of Gram-positive bacteria (Nadagouda et al., 2022).

ANTIBIOFILM ACTIVITY OF AATEABS

Crystal violet assay underlined the antibiofilm effect of the salts, which was found to be related by the length of the salt carbon chain for both MSSA and MRSA. Thus, as the chain length increased, the inhibitory effect in the biofilm production

increased, even as the tested concentration decreased. No significant differences attributable to the resistance phenotype were found. For the MSSA strain, AHTEAB did not present concentrations capable of completely eradicating the biofilm, while for ANTEAB the doses inhibiting the production of biofilm were included in the range 800-400 μg mL^{-1}, for AUTEAB in the range 800-100 μg mL^{-1} and for ADTEAB in the range 800-50 μg mL^{-1}. AHTEAB did not present concentrations capable of eradicating the biofilm also for MRSA, while ANTEAB was found to totally inhibit biofilm production in the concentration range from 800 -200 μg mL^{-1}, AUTEAB and ADTEAB in the range 800-100 μg mL^{-1}. With regard to the susceptible strain, significant differences in antibiofilm activity were found only for ADTEAB ($p<0.0001$) compared to AHTEAB while significant differences were also found for the resistant strain between ANTEAB, AUTEAB and ADTEAB respect AHTEAB ($p<0.0001$). (Figure 10).

These results were also confirmed by observations under CSLM. The antibiofilm activity of the salts is dose-dependent for all the analysed salts, confirming no significant differences between MSSA and MRSA strains (Figure 11). The antibiofilm effect appears to be influenced by the length of the alkyl chain of the AATEABs, which impact on the surface activity. In fact, ADTEAB (the salt with highest surface activity among those tested) better inhibits biofilm formation on the polystyrene plate (Odžak et al., 2023).

ANTIBACTERIAL PROPERTIES OF FUNCTIONALISED TITANIUM DISCS

Using MSSA and MRSA strains, biological tests were conducted to assess the suitability of the proposed functionalisation strategy for the development of intrinsic antimicrobial titanium devices. *S. aureus* was used as a model organism because it is an important bacterial human pathogen that causes a wide range of clinical manifestations (Turner et al., 2019). The bacterial growth inhibition activity, compared to the growth of the used standard strains in basal conditions, showed significant antibacterial activity ($p<0.05$) for all the samples with some differences among them. For non-functionalized titanium disc, the antibacterial activity was practically superimposable for both the strains (-80.7% and -78.2% for MSSA and MRSA respectively), but lower than that showed by functionalized

discs. The antibacterial activity was correlated with the chain length of the linked AATEABS. In fact, among functionalized titanium discs, the longest chain compound (Ti- ADTEAB) showed the highest percentage decrease for both the strains (-87.5 vs -86.6% for MSSA and MRSA respectively) (Figure 12-13).

ANTIBIOFILM PROPORTIES OF FUNCTIONALISED TITANIUM DISCS

Functionalised titanium discs demonstrated anti-biofilm activity in our test circumstances, and this was observed for both the strains that were used. The obtained results reported a number of colonies higher in the sonicated solution collected from non-functionalized discs compared to that collected from functionalized ones. These results were observed for both the strains and were particularly accentuated comparing the non-functionalized disc with Ti-ADTEAB (p<0,001). In particular, the number of colonies was equal to 1,950 x 10^6 CFU/mL (non-functionalized disc) *vs* 437 x 10^6 CFU/mL (Ti-ADTEAB) for MSSA and 1,995 x 10^6 CFU/mL (non-functionalized disc) *vs* 845 x 10^6 CFU/mL (Ti-ADTEAB) for MRSA with a percentage decrease compared to nonfunctionalized disc of -77.5% for the sensible strain and -57.6% for the resistant one (Figure 14). For the other salts the decreases were -30.1% for AHTEAB, -35.5% for ANTEAB, and -37.8% for AUTEAB in the sensitive strain; for MRSA, the decreases were more modest, with -3.1% for AHTEAB, -17.1% for ANTEAB, and -21.9% for AUTEAB. The decreases appear statistically significant when correlated with the length of the alkyl chain of the salts under examination. Significant differences in antibiofilm activity were found only for AUTEAB (p<0.05) and ADTEAB (p<0.001) compared to AHTEAB in MSSA strain while for MRSA significant differences respect to AHTEAB were present for ANTEAB, AUTEAB, ADTEAB (p<0.0001).

These differences were confirmed by the CLSM images (Figure 15). Overall, both the antibacterial and antibiofilm tests show greater activity of the functionalised

titanium discs than the non-functionalised ones. However, some differences between sensitive and resistant strains should be noted. In fact, while for antibacterial activity we obtained superimposable results against the two different types of used strains, in the anti-biofilm activity experiments, the latter was less marked for the resistant strain, which was more capable of producing biofilm on the functionalised disc than the sensitive strain, as reported by previous literature data (McCarthy et al., 2015; Manandhar et al., 2018). However, titanium discs functionalised with ADTEAB, have shown strong anti-biofilm efficacy even against resistant strains. This is an intriguing discovery, considering that antibiotic-resistant bacteria are currently responsible for a large number of implant infections that have serious clinical consequences. Therefore, the antimicrobial and anti-biofilm effects even against antibiotic-resistant bacteria are an important goal to reduce the risks of infection of titanium implants, making the functionalisation of titanium with ADTEABs an excellent strategy to counteract HAIs resulting from prosthetic implantation.

