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CHARACTERIZATION OF A NOVEL ANTIMICROBIAL SURFACE COATING AND ITS EFFECTIVENESS AGAINST IMPORTANT PATHOGENS

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Thesis presented to Escola Superior de Biotecnologia of the Universidade Católica Portuguesa to fulfil the requirements of Master of Science degree in Applied Microbiology

By

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Abstract

The survival and spread of resistant foodborne and nosocomial-associated bacteria through high-touch surfaces is not always prevented by the employment of cleaning protocols. Antimicrobial surface coatings surge from the need to eradicate pathogenic bacteria and prevent future infections and even outbreaks.

This study aimed to characterize a novel QAC-based coating in terms of cytotoxicity, kinetics, and durability and to determine its ability to inhibit important health-associated pathogens on different surface materials (polyvinyl chloride, glass, and stainless steel).

Preliminary efficacy of the novel antimicrobial coating was conducted against several Gram-positive and -negative bacteria. Their growth was inhibited after direct contact, and three relevant pathogens were selected to proceed with the study - *Acinetobacter baumannii* ESB260, *Escherichia coli* ATCC 25922, and *Listeria monocytogenes* Scott A. Both *E. coli* and *L. monocytogenes* were sensitive to all antibiotics tested, unlike *A. baumannii* that was resistant. In addition, all the pathogens presented similar temperature and pH susceptibility profiles. The antimicrobial activity of the coating and its durability on tested surfaces were assessed through an international standard developed by the International Organization for Standardization, (ISO). None of the pathogens were able to survive within 1-minute of contact time on each coated surface. However, despite its quick contact killing time, the durability of the antimicrobial coating on each surface, after 7 days, was not promising. No antimicrobial activity was observed against all the pathogens on the coated surfaces after cleaning with a wet cloth, bleach, and a commercial degreaser, except *E. coli* and *L. monocytogenes* which were both inhibited on glass surfaces treated with the commercial degreaser. Bactericidal activity and minimum inhibitory concentrations for the product were also assessed. Cytotoxicity of the compound was investigated through MTT assay; concentrations as low as 5% (v/v) were revealed to be cytotoxic towards both human keratinocyte cells and mouse fibroblast cells. However, no vestigial concentrations of antimicrobial coating (≤0.3125% v/v) were cytotoxic to human colorectal adenocarcinoma cells.

Further tests are required, especially regarding the treatments to assure the coating's durability, but it is clear that this novel coating is promising to eliminate clinically relevant pathogens.

Keywords: Antimicrobial activity; Quaternary ammonium compounds; touch surfaces.

Resumo

A sobrevivência e disseminação de bactérias resistentes de origem alimentar e nosocomial através de superfícies nem sempre são evitadas pelo uso de protocolos de limpeza. Os revestimentos de superfície antimicrobianos surgem da necessidade de erradicar bactérias patogénicas e prevenir infecções futuras e até mesmo surtos.

Este estudo teve como objetivos caracterizar um novo revestimento baseado em CQA em termos de citotoxicidade, cinética e durabilidade e determinar a sua capacidade de inibir importantes patogénicos associados à saúde em diferentes materiais de superfície (cloreto de polivinil, vidro e aço inoxidável).

A eficácia do novo revestimento foi testada inicialmente contra várias bactérias Gram-positivas e-negativas. Após a sua inibição por contacto direto, três patogénicos relevantes foram selecionados para prosseguir com o estudo - *Acinetobacter baumannii* ESB260, *Escherichia coli* ATCC 25922 e *Listeria monocytogenes* Scott A. Tanto *E. coli* como *L. monocytogenes* foram sensíveis a todos os antibióticos testados, ao contrário da total resistência de *A. baumannii*. Além disso, todos os patogénicos apresentaram perfis de sensibilidade a temperatura e pH semelhantes. A atividade antimicrobiana do revestimento e a sua durabilidade nas superfícies testadas foram avaliadas segundo a ISO 22196 (2011). Nenhum dos patogénicos sobreviveu após 1 minuto de contato em cada superfície revestida. No entanto, apesar do seu tempo de contacto curto, a sua durabilidade após 7 dias em cada superfície não foi promissora. Não foi observada atividade antimicrobiana contra os patogénicos nas superfícies revestidas e limpas com um pano húmido, lixívia e um desengordurante comercial. No entanto, tanto *E. coli* quanto *L. monocytogenes* foram inibidas no vidro tratado com o desengordurante comercial. A atividade bactericida e as concentrações inibitórias mínimas do produto também foram avaliadas pela EN 1276 (2009). A citotoxicidade do composto foi avaliada pelo ensaio MTT e concentrações tão baixas quanto 5% (v / v) foram citotóxicas para células de queratinócitos humanos e de fibroblastos de camundongo. No entanto, nenhuma concentração vestigial de revestimento antimicrobiano (≤ 0,3125% v / v) foi citotóxica para células de adenocarcinoma colorretal humano.

Mais testes são necessários, especialmente em relação aos tratamentos necessários para garantir a durabilidade do revestimento, mas está claro que este novo revestimento é promissor para eliminar patogénicos clinicamente relevantes.

Palavras-chave: Actividade antimicrobiana; compostos de amónio quaternário; superfícies de contacto.

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Contents

List of Abbreviations

AMCs	Antimicrobial Coatings
A\A	Antibiotic\Antifungal
AMP	Antimicrobial Peptides
ATCC	American Type Culture Collection
CAMHB	Cation Adjusted Muller-Hinton Broth
CDC	Centre for Disease Control
CFU\mL	Colony Forming units per millilitre
CLSI	Clinical and Laboratory Standards Institute
D\E	Dey-Engley Neutralizer
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
ECDC	European Centre for Disease Prevention and Control
ECHA	European Chemical Agency
EN	European Standard
EPA	Environmental Protection Agency
FBS	Fetal Bovine Sera
GFSI	Global Food Safety Initiative
GLASS	Global Antimicrobial Resistance and Use Surveillance System
HCAIs	Healthcare-associated infections
HCl	Hydrochloric acid
HGT	Horizontal gene transfer
ISO	International Standard Organization
LHB	Lysed Horse Blood
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
MRLs	Maximum Residue Levels
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTT	3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
NB	Nutrient Broth
NCTC	National Collection of Type Cultures

nm	Nanometre
OCDE	Organisation and Economic Co-operation and Development
PCA	Plate Count Agar
PVC	Polyvinyl Chloride
QACs	Quaternary Ammonium Compounds
spp.	Species
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TSC	Tryptone Sodium Chloride
YE	Yeast Extract

1. Introduction

The spread of multidrug-resistant bacteria (MDR) is one of the global challenges of the 21st century. An ever-increasing range of infections caused by bacteria, viruses, fungi or parasites have their treatment, as well as effective prevention, menaced because of their decreased susceptibility to commonly used antimicrobials, mainly antibiotics and disinfectants, that once were effective (Prestinaci et al., 2015; Vrancianu et al., 2020; Mc Carlie et al., 2020). By definition, bacteria are considered resistant to a particular antimicrobial compound/drug when it cannot effectively inhibit their growth nor kill them after being administrated the prescribed recommended dose of the drug. This, taking into consideration the minimum inhibitory concentration (MIC) to precisely determine the concentration of antimicrobial agent necessary to inhibit the growth of the pathogen that is being studied (Schwarz et al., 2017).

The discovery and subsequent evolution of antibiotics throughout the 20th century led to the belief that humanity had conquered the age-old battle against infectious diseases, proven wrong by the emergence of new pathogens as well as multidrug-resistant pathogens due to the overuse of these antibacterial substances (Spellberg et al., 2013). Resistance to antimicrobial agents has a rising tendency, reaching dangerously high levels worldwide, threatening the treatment of common infectious diseases casting a severe health crisis problem (Reygaert, 2018). Emerging and re-emerging nosocomial and food pathogen-related diseases have significantly increased over the last decades, having a significant impact on public health, both socially and economically.

The three main mechanisms of antimicrobial compound resistance by bacteria can be summarized in: (1) changes in membrane permeability; (2) alteration of the proteins that are antimicrobial targets; and (3) enzymatic degradation of the antimicrobial agent. Resistance to antimicrobial agents can be acquired through horizontal gene transfer (HGT) or evolve through *de-novo* mutations (Pietsch et al., 2020).

Regarding antimicrobial surfaces, the risk of spreading MDR lies not only in developing resistance to a specific antimicrobial agent but also in the occurrence of two phenomena, cross-resistance and co-resistance. Cross-resistance happens when a single

molecular mechanism is capable of mediating resistance to more than one antimicrobial agent. Co-resistance can occur when mechanisms encoding resistance or reduced susceptibility are genetically linked; thus, the selective pressure conferred by the presence of one antimicrobial co-selects for the other and ensures the retention of all of these within the population (Cassidy et al., 2020; Pietsch et al., 2020).

MDR is not a new phenomenon but has recently had its natural progression hastened by the indiscriminate use and overuse of antibiotics, being its inappropriate prescription a determining cause of this rise (Vrancianu et al., 2020). Studies showed that between 30% and 50% of all prescribed antibiotics are not correctly applied (Vázquez-López et al., 2020), as well as the indiscriminate use of disinfectants, having been reported acquired insusceptibility to disinfectants that can be conferred by newly acquired genes or by mutations (Schwaiger et al., 2014). Resistance triggered by biocides has been a poorly studied subject in the past due to its classification as clinically negligible and as a rare phenomenon (Fraise, 2002).

Infections may occur via touch surfaces contaminated with pathogens; thus, antimicrobial surfaces surge from the need to combat pathogenic bacteria and possibly control and prevent future infections and even outbreaks (Cassidy et al., 2020; Villapún et al., 2016). The proper use of this technology imposes a significant advance in maintaining clean and sterile surfaces, a key feature for hospitals, food-related facilities, buses, and any other high-risk areas for contamination (Adlhart et al., 2018). Antimicrobial surfaces have been primarily introduced in healthcare settings with the target goal of reinforcing hygiene procedures already in use and also being employed to deter the surge of MDR worldwide (Pietsch et al., 2020). However, the indiscriminate use of these biocidal surfaces may lead to problems related to our microbiome development, which may impair our immunes system proper function, as well as concerns over potential selection pressure that may drive the evolution of MDR instead of deterring it (Pietsch et al., 2020; Cassidy et al., 2020).

Just as well as antibiotic stewardship already in motion in most hospitals all over the world, a need for antimicrobial surfaces stewardship is needed to improve its use, in such a manner at which it is possible to achieve its original goal with as few setbacks and harmful consequences as possible for human life.

1.1. Pathogens and antimicrobial resistance

Community-associated pathogens are disease-causing bacteria that significantly impact human health, posting a major concern over the blurred lines between molecular and epidemic boundaries between healthcare and community-associated infections (Balkhy et al., 2006; Dupper et al., 2019). A large number of these have emerged from hospital-associated pathogens and associated foodborne pathogens such as *Escherichia coli*, *Campylobacter* spp., *Salmonella* spp., methicillin-resistant *Staphylococcus aureus* (MRSA), *Listeria monocytogenes*, *Acinetobacter* spp., *Pseudomonas aeruginosa*, amongst others (Khan et al., 2020).

Escherichia coli is the most common cause of urinary tract infections (UTIs) in adult women and intestinal infections. Typically, these infections are usually uncomplicated. Antibiotics such as fluoroquinolones are prescribed daily for the treatment of these infections. The overall continued prescription of antibiotics accounts for increasing multidrug-resistant *E. coli* strains worldwide (Nicolle et al., 2013; Nji et al., 2021). Reported resistance to commonly used antibiotics for urinary tract infections such as ciprofloxacin and co-trimoxazole was found to be high for *E. coli*, 43.1% and 54.4%, respectively, data from Global Antimicrobial Resistance and Use Surveillance System (GLASS), not implemented in Portugal (WHO, 2021). Plasmid-borne carbapenemases that confer resistance to almost all antibiotics have been identified in *Enterobacteriaceae*, which severely compromises the treatment of patients (Araújo et al., 2019). Regarding resistance to disinfectants, the presence of quaternary ammonium compounds (QACs), commonly and widely used as a disinfectant, resistant genes were found in *E. coli* isolated from meat samples (Zou et al., 2014).