CONCLUSIONS

The results obtained during these years of doctoral research have demonstrated that the synthesis and use of innovative materials with intrinsic antimicrobial activity could serve as a valuable tool to counteract one of the major public health concern, HAIs related to implantable prosthesis. This novel aspect could especially help to overcome the difficulty of discovering or synthesizing new antibiotics to fight drug-resistant bacteria able to form impenetrable biofilms on prostheses, that is currently one of the main problem to manage. In fact, if a prosthesis becomes infected, the only viable solution to ensure patient safety is its removal, which entails the risks of a new surgery and significant economic costs for the healthcare system. The development of materials for use as scaffolds or bone prosthetics with antibacterial and antibiofilm activity could be a strategy for combating and preventing such infections. In this context, both CS-POSS hydrogels and the functionalization of medical titanium with AATEABs represent feasible strategies. Specifically, the hydrogel would provide an antimicrobial, antibiofilm scaffold that is fully

biocompatible and potentially capable of inducing bone regeneration and osteointegration. Experiments have shown that the addition of POSS enhances the stability of the hydrogel, facilitating its in vivo application without compromising its antibacterial activity against hospital strains or its biocompatibility. On the other hand, the functionalization of titanium—a widely used and biocompatible material—with QAS has demonstrated good efficacy against one of the major responsible of prosthetic infections, e.g. *S. aureus*, regardless of its resistance phenotype. Utilizing these materials could also help to reduce the excessive amounts of antibiotics currently used to treat and manage these infections. Therefore, the production and use of these low-cost materials could significantly benefit patients by reducing both morbidity and mortality from HAIs, while also mitigating the need for prolonged antibiotic treatments, which often have side effects, particularly in vulnerable patients, and contribute to the spread of antimicrobial resistance. Future research in the field of HAIs will increasingly focus on these new materials, promising substantial positive impacts on healthcare and global public health.

TABLES AND FIGURES

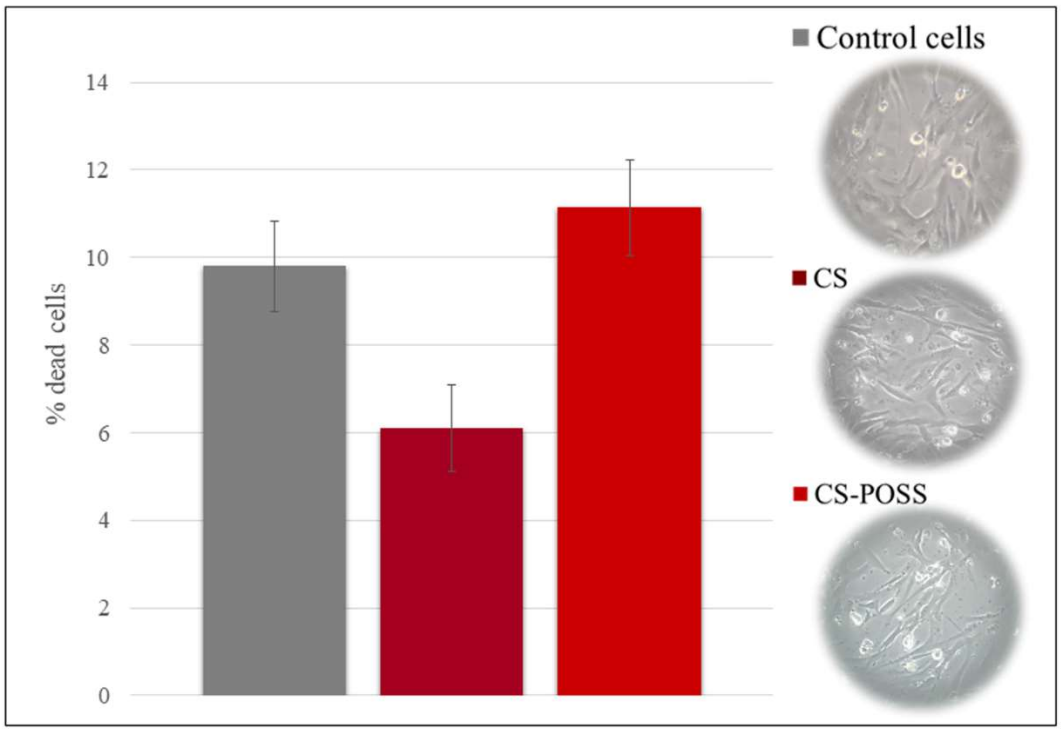

FIGURE 1.

RESULTS OF P.I. TEST FOR THE ASSESSMENT OF THE ASSAYED HYDROGEL BIOCOMPATIBILITY IN HFOB 1.19. EACH VALUE REPRESENTS THE MEAN (±SD) OF THE PERCENTAGE OF DEAD CELLS IN THE EXPERIMENTS MADE IN TRIPLICATE. (PARTIALLY MODIFIED HTTPS://WWW.MDPI.COM/1996-1944/15/22/8208).

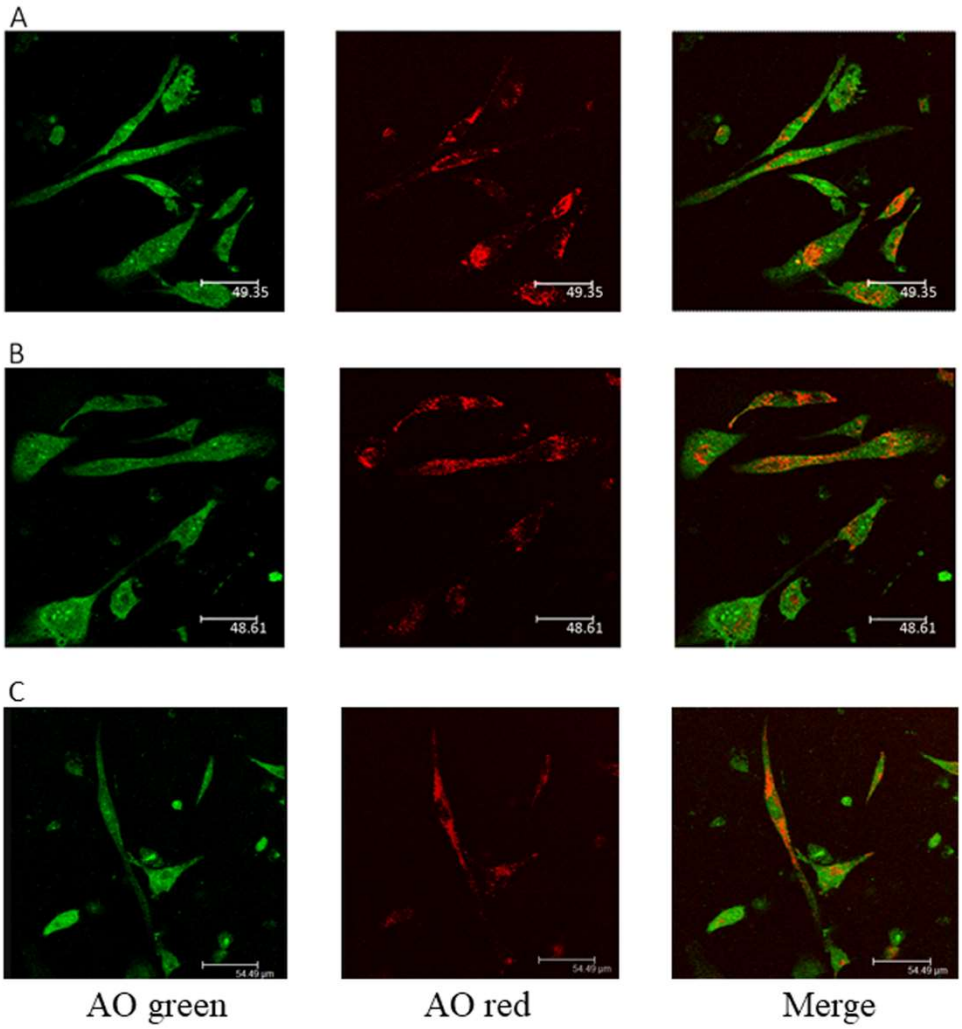

FIGURE 2

*CLSM IMAGES OF HFOB 1.19 CELLS LABELLED WITH THE METACHROMATIC FLUOROPHORE AO. (**A**) CONTROL CELLS; (**B**) OSTEOBLASTIC CELLS GROWN IN THE PRESENCE OF CS HYDROGEL; (**C**) OSTEOBLASTIC CELLS GROWN IN PRESENCE OF CS-POSS HYDROGEL IN ACIDIC COMPARTMENT OF CYTOSOL (INTACT LYSOSOMES AND MATURE ENDOSOMES), THE FLUOROPHORE IS SEQUESTERED THANKS TO THE PROTON PUMP AND EMITS A RED FLUORESCENCE, WHILE IT EMITS GREEN FLORESCENCE IN THE ABSENCE OF A LOW PH. THE CELLS RETAINED THEIR MORPHOLOGY (**A**–**C**) (PARTIALLY MODIFIED FROM HTTPS://WWW.MDPI.COM/1996-1944/15/22/8208).*

	MSSA (7)	MRSA (6)	MSSE (4)	MRSE (9)	VSE (5)	VRE (7)	Non-MDR PSEAER (3)	MDR PSEAER (3)
Amikacin	--	--	--	--	--	--	0%	0%
Amoxicillin Clavulanate	--	--	--	--	50%	100%	--	--
Ampicillin	--	--	--	--	50%	100%	--	--
Ampicillin/Sulbactam	--	--	--	--	50%	100%	--	--
Benzylpenicillin	71.4%	85.7%	--	--	--	--	--	--
Cefepime	--	--	--	--	--	--	0%	50%
Cefoxitin	--	100%	--	--	--	--	--	--
Ceftaroline	0%	0%	--	--	--	--	--	--
Ceftazidime	--	--	--	--	--	--	0%	50%
Ceftazidime/Avibactam	--	--	--	--	--	--	0%	0%
Ciprofloxacin	--	--	--	--	50%	100%	33.3%	0%
Clindamycin	28.6%	71.4%	0%	55.6%	--	--	--	--
Colistin	--	--	--	--	--	--	0%	0%
Co-trimoxazole	0%	0%	0%	0%	--	--	--	--
Daptomycin	0%	0%	0%	0%	--	--	--	--
Erythromycin	28.6%	71.4%	50%	77.8%	--	--	--	--
Fosfomycin	--	--	--	--	--	--	33.3%	--
Fusidic acid	14.3%	0%	25%	66.7%	--	--	--	--
Gentamicin	0%	0%	50%	66.7%	0%	71.4%	0%	0%

Imipenem	--	--	--	--	50%	100%	0%	100%
Kanamycin	--	--	--	--	50%	71.4%	--	--
Levofloxacin	0%	28.6%	0%	88.9%	50%	100%	--	--
Linezolid	0%	0%	0%	11.1%	0%	0%	--	--
Meropenem	--	--	--	--	--	--	0%	0%
Mupirocin	0%	0%	--	--	--	--	--	--
Oxacillin	0%	100%	0%	100%	--	--	--	--
Piperacillin/Tazobactam	--	--	--	--	--	--	0%	50%
Quinupristin/Dalfopristin	--	--	--	--	25%	0%	--	--
Rifampicin	0%	0%	0%	11.1%	--	--	--	--
Streptomycin	--	--	--	--	50%	71.4%	--	--
Teicoplanin	0%	0%	0%	44.4%	0%	100%	--	--
Tetracycline	0%	14.3%	75%	22.2%	--	--	--	--
Tigecycline	0%	0%	0%	0%	0%	14.3%	--	--
Tobramycin	--	--	--	--	--	--	0%	0%
Vancomycin	0%	0%	0%	0%	0%	100%	--	--
AVERAGE VALUE	**8.4%**	**26.2%**	**14.3%**	**38.9%**	**30.4%**	**73.5%**	**5.5%**	**22.7%**

TABLE 1

ANTIMICROBIAL RESISTANCE PATTERNS OF THE BACTERIAL STRAINS USED FOR TESTING ANTIMICROBIAL PROPERTIES OF CS- AND CS-POSS- HYDROGELS. (PARTIALLY MODIFIED FROM HTTPS://WWW.MDPI.COM/1996-1944/15/22/8208)