Listeria monocytogenes is an opportunistic, foodborne pathogen that causes severe infections such as meningitis or septicaemia in newborns, immunocompromised patients, and the elderly or leads to abortion (Charpentier et al., 1999). It can be found in contaminated foods and the environment. Most *L. monocytogenes* strains show intrinsic resistance to cephalosporins, especially third and fourth generations, and fluoroquinolones (de Vasconcelos Byrne et al., 2016). Most human strains of *L. monocytogenes* are sensitive to several antibiotics such as vancomycin, imipenem, rifampicin, gentamicin, penicillin, amoxicillin, and penicillin (de Vasconcelos Byrne et al.,

2016). Some isolates of *Listeria* spp. have a low overall prevalence of biocide tolerance, although a reduced susceptibility to QACs has been reported (Roedel et al., 2019).

Acinetobacter is commonly found in the environment. *Acinetobacter baumannii* accounts for most of the infections caused by this group of bacteria (CDC, 2019). These bacteria can cause a multitude of infections, mostly involving the respiratory tract (ventilator-associated pneumonia). Wound infections and cases of meningitis and bacteraemia have also been reported (Manchanda et al., 2010). In a recent study, 73.6% of the clinical isolates analysed were resistant to quinolones, 71.3% were resistant to sulphonamides, and more than half were resistant to cephalosporins, beta-lactams/beta-lactamase inhibitors, and carbapenems (Chen et al., 2017). No resistance to colistin has been observed (Chen et al., 2017; Vázquez-López et al., 2020). This broad spectrum of antimicrobial agents' resistance is associated with a higher mortality rate as well as longer hospital admissions when compared to other *Acinetobacter* species. Multidrug-resistant *Acinetobacter* strains have been proven to also have a lower susceptibility to disinfectants, especially QACs and chlorhexidine, due to the presence of resistance encoding genes (Ivanković et al., 2017).

Antimicrobial agents such as QACs are among the most commonly used disinfectants, with an increase in bacterial tolerance to QACs reported. The potential development of antimicrobial resistance is still understudied, and the lack of standardized biocide testing is a setback (Gregorchuk et al., 2020). In addition, bacterial responses to different biocides will differ accordingly to their cellular anatomy and physiology (Bragg, 2014). Literature review names two different mechanisms of resistance for disinfectants, intrinsic and acquired. Intrinsic resistance means that bacteria have an innate ability to resist the action of a certain biocide and is most commonly observed in Gram-negative bacteria such as *Pseudomonas aeruginosa* strains (Bragg, 2014; Pachori et al., 2019). The intrinsic resistance mechanism is associated with the low permeability of the external membrane mediated by lipopolysaccharides and efflux bombs (Pachori et al., 2019). The presence of efflux systems from all five *Pseudomonaceae* superfamilies' in *P. aeruginosa*, and its capacity to overexpress more than one system simultaneously may explain the high rates of disinfectant resistance (Beier et al., 2015). Acquired resistance occurs when a previously susceptible organism acquires resistance to a particular compound. This may be a result of mutations or

horizontal gene transfer that allows for bacterial survival under adverse conditions, which is commonly observed in *Staphylococcus aureus* strains (Bragg et al., 2014).

Multiple bacterial isolates from different sources have been reported to have QACs resistance genes (Zou et al., 2014). In *E. coli*, four resistance genes belonging to the small multidrug resistance (SMR) family have been identified: *sugE*, leads to resistance to a subset of toxic QACs; *emrE*, reported to have a key role in resistance to a wide range of biocides; *ydgE*/*ydgF*, known for conferring resistance to anionic detergents and one belonging to the major facilitator superfamily (MFS); and *mdfA*, reported to confer resistance to cationic detergents such as QACs as well as resistance to extreme alkaline pH (Yerushalmi et al., 1995; Chung et al., 2002; Nishino et al., 2001; Edgar et al., 1997; Lewinson et al., 2004).

Resistance genes such as *qacH*, associated with the export of belzalkonium chloride (BAC), and other *qac* genes such as *qacA,* which confers export mediated resistance against QACs, *bcrB*, and *bcrC*, directly related to BAC resistance, have been identified in *L. monocytogenes*, all belonging to the SMR family (Müller et al., 2013; Cervinkova et al., 2013; Xu et al., 2014; Møretrø et al., 2017).

In *A. baumannii*, *qacΔE1*, *qacE*, *qacG*, *qacA* and *qacB*, multidrug exporters directly related to QACs resistance, have been detected in several isolates (Heir et al., 1999; Mahzounieh et al., 2014; Liu et al., 2018). A novel antimicrobial resistance gene belonging to the SMR family, *abeS* has been brought to light with gene deletion proving its role in resistance development to a different range of antimicrobial agents (Srinivasan et al., 2020).

All the proteins encoded by the resistance genes are located in the inner membrane (Bragg et al., 2014; Srinivasan et al., 2020). Therefore, the primary mechanism of disinfectant resistance is the drug efflux pump that plays a vital role in lowering the intracellular concentration of the biocide by being actively pumped out of the cell by these proteins (Bragg et al., 2014).

Currently, due to the SARS-CoV-2 (COVID-19) pandemic, resistance to QACs-based disinfectants has seen a rise in prevalence and frequency (Mahoney et al., 2021). However, Gerba (2015) has reported that some tolerance to QACs after long-term exposure may be expected; the development of resistance is unlikely due to the non-specific action of the compound. It is reported that increased MIC values are due to

QACs overuse, but that is not correlated with resistance development and that errors such as lousy handling, diluted compounds, and pseudo resistance are the cause for improper designation of antimicrobial resistance (Gerba, 2015). The existence of QAC resistant genes is an important finding to refute this study.

1.2. Bacterial adhesion and antimicrobial surfaces

Bacterial adhesion on surfaces has become a significant concern in hospitals, food factories, industry, and even at home, being responsible for economic losses as well, and more importantly, for threatening human health maintenance, being capable of causing severe health problems and complications (Swartjes et al., 2015). The most commonly used prevention and treatment methods are antibiotics, that due to the ever-increasing bacterial multidrug resistance, are increasingly less effective (Swartjes et al., 2015).

The initial attachment is characterized by planktonic bacteria getting close to the surface by a combination of physical and chemical forces. To strengthen the attachment between the surface and the bacterium, appendages such as pili, fimbriae and flagella are key components of the cell. Environmental aspects, such as pH, temperature, and the hydrophobicity or hydrophilicity of the surface, are also essential influencers for bacterial adhesion (Achinas et al., 2019).

The survival and growth of pathogens may occur in touch surfaces, and thus, its transmission through contact with infected inanimate objects. The employment of cleaning protocols does not always result in the eradication of the pathogens, meaning that these can still be present with enough infectious dose to cause disease (Cassidy et al., 2020).

In the study of Hansen et al. (2007), the authors compiled data from The Outbreak Database to assess rates of closure of medical departments during nosocomial outbreaks, calculated and stratified for medical departments, for causative pathogens, for outbreak sources, and the assumed mode of transmission. With 752 outbreaks out of the reported and compiled 1561 reports, infection through contact with contaminated surfaces has been identified as the most likely route of transmission in hospitals (Hansen et al., 2007). Healtcare-associated pathogens are able to survive up

to several months on inanimate surfaces casting a major source of touch transmitted infections when cleaning and disinfection processes are not carried through or fail (Ellingson et al., 2019). It is important to mention that the detection of the pathogen does not translate to the risk of infection due to degradation of the microorganism and subsequent decrease in infection capability (Cassidy et al., 2020). Pathogens such as *Clostridium difficile*, *Escherichia coli*, *Klebsiella spp*., and *Staphylococcus aureus* have been shown to have a detectability period of over one month on common inanimate surfaces (Cassidy et al., 2020).

Being the process of cleaning and disinfecting of frequently touched surfaces of utmost importance when it comes to deterring healthcare-associated infections (HCAIs), studies show that the majority of near-patient surfaces are not adequately disinfected regularly; thus, the need for improved approaches that not only reduce microbial activity but that also deter the development of MDR (Ahonen et al., 2017).

Studies around the world have shown, per example, worrisome findings regarding what microorganisms can be found on buses. For example, in a Portuguese study by Simões et al. (2011), the authors observed a high prevalence of methicillin-resistant *Staphylococcus aureus* in buses in Porto and Lisbon. Chowdhury et al. (2016) also identified the presence of enteric bacteria, such as *E. coli*, *Salmonella* Typhi and *Shigella* in buses in Bangladesh (Simões et al., 2011; Chowdhury et al., 2016).

The need for novel additional or even replacement methods to the traditional mishandled use of disinfectants and antibiotics to reduce microbial activity on-site, and infection potential is preeminent to reverse the growth of MDR (Adlhart et al., 2018).

1.3. Antimicrobial surface coatings

The prevention of infections caused by these pathogens could be effectively carried out by using antimicrobial surface coatings that do not allow the viable bacteria to adhere to the surface and/or inhibit its growth.

The technology behind antimicrobial surfaces has been developed over the last few years, being introduced mainly in healthcare settings to complement pre-existing hygiene protocols to prevent infections from occurring and thus diminish the use of antibiotics to help fight the ever-increasing antimicrobial resistance. However, a concern

that rises from this alternative is the selection pressure that may be exerted, which may cause further spread of antimicrobial resistance instead of its aim to reduce it (Pietsch et al., 2020).

The increasing demand for stringent measures to control and prevent future outbreaks and to suppress the growth of MDR, as well as the rising awareness regarding health maintenance, has led to a rising tendency to the development of antimicrobial surfaces, with the industry anticipating an increase in demand, as reported by Global Market Insights (Global Market Insights 2021).

There are two main mechanisms when it comes to how effective antimicrobial surface coatings work. Its function may rely on anti-adhesive proprieties that do not allow bacterial adhesion to surfaces, or on bactericidal proprieties, that kill the bacteria before or after contact. A combination of both mechanisms is also widely used to achieve optimum results (Swartjes et al., 2015). The preeminent types of antimicrobial surface coating are antimicrobial peptides, antibacterial enzymes, nanoparticle-containing coatings such as gold or silver, quaternary ammonium compounds, anti-adhesive polymers via super hydrophobic coatings and chitosan-based coatings (Swartjes et al., 2015).

Antimicrobial peptides (AMPs) are small molecular weight proteins with broad-spectrum antimicrobial activity, composed of amino acids, and suitable for surface attachment. It has been shown to be effective against strains with high antimicrobial resistance with relatively little induction of resistance among its target organisms (Swartjes et al., 2015).

Antibacterial enzymes are commonly used in detergents and industrial settings, mainly in the food industry, due to their non-toxicity. Enzymes interfere with bacterial adhesion on surfaces and may act as a biocide (Swartjes et al., 2015).

Gold, silver and copper nanoparticles have been shown to act as biocides, having been widely used throughout the years to prevent bacterial adhesion and biofilm formation. Mammalian cells studies are important to access toxicity against human cells of these nanoparticles (Swartjes et al., 2015). In addition, environmental studies have shown that copper-based antimicrobial coatings (AMCs) can co-select for MDR under submerged conditions (Pietsch et al., 2020).

Chitosan-based coatings already possess antibacterial properties by themselves, but additional antimicrobial compounds to increase the biocidal effect, such as QACs, are usually added. Its biocompatibility and modification ease make it an enticing component to antimicrobial coatings development (Swartjes et al., 2015).