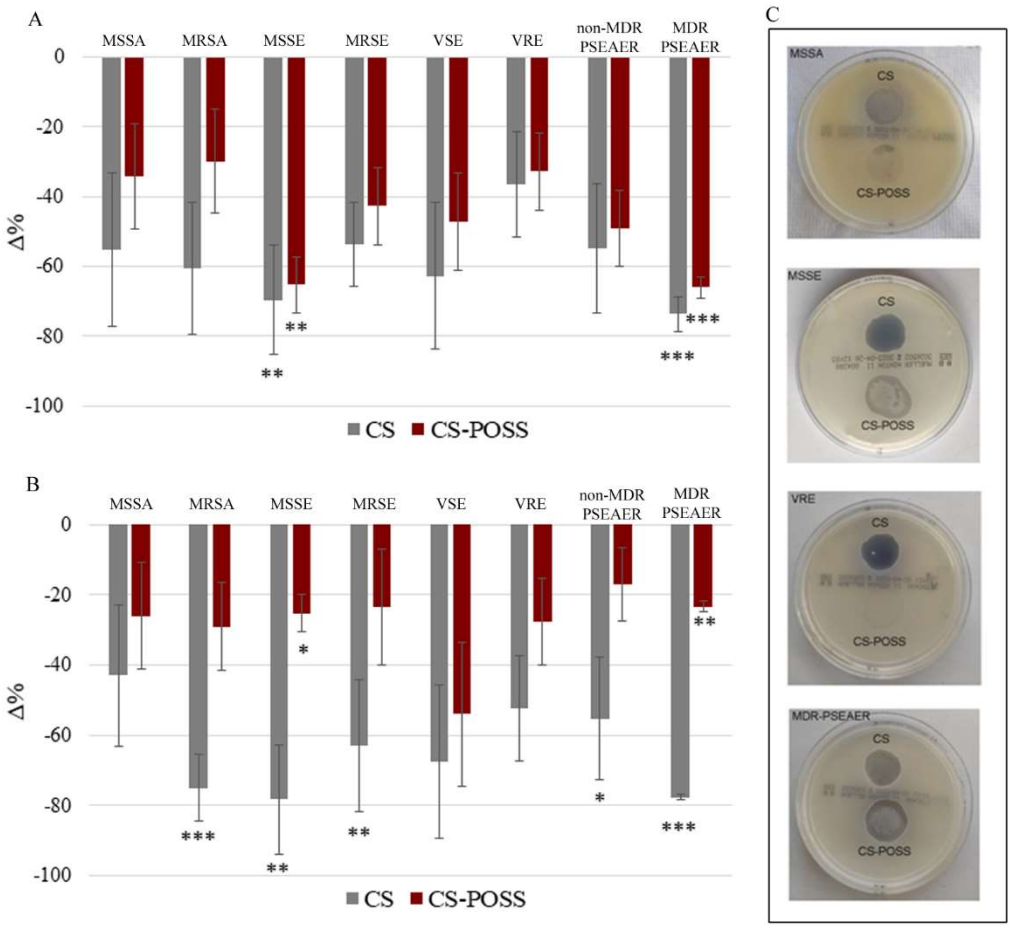

FIGURE 3

*DECREASED BACTERIAL GROWTH CAUSED BY CS AND CS-POSS TREATMENT, COMPARED TO BASELINE CONDITIONS, IN THE MHB SUSPENSION (**A**) AND MULLER–HINTON AGAR MEDIUM PLATES (**B**). BACTERIAL GROWTH IN PLATES WITH CS AND CS-POSS HYDROGELS (**C**). *, **, AND *** P < 0.05, 0.01 AND 0.001, RESPECTIVELY. (PARTIALLY MODIFIED FROM HTTPS://WWW.MDPI.COM/1996-1944/15/22/8208).*

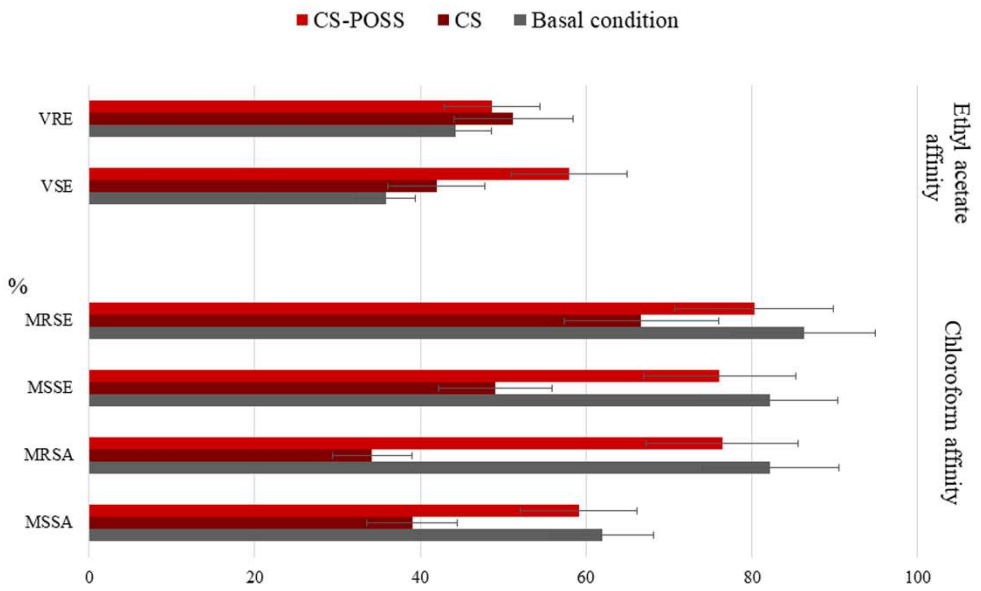

FIGURE 4

CHANGES OF CHARGES ON THE BACTERIAL CELL SURFACE EVALUATED BY AFFINITY TO ETHYL ACETATE (DONATING ELECTRON) AND CHLOROFORM (ACCEPTING ELECTRON), AFTER CS OR CS-POSS TREATMENT. (PARTIALLY MODIFIED FROM HTTPS://WWW.MDPI.COM/1996-1944/15/22/8208).