Lastly, quaternary ammonium compounds are widely used in disinfectants, being the lysis of the bacterial cell its primary mechanism of action. Its high stability allows the coating to remain intact due to its bacteria disintegrating mechanism, which does not allow disturbance of the coating proprieties (Swartjes et al., 2015). From the view of disinfection, they are bactericidal for a wide range of pathogens such as *E. coli*, *S. aureus*, *Streptococcus mutans*, *Bacillus subtilis*, *P. aeruginosa* and the fungal pathogen *Candida albicans* (Villapún et al., 2016). QACs were initially used in common liquid disinfectant solutions, but they are currently studied for use in antimicrobial-coated surfaces. This is due to the fact that these compounds have been proven to be very stable, mostly unaffected by pH, easily dissolved in water, do not evaporate, and solutions containing QACs, when dry, leave a solid residue that allows having prolonged activity on contact surfaces for a long time (Swartjes et al., 2015; Chauret, 2014; Lee et al., 2020). This ability to maintain antimicrobial proprieties effective on contact surfaces for long periods derives from the highly stable contact-killing mechanism that does not disrupt the chemical bounds of the compound due to rapid disintegration of the bacteria, allowing the surface treated with the antimicrobial coating to remain active (Swartjes et al., 2015).

Disinfectants based on QACs are considered relatively safe and not harmful to humans if concentrations are kept within the values established by the Environmental Protection Agency (EPA).

Overall, each different surface coating technology can be applied for different real-life scenarios. Its employment depends on the intended use of the surface to treat and on the expectation of the duration of the antimicrobial agent being short or long term (Swartjes et al., 2015).

1.4. Antimicrobial surfaces technology and antimicrobial stewardship

A total of 4.5 million healthcare-associated infections have been estimated to occur each year in European hospitals, as reported by the European Centre for Disease Prevention and Control (ECDC, 2018), with more than half of these infectious diseases being preventable. Alone, these numbers forward the public health risks, as well as the economic burden, that arise from HCAIs, being its danger exacerbated by the presence of multi-drug resistant organisms; ECDC reported that one-third of HCAIs causing bacteria is resistant to antibiotics as well as other antimicrobials (ECDC, 2018).

In 2016, COST Action "AMiCI - Anti-Microbial Coating Innovations to prevent infectious diseases" was established to evaluate the impact of AMCs in the healthcare bubble due to the ever-rising need to deter the prevalence of MDR. Acknowledgement of the benefits that AMCs may present in prevention and control of infectious diseases has increased over the years, being helpful when in combination with already employed disinfection and cleaning of surfaces as well as hand hygiene to hinder the spread of infectious diseases through high-touch surfaces (Dunne et al., 2020). However, and despite the promising technology, AMC usage has not been established in healthcare settings, being absent from 2019/2020 WHO guidelines to prevention and control of HCAIs (WHO, 2019). This derives from the lack of a credibility threshold derived from: lack of significant, systematic and international research on the benefits or hindrance of AMC in healthcare settings; lack of know-how regarding the use and commercial availability; lack of information regarding the possible side effects of this technology, such as MDR development and environmental impact and lack of standardized testing procedures that emulate real life conditions of healthcare settings (AMICI Action Network).

Standardized antimicrobial efficacy testing methods such as ISO 22196 (2011) specifies an *in vitro* method of evaluating the antibacterial activity of antibacterial-treated plastics, and other non-porous surfaces, being the most used testing protocol in the industry (International Standards Organization, 2021; Campos et al., 2016). This method is used to quantitatively assess the biocidal or bacteriostatic effects through direct contact of the liquid bacterial culture with control and test surfaces, covered with plastic film and incubated for 24h at high relative humidity (no less than 90%). Recovery of bacteria from test specimens is performed immediately after inoculation (T0) for

control surfaces and after 24h incubation (T24) for control and test surfaces (International Standards Organization 2021). This standard has been proven reliable for testing the biocidal activity of active materials and surface coatings. However, this standard has also been proven to not reflect real-life scenarios due to its artificial experimental conditions, such as high incubation temperature, high incubation relative humidity, and the surface´s direct contact with high concentrations of bacterial culture, making it difficult to extrapolate results to real-life clinical or industrial settings (Wiegand et al., 2018; Ojeil et al., 2013).

The European Standard EN 1276:2019 specifies a suspension test for assessing the antimicrobial activity of chemical disinfectants used in food, industrial, domestic and institutional areas, excluding areas and situations where disinfection is medically indicated and excluding products used on living tissues except for hand hygiene in the food, industrial, domestic and institutional areas (European Standards 2009). In this method, a diluted product sample is added to the bacterial test suspension with the addition of an interfering substance mimicking dirty conditions, bovine albumin. Interfering substances are used to assure that all parameters found in real-life settings are accounted for, such as soiling with organic matter that can affect the antimicrobial proprieties of the test product (Araújo et al., 2013; Lambert et al., 2001). At the end of the established contact time between the sample and the test organism and interfering substance, the aliquot is neutralized, and the bactericidal and/or bacteriostatic activity of the product is assessed (European Standards, 2009). Disadvantages of this standard have been set on possible difficulty of repeatability and reproducibility of the test and on the fact that time allowance for contact times is short, which will require training to ensure proper time management. Advantages lie in the low difficulty of the procedure as well as well-defined thresholds and reliable results reports (Taylor et al., 1999).

One of the pitfalls of AMCs is the lack of peer-approved guidelines that will validate the product and make it marketable and profitable. To mend this, it is necessary to implement standard tests that will echo safe end-use, real-life *in situ* conditions, with regulatory guidance being available to researchers and commercial stakeholders that are interested in providing AMCs products in order to benefit public health (Dunne et al., 2020; Ojeil et al., 2013).

Even though that studies on the field are still scarce, a study by Piestch et al. (2020) has shown that metal-based AMCs can account for an uncontrollable selective environment that may exacerbate the spread of MDR in healthcare settings instead of containing it via co-resistance and cross-resistance methods. Regarding non-metal-based coatings, the risk does not only lie on resistance that may develop to the specific antimicrobial but on the occurrence of cross-resistance and/or co-resistance (Piestch et al., 2020; Cassidy et al., 2020).

Development and evolution of standardized tests are necessary to validate results, being that many studies to date show the efficacy of AMCs under high-moisture content or in solution instead of under environmentally relevant conditions. This fact is crucial in understanding why AMCs are not employed to date in healthcare and community settings, lack of studies with real-life conditions that validate the effectiveness and benefits of the technology that may overshadow the risk of MDR spread (Piestch et al., 2020).

Another concern that arises from the use of AMCs is the consequences of low microbe exposure on our microbiomes. These microbe communities are well organized and respectively adjusted to specific environments. When at equilibrium, conditioned by factors such as pH, nutrient availability, and host conditions, this microbiome is responsible for maintaining health. If a disruption to that equilibrium occurs, and if the conditions for pathogen manifestation are favoured, infection and inflammation may occur, putting our immune system at risk. With radical changes in hygiene, such as seen with the Covid-19 pandemic, the decreased exposure to a diverse set of microbes combined with indiscriminate use of antibiotics and antimicrobials, can result in the impairment of our microbiomes (Bloomfield et al., 2016; Cassidy et al., 2020).

Antimicrobial stewardship is one of the most pressing human public health matters nowadays that embodies the ethical planning, employment, and management of antimicrobial use.

1.5. Cytotoxicity of antimicrobial surface coatings

As with all disinfectants, antimicrobial coatings may be prone to some residual toxicity when in direct contact with the skin or any other mucous membrane (Hoh et al., 1993).

Cytotoxicity assays are essential to measure the cellular or metabolic changes associated with viable or nonviable cells caused by toxic effects of chemicals and its different mechanisms of toxicity such as cell membrane disintegration, inhibition of protein synthesis, amongst others. Different types of tests must be employed according to different parameters, being specificity and sensitivity the major factors. The nature of the sample and its intended use will determine which test is most appropriate to be carried out to obtain accurate and reliable results. A broad range of tests is used, such as dye exclusion assays, colorimetric assays, fluorometric assays, and luminometric assays (Woolfson et al., 2009; Aslantürk, 2017).

Cytotoxicity tests should primarily be reliable and easily reproducible as well as cost-effective (Aslantürk, 2017). For AMCs development and employment, cytotoxicity assays are essential and important to verify the safety of antimicrobial compounds for use and for public health maintenance (Dominguez et al., 2019).

Cytotoxicity colorimetric assays such as the [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] metabolic assay, also known as MTT assay, is widely used to measure cellular metabolic activity as an indicator of cell viability or cell damage, being referred to as the "gold standard" of cytotoxicity assays (Aslantürk, 2017). This assay is based on the reduction of a yellow tetrazolium salt into purple formazan crystals by metabolically active cells. The coloured solution that results from the dissolution of the formazan crystals with dimethyl sulfoxide (DMSO), is then quantified by measuring the absorbance between 500-600 nm, using a microplate spectrophotometer (Mosmann, 1983; van Meerloo et al., 2011). The darker the final solution, the greater the number of metabolically active cells (Mosmann, 1983).

Immortalized human epidermal keratinocyte line cells, HaCaT, are often used for *in vitro* assays as a paradigm for epidermal cells, essential to assess the toxicity of frequently used disinfectants, in the case of this study. Epidermal cells are mainly composed of keratinocytes, up to 95%, playing an essential part in the structure of the epidermis and as a barrier, being the first to be affected by toxic substances. Therefore,

other authors have reported that these should be considered the most fitting target cells for assessing the sensitivity of the skin to toxicants that may be present in disinfectants such as AMCs (Colombo et al., 2017; Hoh et al., 1993). Skin inflammatory and immunological responses are initiated in the keratinocytes and are important in the process of wound regeneration. Its use in cytotoxicity assays is crucial to help decipher skin irritation processes when coupled with immunology studies (Colombo et al., 2017).

Mouse fibroblast cells, L929, a cell line derived from the normal subcutaneous connective tissue of an adult C3H strain mouse, are widely used in *in vitro* assays due to their morphological characteristics (rapid growth) and ease of use and accessibility (Qiu et al., 2016). As well as keratinocytes, fibroblasts are essential in wound healing and are used as a model for assessing potential cytotoxicity (Wang et al., 2013; Cavallaro et al., 2015).

With AMCs being products that act on surfaces, it is important to verify if vestigial concentrations of the product that may be transferred to food products may have an adverse effect on human health if ingested. For that, immortalized human colorectal adenocarcinoma (Caco-2) cells are used as a model for the intestinal epithelial barrier (Lea, 2015).

Antimicrobial substances migration from food contact surfaces to food products is considered a failure in the antimicrobial coating design and also considered as food contamination. Easy detachment of the antimicrobial agent from the surface and into food products directly affects the durability and antimicrobial activity of the AMCs. Toxicology reports of the antimicrobial agents used in AMCs are crucial for the development and safe end-use of this technology, being important to set a threshold for the maximum residue levels (MRLs) of the antimicrobial coating that may be present in the treated surface after the disinfection process (Dominguez et al., 2019). The MRLs for quaternary ammonium compounds have been set at 0.1 mg/kg (European Standing Committee in 2014).

1.6. Aim of this study

The main objective of this work was to determine the ability of a commercial surface coating compound to inhibit important health-associated pathogens. For this, the pathogens *Escherichia coli* ATCC 25922, *Listeria monocytogenes* Scott A and *Acinetobacter baumannii* ESB260 were firstly characterized regarding their resistance profiles to different pH's, temperatures and antibiotics, and it was evaluated their inhibition when in contact with the commercial coating on different commonly used surfaces. In addition, the durability and kinetics of the coating on each surface were also tested, as well as its cytotoxic activity on relevant target cells.