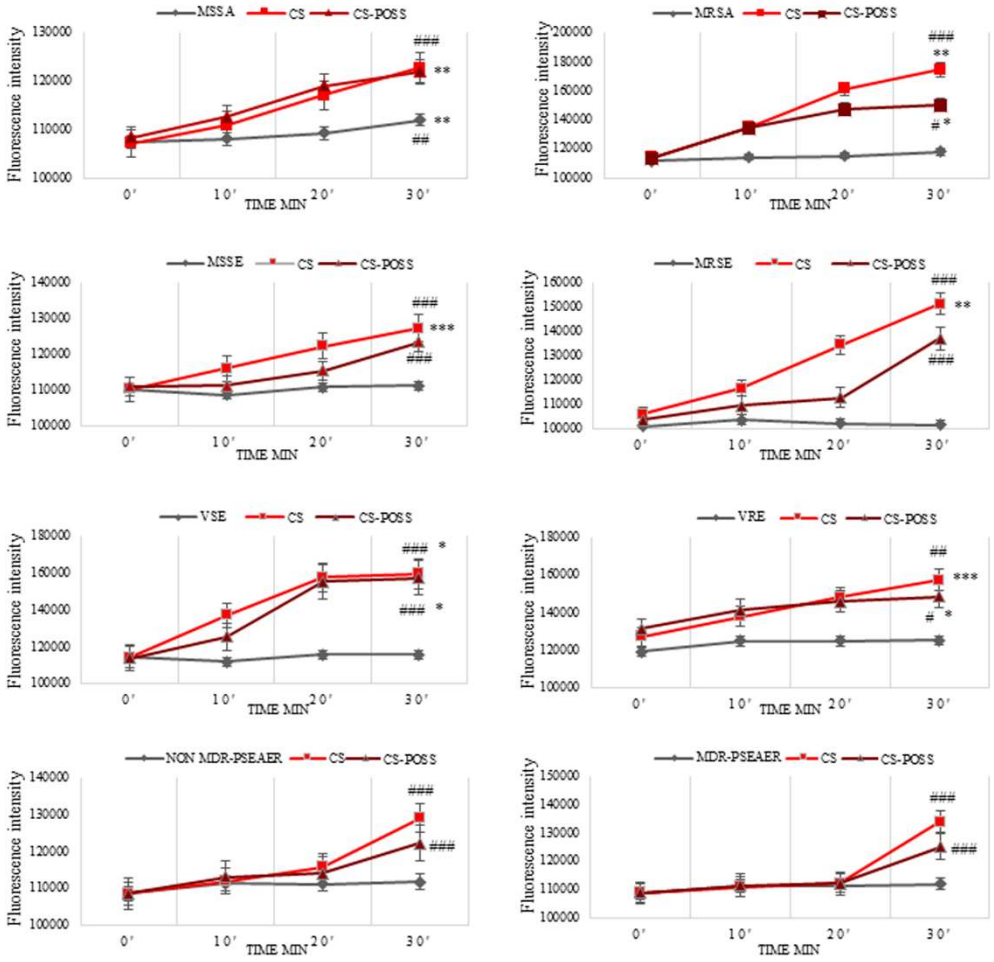

FIGURE 5

PROPIDIUM IODIDE FLUORESCENCE INTENSITY IN BASAL CONDITION AND AFTER 10', 20', AND 30' OF CS AND CS-POSS TREATMENT (P < 0.05, ** P < 0.01, *** P <0.001; # P < 0.05, ## P < 0.01, ### P <0.001)."*" TIME CORRELATION AND "#" 30' VS 0'. (PARTIALLY MODIFIED FROM HTTPS://WWW.MDPI.COM/1996-1944/15/22/8208).*

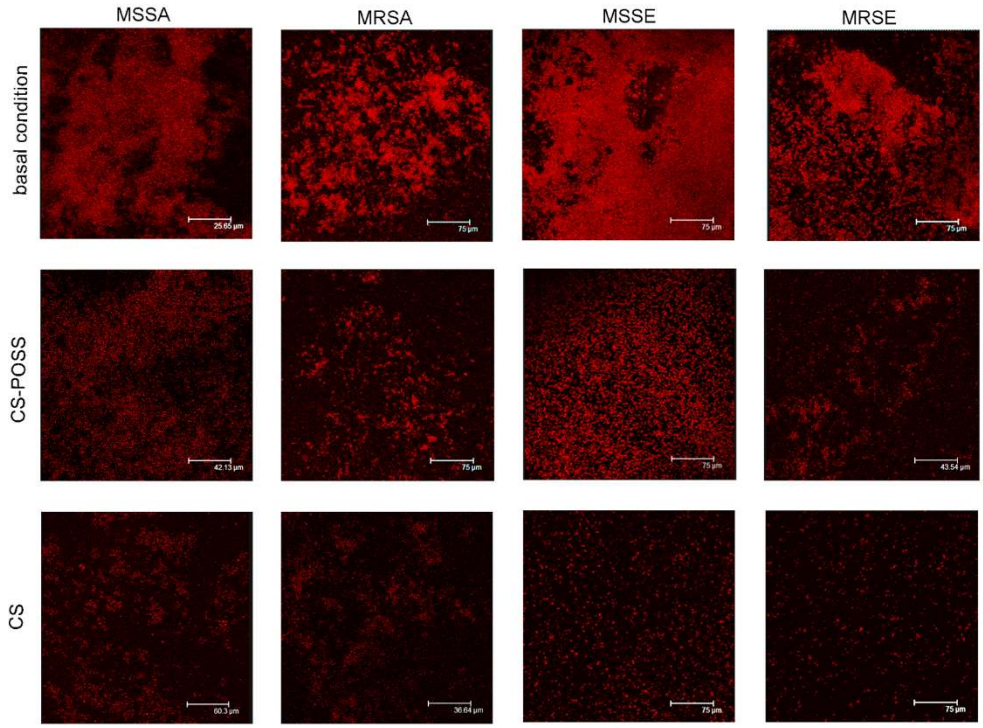

FIGURE 6

CONFOCAL LASER IMAGES OF BIOFILM FORMED BY STAPHYLOCOCCI *IN BASAL CONDITION AND WITH CS AND CS-POSS HYDROGELS. (FROM HTTPS://WWW.MDPI.COM/1996-1944/15/22/8208).*

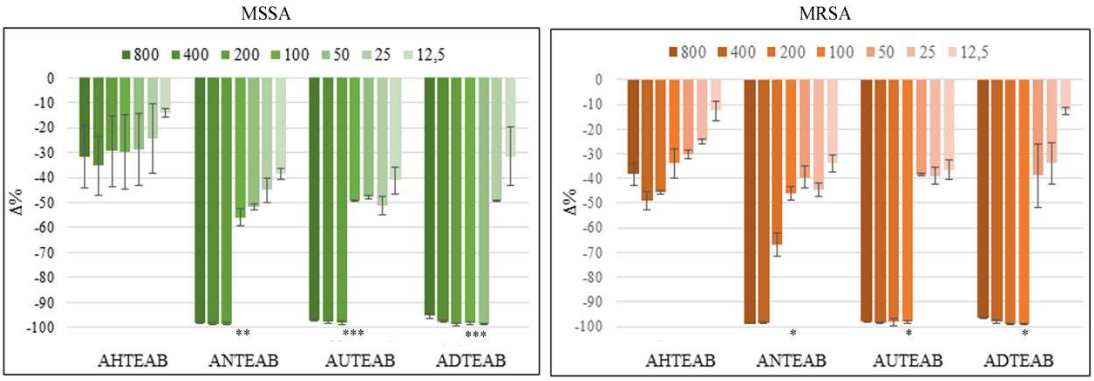

FIGURE 7

*INHIBITION OF BACTERIAL GROWTH FOLLOWING EXPOSURE TO DIFFERENT AATEABS COMPARED TO THE STRAIN UNDER BASAL CONDITIONS, FOR MSSA AND MRSA (*P<0.05, ** P<0.01, ***P<0.001).*

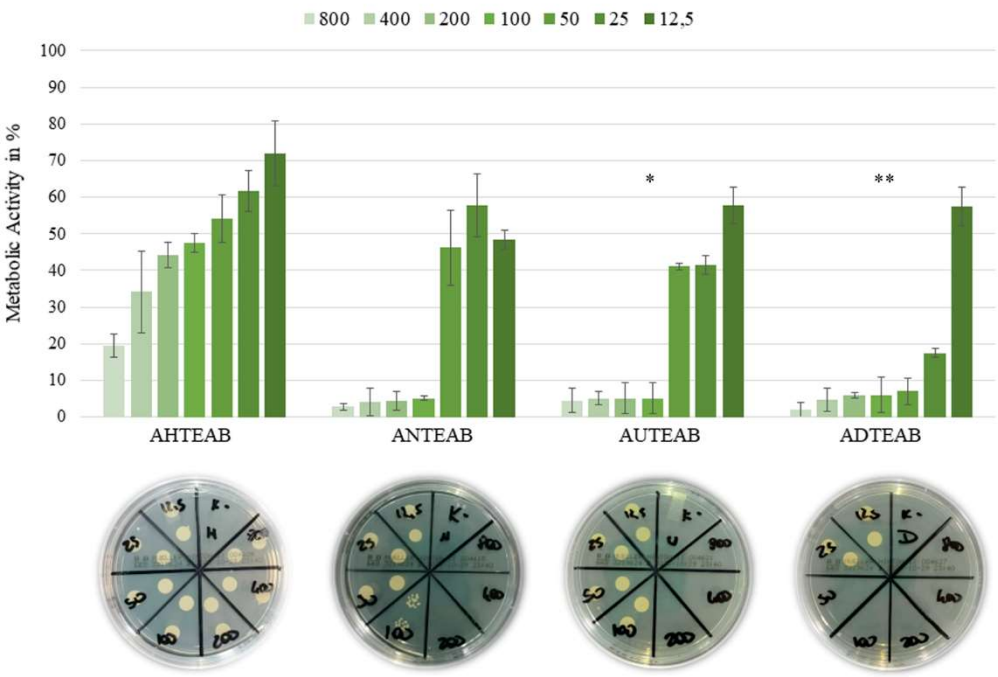

FIGURE 8

*METABOLIC ACTIVITY OF THE MSSA STRAIN AFTER EXPOSURE TO DIFFERENT SALTS AT DIFFERENT CONCENTRATIONS AND THE CORRESPONDING MBC TEST (*P<0.05, ** P<0.01).*

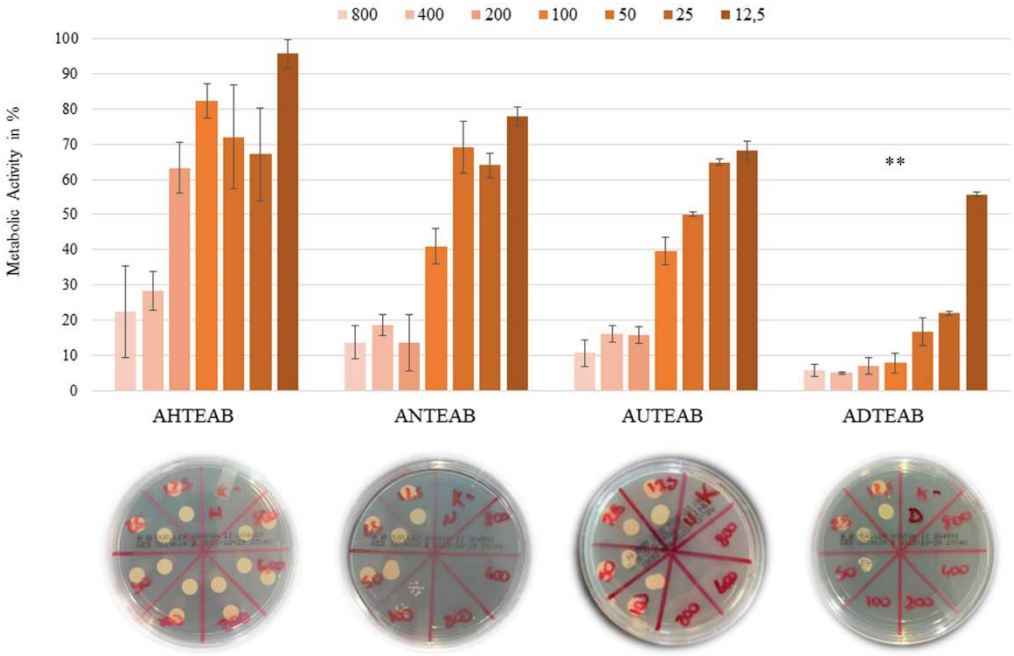

FIGURE 9

*METABOLIC ACTIVITY OF THE MRSA STRAIN AFTER EXPOSURE TO DIFFERENT SALTS AT DIFFERENT CONCENTRATIONS AND THE CORRESPONDING MBC TEST (** P<0.01).*