2. Materials and methods

In this section, all the methods carried in this work will be described.

2.1. Preliminary antimicrobial coating efficacy

2.1.1. Coating compound and microorganisms used in this study

Antimicrobial coating compound (solution) was provided by CTEQ Indústria Química, S.A. (Santo Tirso, Portugal).

Several microorganisms (listed in Table 2.1.1.1) were grown twice at 37 ℃ (under aerobic or anaerobic conditions) during 18 hours in TSAYE (Tryptic Soy Agar (TSA)) (Biokar Diagnostics, Beauvais, France) supplemented with 6g/L Yeast Extract (YE) (Biokar Diagnostics)).

Table 2.1.1.1 Microorganisms used in this study

Microorganisms	Strain	Source
Gram-positive	*Staphylococcus aureus 18N (Methicillin-resistant Staphylococcus aureus—MRSA)*[1]	*Isolates from Culture Collection of Escola Superior de Biotecnologia (Porto, Portugal)*
	Staphylococcus aureus 2037M1 (MSSA)[1]	
	Bacillus cereus ESB14[1]	
	Bacillus subtilis ESB15[1]	
	Clostridium sporogenes 1.31[1]	
	Clostridium sporogenes 1.34	
	Clostridium sporogenes 1.61[1]	
	Clostridium perfringens 1.16[1]	
	Clostridium perfringens 1.19[1]	
	Clostridium perfringens 1.22[1]	
	Enterococcus faecalis ATCC 29212[1]	*American Type Culture Collection (ATCC; Manassas, VA, USA)*
	Staphylococcus aureus ATCC 29213[1]	
	Staphylococcus aureus ATCC 6538	
	Enterococcus faecalis DSMZ 12956[1]	*Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany)*
	Enterococcus faecium DSMZ 13590[1]	

	Enterococcus flavescens DSMZ 7370	
	Enterococcus gallinarum DSMZ 20628	
	Enterococcus casseliflavus DSMZ 20680	
	Listeria innocua 2030c	*Public Health Laboratory Service (PHLS; London, UK)*
Gram-negative	*Salmonella Braenderup ESB7*	*Isolates from Culture Collection of Escola Superior de Biotecnologia (Porto, Portugal)*
	Salmonella Enteritidis ESB8	
	Salmonella Typhimurium ESB9	
	Klebsiella pneumoniae ESB11	
	Pseudomonas aeruginosa ESB13	
	Yersinia enterocolitica ESB24	
	Acinetobacter baumannii R ESB28	
	Acinetobacter baumannii S—1 ESB29	
	Acinetobacter baumannii S—2 ESB32	
	Acinetobacter calcoaceticus R ESB30	
	Acinetobacter calcoaceticus S ESB31	
	Yersinia enterocolitica NCTC 10406	*National Collection of Type Cultures—Culture Collection of Public Health England (NCTC; Salisbury, UK)*
	Escherichia coli ATCC 29215	*American Type Culture Collection (ATCC; Manassas, VA, USA)*
	Pseudomonas aeruginosa ATCC 15442	

To perform the bacterial suspensions of each inoculum, colonies were transferred via a sterile loop to a tube containing Ringer (Biokar Diagnostics) solution. Cell density was adjusted to 0.5 McFarland scale to obtain a final inoculum concentration of approximately 10^7 to 10^8 Colony Forming Units (CFU)/mL, depending on the genera. Experiments were made in duplicate.

2.1.2. Antimicrobial activity spectrum of the coating compound studied

The coating compound was diluted in sterile deionized water and tested at three concentrations 100%, 50% and 25% (v/v).

Bacterial suspensions standardized to 0.5 McFarland scale were separately spread on Müeller-Hinton agar (Biokar Diagnostics) using sterile swabs. For each plate, 20 µL of each compound at different concentrations was dropped on the top. Plates were incubated at 37 ℃ overnight. After incubation, the diameter of each inhibition halo was measured.

2.2. Temperature and pH susceptibility profiles

2.2.1. Bacterial suspension

One single colony of each isolate was transferred to TSBYE (Tryptic Soy Broth (TSB; Biokar Diagnostics) supplemented with 6g/L YE) and incubated at 37 ℃ for 18 h. This culture was subsequently diluted at 1:100 in TSBYE and incubated in the same conditions. Cells were washed by centrifuging the inoculum at 11000 rpm for 5 minutes (Eppendorf Mini spin, Hamburg, Germany). The supernatant was discarded, followed by resuspension in Nutrient Broth (NB, Biokar Diagnostics), and centrifuged again at the same conditions. Again, the supernatant was discarded, and the washed cells were resuspended in NB at the initial volume. An optimal bacterial concentration of 10^9 CFU/mL for the bacterial suspension was achieved.

2.2.2. Temperature susceptibility profile

Nutrient Broth at pH 7.0 was inoculated with each bacterial suspension and incubated at 37 ℃ (control), 60 ℃, and 65 ℃ for 10 seconds, 5, 10, 15 and 20 minutes. After exposure, each sample was inoculated on TSAYE using an easySpiral Automatic plater (Interscience, Saint Nom la Bretèche, France) and the plates were incubated at 37 ℃ for 24h. Two independent replicas were performed.

Plates containing 30 to 300 colonies were counted, and the number of viable bacteria in CFU/mL was determined. Bacterial counts were transformed to logarithmic reductions following the equation: log N/N0, being N0 the initial bacterial count and N the bacterial count at a given time.

2.2.3. pH susceptibility profile

Nutrient Broth adjusted with 1M hydrochloric acid (HCl) at pH values of 7.0 (control), 4.0 and 2.0, was inoculated and incubated at 37 ℃ for 10 seconds, 20 and 60 minutes. Two independent replicas were performed.

Samples were plated, incubated and colonies counted as described above. Bacterial counts were transformed to logarithmic reductions as described previously.

2.3. Antibiotic susceptibility

Antibiotic resistance of *E. coli* ATCC 25922, *L. monocytogenes* Scott A and *Acinetobacter baumannii* ESB260 was assessed through broth dilution method according to Clinical and Laboratory Standards Institute (CLSI, 2017). Each bacterial suspension was prepared on TSBYE as described previously. Then, dilution of the washed cells was performed on Ringer's solution to achieve the goal bacterial concentration of 10^8 CFU/mL.

2.3.1. Microdilution broth method

Cation adjusted Müeller-Hinton broth (CAMHB; Sigma Aldrich, Missouri, USA) was supplemented with 2.5% (v/v) lysed horse blood (LHB, Thermo Fisher Scientific, Massachusetts, United States). Stock solutions of each antimicrobial agent were made, and several dilutions were performed (Tables 2.3.1; 2.3.2 and 2.3.3) to the minimum inhibitory concentration (MIC) Breakpoints (μg/mL) established by CLSI (2017).

Table 2.3.1.1. Antibiotics and concentration ranges tested for *Acinetobacter* 260.

Antibiotic	Concentration range (μg/mL)
Ampicillin	32 - 8192
Ceftazidime	8 - 2048
Gentamycin	4 - 1024
Tetracycline	4 - 1024
Trimethoprim - Sulfamethoxazole	0.25/4.75 - 256/4864
Ciprofloxacin	0.125 - 256

Table 2.3.1.2 Antibiotics and concentration ranges tested for *E. coli*.

Antibiotic	Concentration range (μg/mL)
Ampicillin	0.25 - 512
Ceftazidime	0.125 - 256
Gentamycin	0.125 - 256
Tetracycline	0.125 - 256
Nalidixic acid	0.25 - 512
Ciprofloxacin	0.25 - 512

Table 2.3.1.3. Antibiotics and concentrations tested for *L. monocytogenes*.

Antibiotic	Concentration range (μg/mL)
Penicillin	0.25 - 128
Ampicillin	0.25 - 128
Trimethoprim - Sulfamethoxazole	0.25/4.75 - 256/4864

Equal volumes of bacterial suspension and the diluted antimicrobial solution were added to each well in a 96-well microplate. Microplates were incubated at 37 ℃ for 18h. Positive control of bacteria grown without antimicrobial agents, and negative control with just CAMHB (Sigma) with 2.5% (v/v) LHB, were performed. For all antimicrobials, *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 were used as quality control bacteria for MIC as recommended by CLSI (2017).

Plates were checked for bacterial growth, identifiable by turbidity of the well. Validity of the controls and quality control was also assessed. Two independent replicas were performed.

2.4. ISO 22196:2011 Measurement of antibacterial activity on plastics and other non-porous surfaces

2.4.1. Surfaces used in this study

Slides of glass, polyvinyl chloride (PVC), and stainless steel measuring approximately 25mm x 75mm were tested. Each surface was previously sterilized using 70% (v/v) bleach, washed three times with deionised sterile water to remove any remaining bleach and impurities, and dried at 60 ℃ overnight.

For each surface, testing was performed on nine specimens: three untreated to measure viable cells immediately after surface inoculation, three untreated to measure viable cells after 24h of incubation of the inoculated surface, and three treated specimens with the compound to measure the viable cells present on the surface after 24h incubation.

2.4.2. Preparation of bacterial suspensions/ test inoculum

Escherichia coli ATCC 25922, *Listeria monocytogenes* Scott A and *Acinetobacter baumannii* ESB260 were cultured on TSAYE, as described previously. After incubation, cells were centrifuged at 11000 rpm for 5 minutes, washed, and resuspended at the initial volume in 1:500 NB. Serial dilutions were performed to achieve the required bacterial concentration between $2.5x10^5$ – $1.0x10^6$ cells/mL.

2.4.3. Measurement of antimicrobial activity

Sterile surfaces were placed in Petri dishes and inoculated with 200μL of the test inoculum. Sterile pieces of Stomacher bag (Interscience, Saint Nom, France) measuring 15mm x 65mm were used to cover the test inoculum and gently pressed down to the edges of the film, without any leaking to the edge of the surface. Immediately after inoculation, the recovery of bacteria from three of the untreated specimens was determined by washing the inoculated surface with 10mL of Dey-Engley (D/E) neutralizer (BD Difco, New Jersey, USA), which was used as the initial suspension ten-fold diluted. Other two-fold serial dilutions were performed in 1:500 NB and 100 μL of

each dilution was spread on Plate Count Agar (PCA, Biokar Diagnostics) and incubated for 48h at 37 ℃.

Half of the inoculated untreated specimens and the inoculated treated specimens were incubated at 22 ℃, for 24h, and Petri dishes were covered with damp sterile gauze to achieve a relative humidity of no less than 90% to prevent the drying of the inoculum. Recovery of the viable bacteria was performed as mentioned above.

After incubation, plates containing 30 to 300 colonies were counted and the number of viable bacteria in cells per cm^2 was determined according to the standard parameters for test validation. Three independent replicas were performed.

2.4.4. Antimicrobial coating kinetics

Antimicrobial coating kinetics were assessed through ISO 22196 (2011). Each bacterial inoculum was exposed to the antimicrobial coating, and different times of exposure were used to assess the compound kinetics: 1, 10 and 20 minutes. Three independent replicas were performed.

2.4.5. Antimicrobial coating durability

The surfaces treated with the antimicrobial coating were lightly cleaned using four different methods, including i) swiped with bleach, ii) scrubbed with water, iii) swiped with a commercial surface disinfectant, and iv) swiped with a commercial degreaser commonly used to clean surfaces. All samples were swiped with a wet cloth after each treatment and were allowed to dry in a sterile Petri dish. The durability of the compound was assessed by contaminating the surface with inoculum and performing the standard ISO protocol one week after the original application of the compound and after the cleaning process. Three independent replicas were performed, and surfaces treated with the coating and left untouched for one week were used as control.

2.5. EN 1276:2019 Evaluation of the bactericidal activity of chemical disinfectants

2.5.1. Bacterial suspension

Bacterial suspensions of *E. coli, L. monocytogenes* and *Acinetobacter 260* were prepared on TSBYE as described previously. Cells were washed and resuspended at the initial volume in Tryptone Sodium Chloride solution (TSC, Biokar Diagnostics). Serial dilutions were performed to achieve the optimal bacterial concentration between $1.5x10^8 – 5.0x10^8$ CFU/mL for the test suspension, and $3.0x10^2 – 1.6x10^3$ CFU/mL for the validation suspension.

2.5.2. Test method

The determination of bactericidal concentrations was carried out using the dilution-neutralization method. The contact time tested was 5 min $\pm$ 10s at 20 ℃, with the chosen interfering substance being bovine albumin (Merck, Darmstadt, Germany) mimicking dirty surface conditions. Ten concentrations of the sample product were tested: 25%, 12.5%, 6.25%, 3.25%, 1.56%, 0.78%, 0.39%, 0.19%, 0.09% and 0.048% (v/v). For each concentration, the sample product was added to a mix of test suspension and interfering substance and placed in a water-bath at 20 ℃ for the chosen contact time. The mixture was then neutralized with the Dey-Engley neutralizer and again placed in a temperature-controlled water bath for the chosen contact time. PCA plates were inoculated with the final test mixture and incubated at 37 ℃ for 24h.

Controls for the experimental conditions, neutralizer and method validation were performed according to the experimental protocol.

Plates containing 30 to 300 colonies were counted and CFU/mL were determined according to the standard parameters for test validation. Three independent replicas were performed.

2.6. Cytotoxicity – MTT assay

2.6.1. Human keratinocytes and mouse fibroblasts exposure to test antimicrobial coating

Cytotoxicity of the compound was assessed using the MTT assay standard protocol. Cultured primary human keratinocytes, HaCat cells (Cell Line Services, 300493, Oppenheim, Denmark), and mouse fibroblast cells (NCTC, ECACC 85103155, Public Health, England) were seeded in a 96-well microplate with a cell concentration of $1.0x10^5$ cells/mL in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Verviers, Belgium) with a glucose content of 4.5g/L (HaCat cells) and 1g/L (fibroblast cells), supplemented with 10% (v/v) fetal bovine sera (FBS) and 1% (v/v) Penicillin-Streptomycin-Fungizone solution (A/A) and incubated for 24h at 37 ℃ with an atmosphere containing 5% CO_2. Sample solutions comprising different proportions of DMEM, FBS, and A/A, and the test compound, at eight concentrations - 5, 10, 15, 20, 25, 30, 35 and 40% (V/V) - were added to each well and incubated at the same conditions. Three independent replicas were performed. A positive control, cells in the growth medium, negative control, cells with 30% (v/v) DMSO, and background control, medium only, were included. After incubation, the medium was discarded, and MTT solution, composed by DMEM and MTT, was added to each well, and the plate was incubated for 2h in the dark. MTT solution was discarded, DMSO was added, and the plate was shaken for 15 minutes in the dark and at room temperature. Two independent replicas were carried out.

2.6.2. Human keratinocytes and mouse fibroblasts exposure to commercial cleaning products

Exposure to four different commercially available disinfectants and one commercial degreaser was performed. Seeding for HaCat cells and fibroblast cells was performed as prior described. Cells were exposed to two concentrations of ethanol, 70% and 95% (v/v), two concentrations of bleach, 25% and 75% (v/v), and one concentration of two commercial disinfectants and one commercial degreaser, 95% (v/v). The microplates were incubated at the same conditions for 24h and MTT solution addition

was followed as described previously. Positive, negative, and background controls were performed. Two independent replicas were made.

2.6.3. Human colorectal adenocarcinoma cells exposure to vestigial concentrations of test antimicrobial coating

Cultured immortalized human colorectal adenocarcinoma (Caco-2) cells (ATCC ECACC 86010202, Manassas, Virginia), at late post-confluence, were seeded in a 96-well microplate with a cell concentration of $9.5x10^4$ cells/mL in DMEM with a glucose content of 4.5g/L, supplemented with 10% (v/v) FBS, 1% (v/v) Penicillin-Streptomycin-Fungizone solution (A/A), 1% (v/v) non-essential amino-acids (Gibco, Grand Island, USA) and 1% (v/v) pyruvate (Gibco), and incubated for 24h at 37 ℃ with an atmosphere containing 5% CO_2. Cells were exposed to vestigial concentrations of the antimicrobial coating, 5% to 0.0003% (v/v), and incubated at the same conditions. Positive, negative, and background controls were performed. After incubation, the medium was discarded and MTT solution, composed of DMEM with 4.5g of glucose and MTT, was added to each well, and the plate was incubated for 2h in the dark. MTT solution was discarded and DMSO was added. Plate was shaken for 15 minutes in the dark and at room temperature. Two independent replicas were performed.

The absorbance of all the wells was measured at 570 nm using a microplate spectrophotometer, and the results were analysed using the following equation:

$$\%\ Metabolic\ Inhibition = \frac{Abs\ Positive\ Control - Abs\ Sample}{Abs\ Positive\ Control} \times 100$$

2.7. Statistical analysis

The average and standard deviation data were calculated using Microsoft OfficeTM 365 Excel 2016. Any statistically significant differences between means were assessed by ANOVA test followed by a post-hoc test (Tukey HSD) calculated in IBM® SPSSTM - IBM Statistic Analytics 28.0. The mean difference was considered significant at the 0.05 level.

3. Results and discussion

3.1. Preliminary antimicrobial coating efficacy

In order to determine if the antimicrobial surface coating was effective against common foodborne and nosocomial pathogens (listed in Table 2.1), their susceptibility to the disinfectant was assessed by direct contact of the bacteria to three different concentrations of the compound: 100%, 50% and 25% (v/v). All microorganisms tested were susceptible to the compound. For most of the tested microorganisms, well-defined inhibition growth zones were observed.

In healthcare and industrial settings, disinfectants are widely used to reduce the spread of infections. Unfortunately, numerous authors have reported that a rise in pathogen resistance to disinfectants is being observed at an alarming rate (Wisplinghoff et al., 2007; Buffet-Bataillon et al., 2012; Bragg et al., 2014; He et al., 2017).

The ever-rising popularity of quaternary ammonium compounds is that it allows for good antimicrobial activity at low concentrations and at short contact times for any hard surface (Norhan et al., 2014). Resistance to QACs-based disinfectants and the mechanism by which it occurs are still a poorly studied field. Gram-negative bacteria are less susceptible to QACs than Gram-positive, being *Pseudomonas spp*. a good example due to high intrinsic resistance compared to other Gram-negative bacteria (Langsrud et al., 2003). Some strains of *Pseudomonas aeruginosa* have been widely reported as being resistant to QACs (Hoff et al., 1986; Langsrud et al., 2003; Tonoyan et al., 2019). Resistance to *Staphylococcus* species is commonly associated with the presence of multi-drug efflux pumps or, more specifically, QAC specific efflux pumps (Mahoney et al., 2021). Authors have hypothesized that *qua* genes are likely behind the resistance mechanisms for this Gram-positive bacterium (Langsrud et al., 2003). Emergence of *Listeria monocytogenes* tolerance or low level resistance to QACs has been shown in several reports (Gerba et al., 2015; Martínez-Suárez et al., 2016). *Escherichia coli*, *Enterococcus faecalis,* and some *Salmonella* serotypes have also been shown a reduced susceptibility to QACs compounds after repeated exposure (Braga et al., 2010; Soumet et al., 2012).

The results obtained in this study showed that the antimicrobial coating was effective even against the pathogens with possible lower susceptibility as described by literature. This finding was significant for the proven effectiveness of the product against significant pathogens as well as for the prospect of commercial use. With the promising results of the preliminary antimicrobial efficacy of the compound, three clinically relevant pathogens, *Escherichia coli* ATCC 25911, *Listeria monocytogenes* Scott A, and *Acinetobacter baumannii* 260, were chosen to carry on with further testing.

3.2. Temperature and pH susceptibility profiles

The three pathogens selected were characterized according to their temperature and pH susceptibility profiles as well as antibiotic susceptibility.

Acinetobacter baumannii ESB260

Clinical isolates of *Acinetobacter baumannii* have been widely studied, and several reports have determined that these bacteria have a temperature growth range of 25 to 45 ℃, being 37 ℃ its optimal growth temperature. Optimum pH values range from 4.5 to 8.0, but growth at pH 3.0 has also been observed (Dekic et al., 2018).

The growth at 60 and 65 ℃ was compared against the control at 37 ℃. The samples were taken every 5 minutes until 20 minutes and logarithmic reductions found are presented in Figure 3.2.1. Each test temperature showed a significant decrease in bacterial growth. After a 20-minute exposure, 4.0 and 5.0 log reduction were observed for 60 ℃ and 65 ℃, respectively. No significant differences were observed between treatments (ρ>0.05).

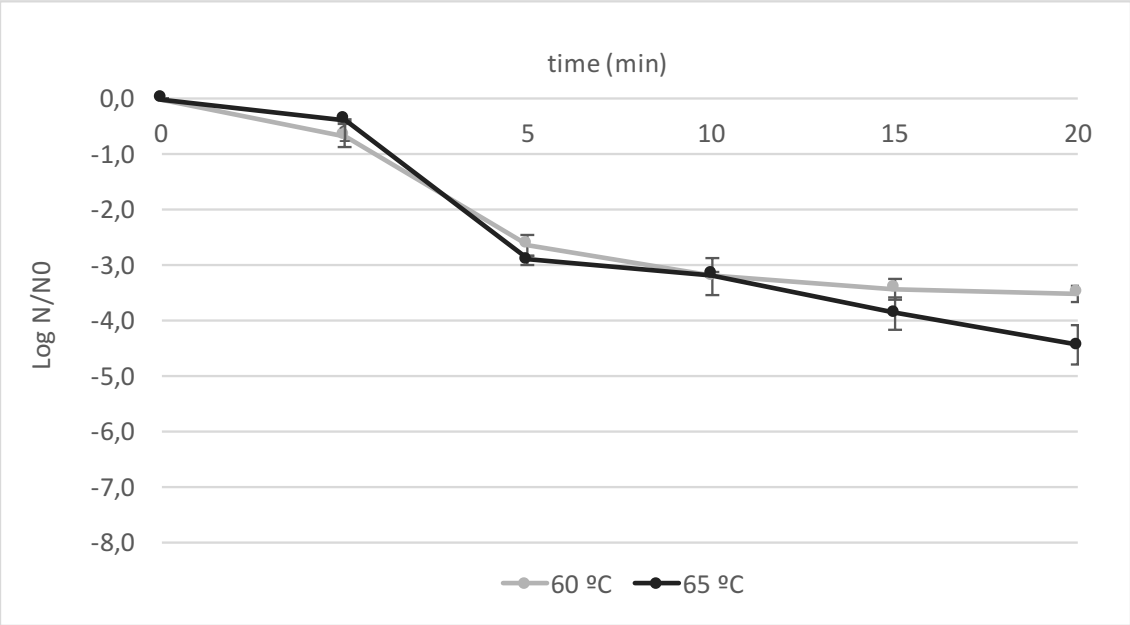

Figure 3.2.1. Temperature susceptibility profile for *Acinetobacter baumannii* ESB260.

Several previous studies support our results. In the study of Campos et al. (2018), the authors showed that several clinical isolates of *A. baumannii* were reduced to values below the detection limit of the enumeration technique at 60 ℃ and between 30 and 45 minutes of exposure (Campos et al., 2018). Dekic et al. (2018) also reported that at 63 ℃, *A. baumannii* isolates were able to survive for up to 2h maximum (Dekic et al., 2018). *Acinetobacter baumannii* is known to easily adapt to environmental stress and causing multidrug-resistant infections, thus the need to better study and understand how to prevent and control its spread (Dekic et al., 2018; De Silva et al., 2017).