FIGURE 10

*BIOFILM FORMATION (%), EVALUATED WITH CRYSTAL VIOLET ASSAY, OF MSSA AND MRSA AFTER 48 H TREATMENT WITH AATEABS, (***P<0.001).*

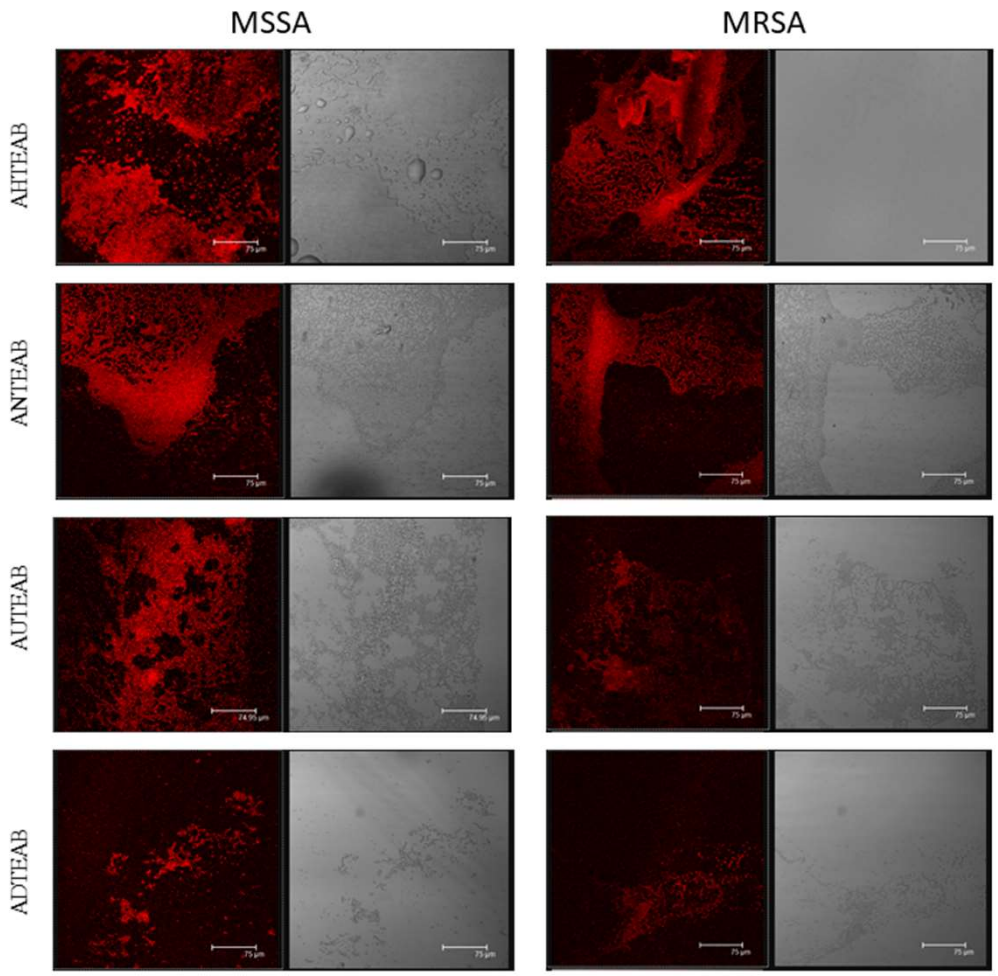

FIGURE 11

CONFOCAL LASER IMAGES OF BIOFILM FORMED MSSA AND MRSAIN BASAL CONDITION AND WITH DIFFERENT AATEABS AT THE CONCENTRATION OF $25\mu g\ mL^{-1}$.

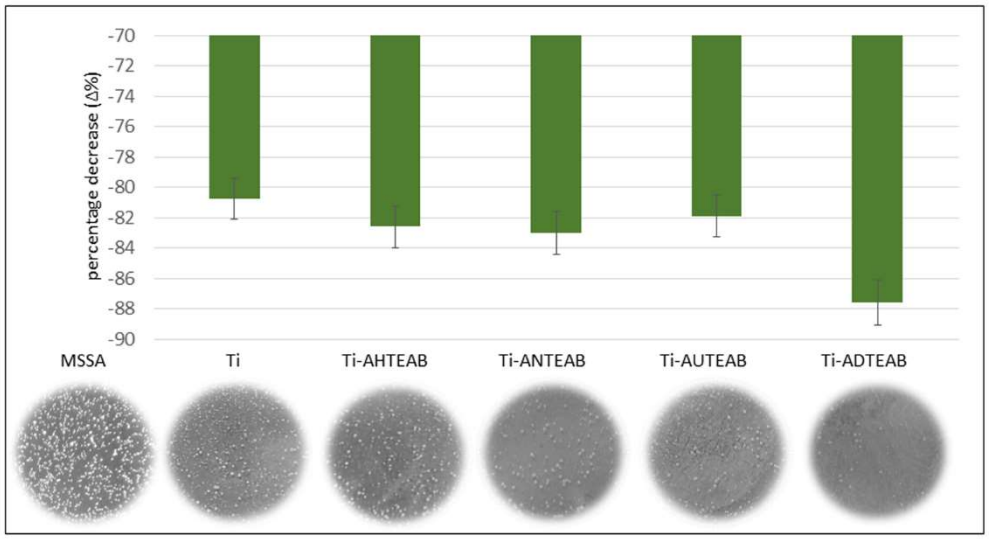

FIGURE 12

ANTIBACTERIAL ACTIVITY OF NON-FUNCTIONALIZED AND FUNCTIONALIZED TITANIUM DISCS AGAINST MSSA.