Results regarding the survival of A*. baumannii* ESB260 at pH 2.0 and pH 4.0, compared to the control at pH 7.0, are shown in Figure 3.2.2.

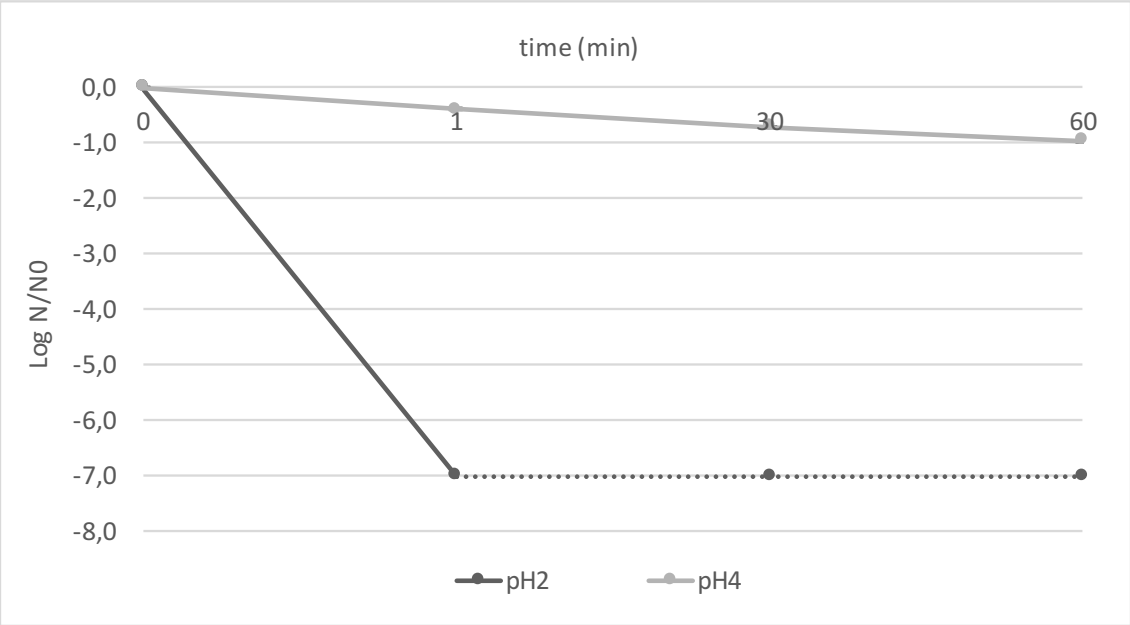

Figure 3.1.2. pH susceptibility profile for *Acinetobacter baumannii* ESB260. The dotted line means that the isolate was reduced to values below the detection limit of the enumeration technique.

No growth was observed at pH 2.0, meaning total inhibition of bacterial growth at very acidic nutrient-rich medium conditions at 37 ℃. At pH 4.0 (37 ℃), *A. baumannii* successfully survived with only a small decrease observed, <1.0 log cycle, as supported by previous studies with other *A. baumannii* strains (Zeidler et al., 2019). Significant differences (ρ<0.05) were observed between *A. baumannii* growth at pH 2.0 and pH 4.0. When exposed to acidic stress, *A. baumannii* has shown no survival for pH lower than 3.0. Campos et al. (2018) observed immediate inhibition of clinical *A. baumannii* strains when exposed to vinegar (pH level between 2.0 and 3.0) (Campos et al., 2018). This finding is important because of the knowledge that disinfection for *A. baumannii* is more effective under acidic conditions (Dekic et al., 2018).

Escherichia coli ATCC 25922

Escherichia coli growth temperature ranges between 30 to 40 ℃, being the optimum growth temperature at 37 ℃. For temperatures lower than 10 ℃ and above 42 ℃, *E. coli* growth rate is severely impaired. Authors have shown that for temperatures above 60 ℃, *E. coli* is able to survive less than 5 minutes, and at 70 ℃, no survival occurs for any length of time (Yang et al., 2020; Groh et al., 1996). In addition,

this bacterium favours neutral pH for growth, with acidic and alkaline pH environments proving its inhibition (Yang et al., 2020).

In Figure 3.2.3 are presented the logarithmic reductions observed at 60 and 65 ℃, compared to the control at 37 ℃.

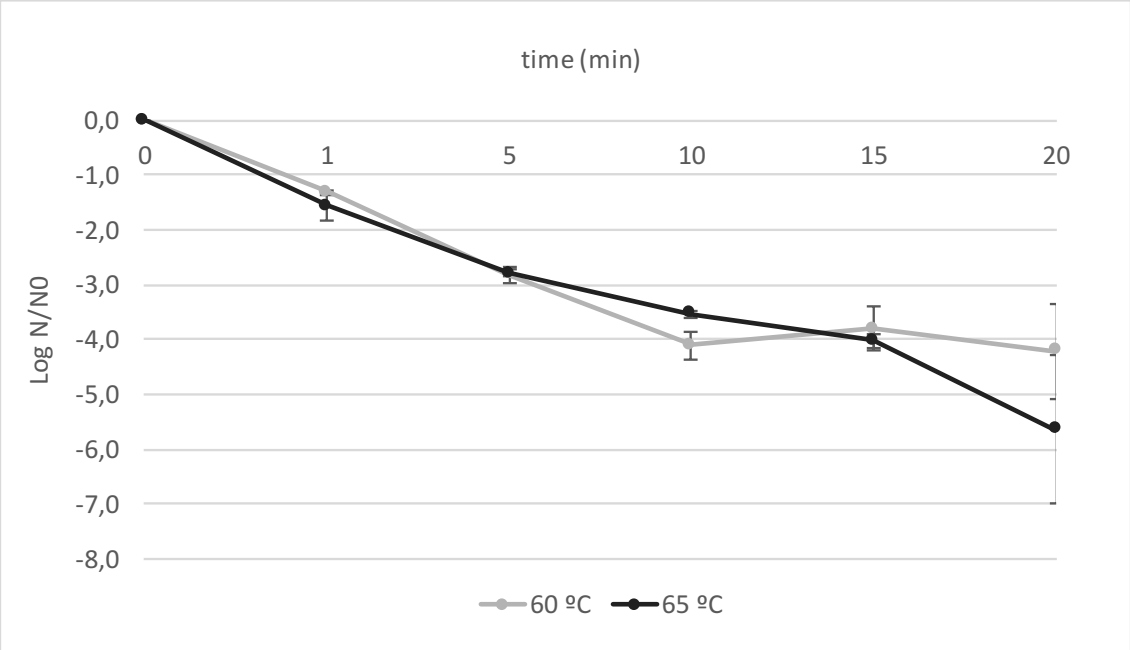

Figure 3.2.2. Temperature susceptibility profile of *Escherichia coli* ATCC 25922.

A significant decrease (ρ<0.05) was observed at 60 ℃ and 65 ℃ after a 20-minute exposure, with a reduction of >4.0 and >5.0 log cycles, respectively. A previous study on the heat resistance of the same *E. coli* strain for 57, 60, and 63 ℃ have shown similar results, with a sharp log decrease for temperatures above 60 ℃ in a short period (Li et al., 2017). No significant differences were observed between both temperatures tested (ρ>0.05).

The survival of *E. coli* at pH 2.0 and pH 4.0 is shown in Figure 3.2.4. Survival rate was 0% at pH 2.0, with a 7.0 log reduction. This result was expected since this bacterium favours neutral pH during growth. Acidic environments have been shown to fully inhibit *E. coli* growth (Yang et al., 2020). For pH 4.0, only a 0.5 log reduction was observed. Since the bacterium was cultured at a highly nutrient-rich medium, its growth at pH 4.0 was similar to growth at pH 7.0 (used as control), as reported by previous studies (Xu et

al.,2020). Significant differences (ρ< 0.05) were observed between growth at pH 2.0 and pH 4.0.

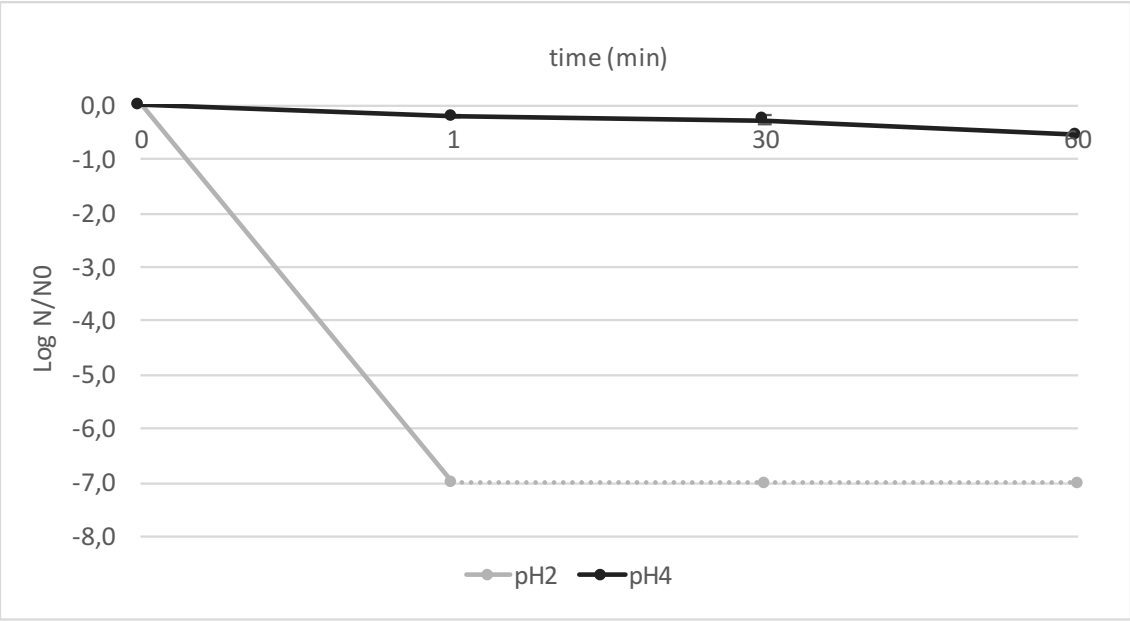

Figure 3.2.3. pH susceptibility profile of *Escherichia coli* ATCC 25922. The dotted line means that the isolate was reduced to values below the detection limit of the enumeration technique.

Listeria monocytogenes Scott A

Strains of *L. monocytogenes* can grow in a wide range of temperatures, from 1 to 45 ℃, with optimal growth temperatures between 30 and 37 ℃. Additionally, it can grow in adverse environmental conditions, such as low pH values between 4.3 and 5.2 (Ita et al., 1990; Cunha et al., 2015). The minimum pH value that allows for *L. monocytogenes* growth has been set at 4.39 by the ICMSF (International Comission on Microbiological Specifications for Foods., 2001).

In Figure 3.2.5, it is possible to observe that *L. monocytogenes* showed the slight variation between both test temperatures, with a <4.0 log reduction after a 20-minute exposure. No significant differences were observed between both temperatures tested (ρ>0.05). Temperatures over 50 ℃ have been proven to be lethal against *L. monocytogenes*, with survival rates decreasing sharply for 60 and 65 ℃ at short exposure times (Monu et al., 2015; Aryani et al., 2015).

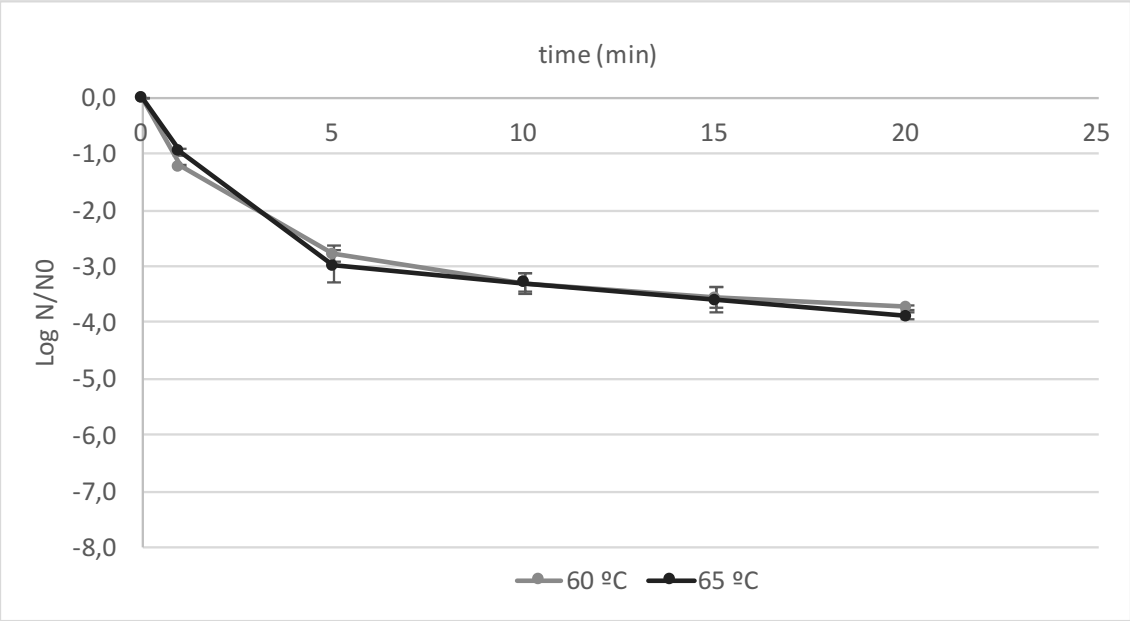

Figure 3.2.4. Temperature susceptibility profile of *Listeria monocytogenes* Scott A.

Following the same tendency as the other tested pathogens, no growth of *L. monocytogenes* was observed at pH 2.0 after a 1-minute exposure (Figure 3.2.6). Significant differences ($\rho < 0.05$) were observed between treatments. At pH 4.0, the growth was not significantly affected, with values similar to those found for the control at pH 7.0. The ability of *L. monocytogenes* to grow at low pH is well documented (Ita el al., 1990; Buchanan et al., 1992; Cunha et al., 2015).

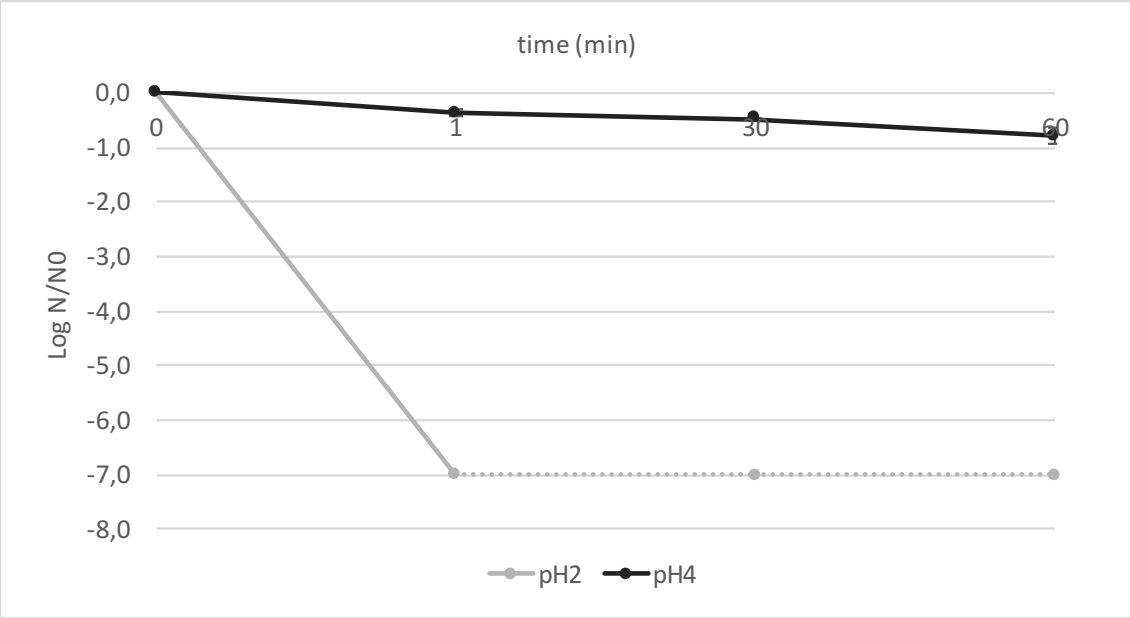

Figure 3.2.5. pH susceptibility profile of *Listeria monocytogenes* Scott A. The dotted line means that the isolate was reduced to values below the detection limit of the enumeration technique.

Listeria monocytogenes strains have been shown to be able to grow at pH values lower than 5.0, but only if the medium pH was gradually decreased (Ita et al., 1990). Cells of *L. monocytogenes* grown at lower pH values (pH 5.0) have been shown to better adapt to acid shock rather than cells grown in neutral pH (Zaika et al., 2003). In the present study, bacteria were directly inoculated in nutrient broth already adjusted to test pH values. Therefore, no survival at pH 2.0 may be due to sudden disruption in cell intracellular homeostasis that may have led to cell death (Miller et al., 2009; Cunha et al., 2015).

All pathogens had similar temperature and pH susceptibility profiles. Temperatures above 50 ℃ have been reported to be lethal towards all three tested pathogens as well as a sharp decrease in survival rate after short-term exposure to temperatures between 60 and 65 ℃ (Dekic et al., 2018; Li et al., 2017; Aryani et al., 2015). Bacterial growth at pH 4.0, similar to the control at pH 7.0, was also expected for all bacteria (Xu et al.,2020). In addition, total inhibition of growth at pH 2.0 was observed for all pathogens, possibly due to pH shock; cells were initially grown in a neutral pH medium and directly inoculated in pH adjusted solution (Zaika et al., 2003; Miller et al., 2009).

3.3. Antibiotic susceptibility

Inadequate prescription of antibiotics and subsequent therapeutic failure have led to the spread of MDR as well as major public health issues. The selection of antibiotics should be based on multiple parameters, especially microbiological, to assure that they are only prescribed in case of a bacterial infection and in such doses that will be efficient towards the end goal of infection treatment. Minimum inhibitory concentration (MIC) is the most used and the best available criterion for bacterial antibiotic susceptibility profiles(Kowalska-Krochmal et al., 2021). Although it does not always reflect *in vivo* studies, *in vitro* testing is an essential first step to assessing bacterial susceptibility to different antibiotics (Barbosa et al., 2009).

Table 3.3.1. Antibiotic susceptibility of *Acinetobacter baumannii* ESB260.

Antibiotic	MIC (µg/mL)	Interpretation
Ampicillin	1024	Resistant
Ceftazidime	128	Resistant
Tetracycline	256	Resistant
Ciprofloxacin	16	Resistant
Trimethoprim/ Sulfamethoxazole	8/152	Resistant

The results from this study are in agreement with the high frequency of multi-drug resistant isolates of *A. baumannii* that has been widely reported (Mak et al. 2009; Chen et al., 2017).

Acinetobacter baumannii is an opportunistic pathogen that, with the indiscriminate and overuse of antibiotics over the last couple of decades, has increased its resistance to a wide range of antibiotics such as fluoroquinolones, beta-lactams, and, most recently, carbapenems, furthering the spread and emergence of multidrug-resistant strains and nosocomial infection cases within clinical settings (Mak et al., 2009; McConnell et al., 2012). With therapeutic treatment options getting limited, it is crucial to keep monitoring this pathogen and to apply antimicrobial stewardship measures to prevent further MDR spread (Mak et al., 2009).

The MIC values obtained for the four antibiotics tested for *E. coli* ATCC 25922 are presented in Table 3.3.1.

Table 3.3.2. Antibiotic susceptibility of *Escherichia coli* ATCC 25922.

Antibiotic	MIC (µg/mL)	Interpretation
Ampicillin	4	Sensitive
Gentamycin	8	Intermediate
Tetracycline	4	Sensitive
Nalidixic acid	8	Sensitive

This strain was susceptible to ampicillin, tetracycline, and nalidixic acid, an interpretation supported by the MIC Breakpoints (μL/mL) for *E. coli* established by CLSI (2017). Previous studies with the same strain showed agreement with the present results for tetracycline and nalidixic acid (Brown et al., 2004; Pormohammad et al., 2019). However, a high prevalence of ampicillin, nalidixic acid, and tetracycline resistance in human isolates has been observed (Puvaca et al., 2018). *Escherichia coli* ATCC 25922 was classified as intermediate resistant to gentamycin with a MIC value of 8 μL/mL (Table 3.3.1). This intermediate resistance to gentamycin has been previously reported in *E. coli* isolates recovered from raw milk and serrano artisanal cheese (Parussolo et al., 2018).

If resistance is observed in *E. coli* isolates, a common denominator in the phenomenon of antibiotic resistance is mainly the overuse of antibiotics, in animals and humans due to the multiple pathways of transmission between both (Puvaca et al., 2021). Therefore, antimicrobial stewardship programs for both animal and human use are imperative to control the spread of the MDR *E. coli* strains as well as constant monitoring of antibiotic susceptibility profiles (Pormohammad et al., 2019).

Due to the high MIC values obtained, *A. baumannii* 260 was classified as resistant to all tested antibiotics (Table 3.3.2).

Table 3.3.2. Antibiotic susceptibility of *Acinetobacter baumannii* ESB260.

Antibiotic	MIC (μg/mL)	Interpretation
Ampicillin	1024	Resistant
Ceftazidime	128	Resistant
Tetracycline	256	Resistant
Ciprofloxacin	16	Resistant
Trimethoprim/ Sulfamethoxazole	8/152	Resistant

The results from this study are in agreement with the high frequency of multi-drug resistant isolates of *A. baumannii* that has been widely reported (Mak et al. 2009; Chen et al., 2017).

Acinetobacter baumannii is an opportunistic pathogen that, with the indiscriminate and overuse of antibiotics over the last couple of decades, has increased its resistance to a wide range of antibiotics such as fluoroquinolones, beta-lactams, and, most recently, carbapenems, furthering the spread and emergence of multidrug-resistant strains and nosocomial infection cases within clinical settings (Mak et al., 2009; McConnell et al., 2012). With therapeutic treatment options getting limited, it is crucial to keep monitoring this pathogen and to apply antimicrobial stewardship measures to prevent further MDR spread (Mak et al., 2009).

Listeria monocytogenes Scott A was susceptible to ampicillin, penicillin and trimethoprim/sulfamethoxazole, as shown in Table 3.3.3.

Table 3.3.3. Antibiotic susceptibility of *Listeria monocytogenes* Scott A.

Antibiotic	MIC (µg/mL)	Interpretation
Ampicillin	2	Sensitive
Penicillin	2	Sensitive
Trimethoprim / Sulfamethoxazole	0.5/9.5	Sensitive

The results from this study are in agreement with published literature for either food-derived isolates or clinical isolates (Barbosa et al., 2013; Olaimat et al., 2018). However, resistance to these antibiotics has also been observed by some scholars with an ever-rising trend (Barbosa et al., 2013; Faezi-Ghasemi et al., 2015; Olaimat et al., 2018). Acquired resistance to commonly used antibiotics for listeriosis poses a severe threat to the effective treatment of this disease and consequently to public health. Therefore, it is imperial to monitor the increase of resistance in the coming years as well as decode environmental stresses and mechanisms that may be underlying in the exacerbation of this issue (Olaimat et al., 2018).