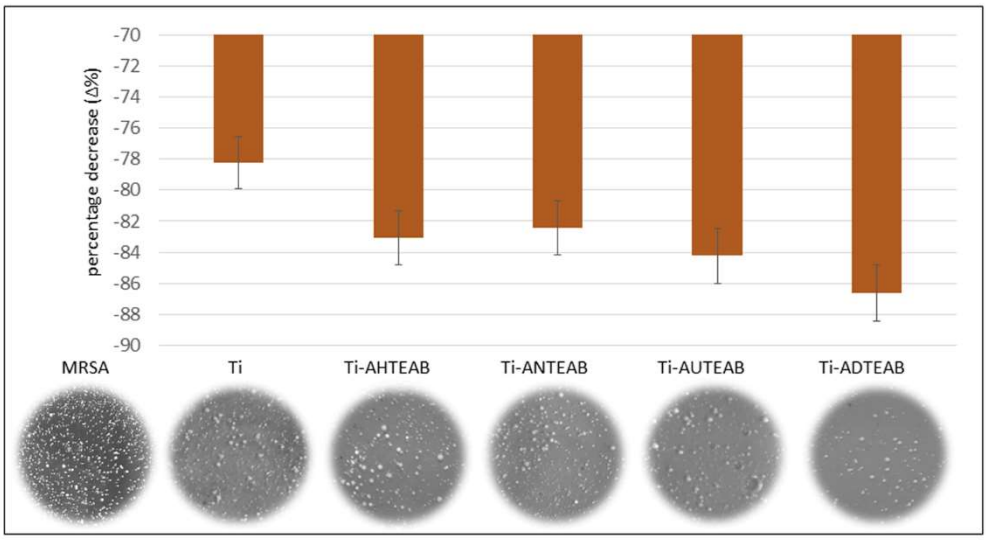

FIGURE 13

ANTIBACTERIAL ACTIVITY OF NON-FUNCTIONALIZED AND FUNCTIONALIZED TITANIUM DISCS AGAINST MRSA.

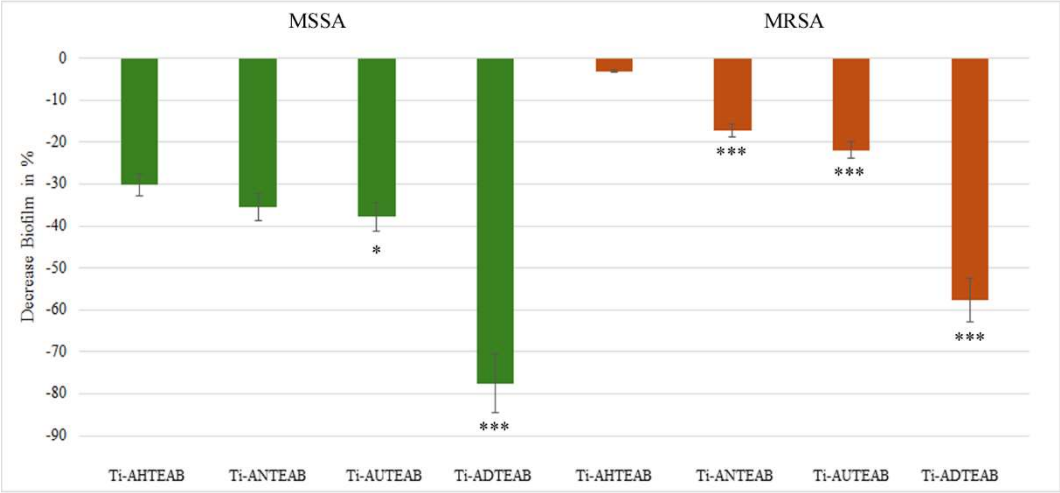

FIGURE 14

*DECREASE BIOFILM FORMATION (%) OF MSSA AND MRSA AFTER 48 H TREATMENT WITH TI-AATEABS (*P<0.05, ***P<0.001).*

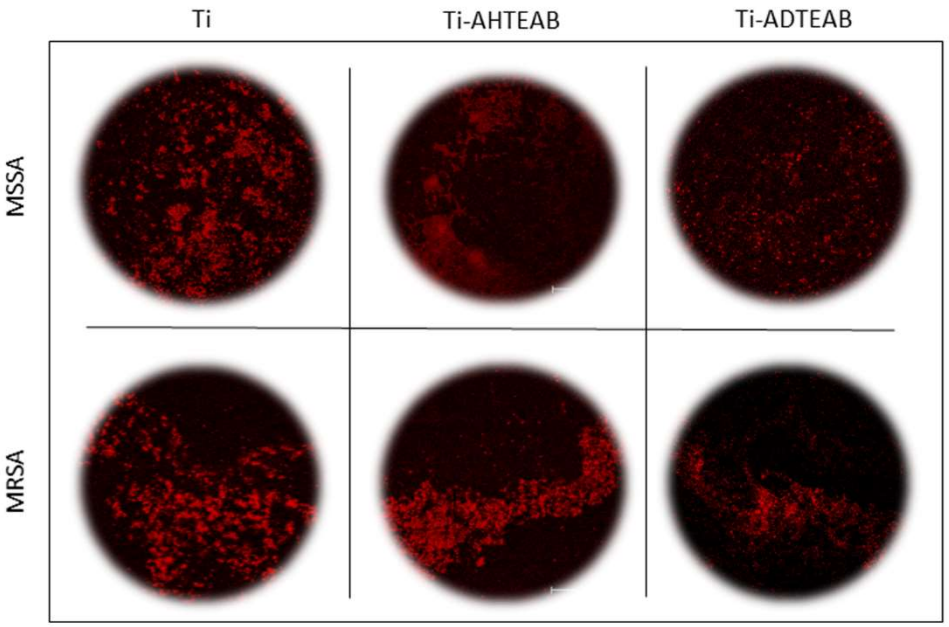

FIGURE 15

CONFOCAL LASER IMAGES OF BIOFILM FORMED MSSA AND MRSA IN TI, TI-AHTEAB AND TI-ADTEAB.

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