3.4. ISO 22196:2011

3.4.1. Antimicrobial activity and kinetics

The effectiveness of an antimicrobial agent can be characterized by the antibacterial activity of the compound, which was measured through ISO 22196 (2011) with some modifications.

Results obtained for *A. baumannii* are presented in Figure 3.4.1.1 and Table 4.1.1.1.

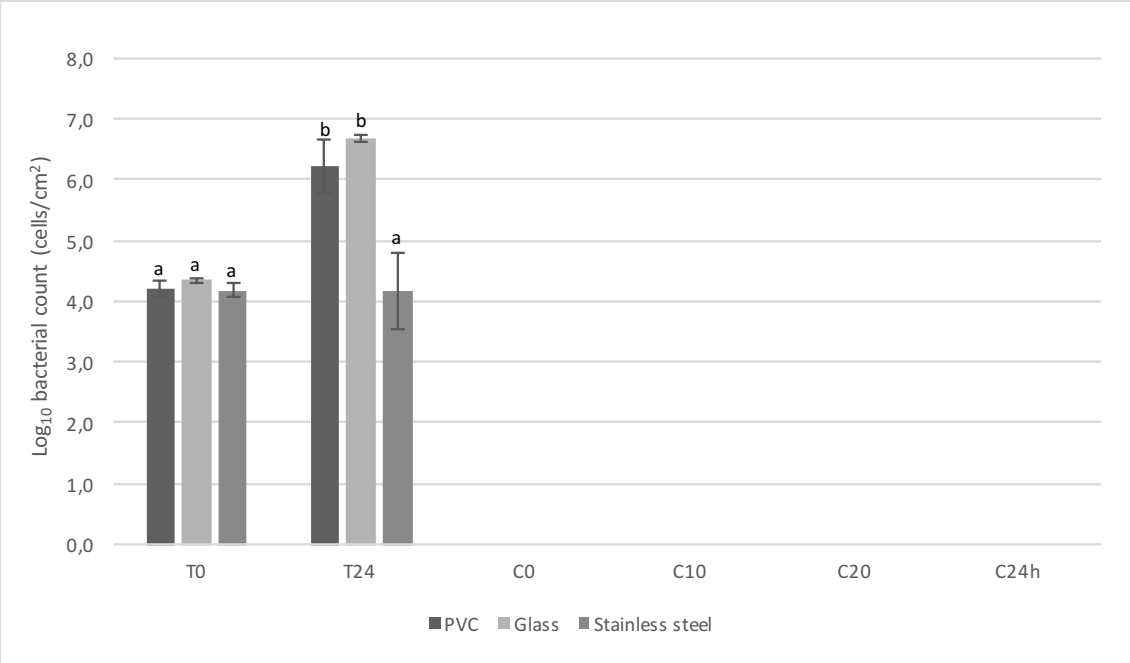

Figure 4.1.1.1. Contact killing time for *Acinetobacter baumannii* ESB260. Recovery of bacteria immediately after inoculation (T0); after 24h incubation (T24) on untreated surface; Recovery of bacteria immediately after inoculation (C0); after 10 minutes (C10), 20 minutes (C20) and 24h incubation (C24) on each treated surface. The results are means based on data from three replicates and standard deviations are indicated by error bars. Equivalent lowercase letters mean no significant differences between each condition (p>0.05).

Table 4.1.1.1 Antibacterial activity (R) for *Acinetobacter baumannii* ESB260.

	PVC	Glass	Stainless steel
C0	6.55 ± 0.18^{a}	6.34 ± 0.55^{a}	4.17 ± 0.63^{b}
C10	6.55 ± 0.18^{a}	6.34 ± 0.55^{a}	4.17 ± 0.63^{b}
C20	6.55 ± 0.18^{a}	6.34 ± 0.55^{a}	4.17 ± 0.63^{b}
C24h	6.55 ± 0.18^{a}	6.34 ± 0.55^{a}	4.17 ± 0.63^{b}

Recovery of bacteria immediately after inoculation (C0), and after 10 minutes (C10), 20 minutes (C20) and 24h (C24) incubation on each treated surface. The results are means based on data from three replicates and standard deviations are indicated by error bars. Equivalent lowercase letters mean no significant differences between each condition (p>0.05).

No significant differences (ρ>0.05) were observed for bacterial recovery immediately after inoculation (T0) for all three surfaces, unlike after 24h for untreated surfaces (T24) (ρ<0.05). All treatments were compared to T24 since it was a control for bacterial recovery after 24h incubation on untreated surfaces. For all three surfaces tested, *A. baumannii* was not able to survive within 1-minute of contact time; reductions of >6.0 log cycles were observed for glass and PVC surfaces, and >4.0 log cycles for stainless steel surface.

In Figure 3.4.1.2 and Table 3.4.1.2 are presented the results obtained for *Escherichia coli* ATCC 25922, respectively.

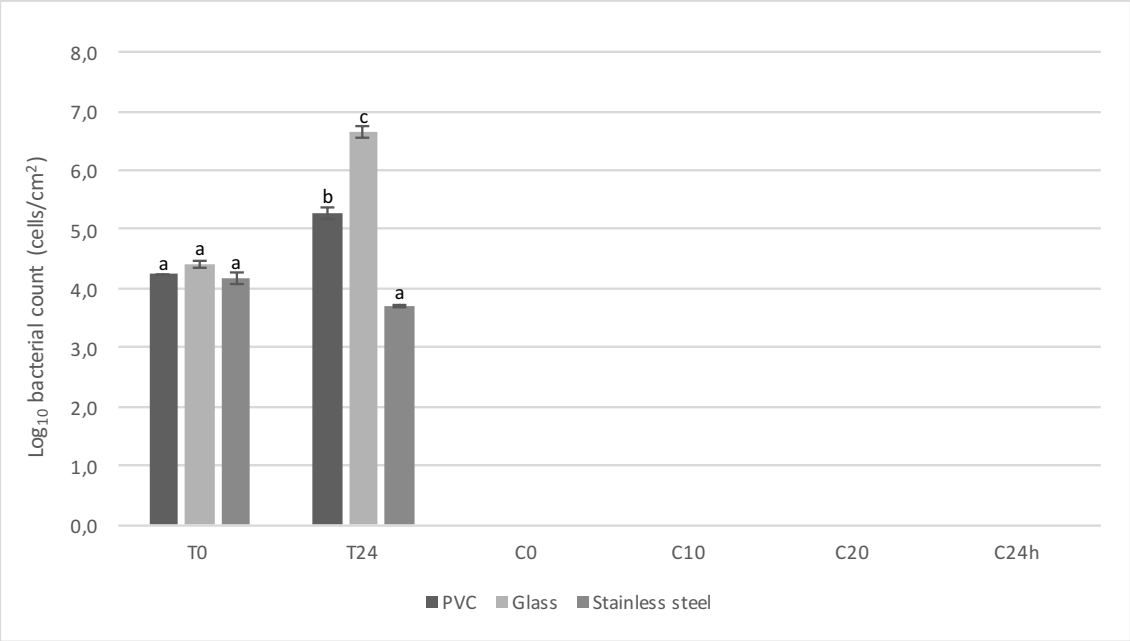

Figure 3.4.1.2. Contact killing time for *Escherichia coli* ATCC 25922. Recovery of bacteria immediately after inoculation (T0); after 24h incubation (T24) on untreated surface; Recovery of bacteria immediately after inoculation (C0); after 10 minutes (C10), 20 minutes (C20) and 24h incubation (C24) on each treated surface. The results are means based on data from three replicates and standard deviations are indicated by error bars. Equivalent lowercase letters mean no significant differences between each condition (p>0.05).

Table 3.4.1.2. Antibacterial activity (R) for *Escherichia coli* ATCC 25922.

	PVC	Glass	Stainless steel
C0	5.27 ± 0.10^{a}	6.64 ± 0.10^{b}	3.70 ± 0.02^{c}
C10	5.27 ± 0.10^{a}	6.64 ± 0.10^{b}	3.70 ± 0.02^{c}
C20	5.27 ± 0.10^{a}	6.64 ± 0.10^{b}	3.70 ± 0.02^{c}
C24h	5.27 ± 0.10^{a}	6.64 ± 0.10^{b}	3.70 ± 0.02^{c}

Recovery of bacteria immediately after inoculation (C0), and after 10 minutes (C10), 20 minutes (C20) and 24h (C24) incubation on each treated surface. The results are means based on data from three replicates and standard deviations are indicated by error bars. Equivalent lowercase letters mean no significant differences between each condition (p>0.05).

Only at T0 no significant differences between surfaces were observed (ρ>0.05). *Escherichia coli* was inhibited within 1-minute of contact time for all three surfaces tested, >6.0 log cycles reduction for glass surface, >5.0 log cycles reduction for PVC surface, and >3.0 log cycles reduction for stainless steel surface.

Results of *Listeria monocytogenes* recovery at T0 and T24 are presented in Figure 3.4.1.3 and Table 3.4.1.3, respectively.

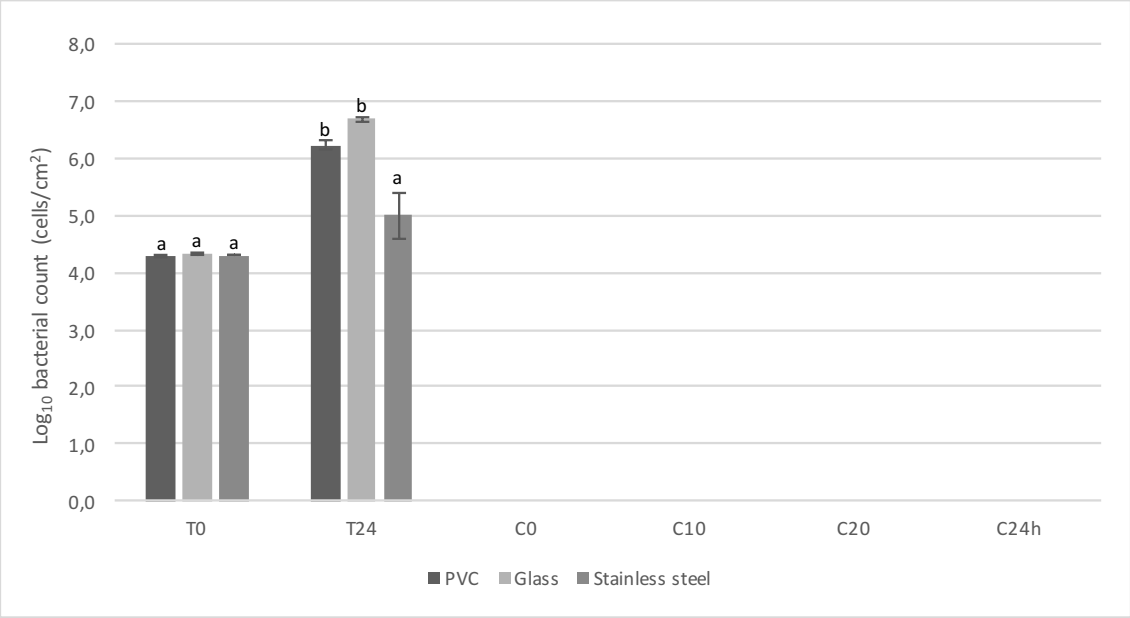

Figure 3.4.1.3. Contact killing time for *Listeria monocytogenes* Scott A. Recovery of bacteria immediately after inoculation (T0); after 24h incubation (T24) on untreated surface; Recovery of bacteria immediately after inoculation (C0); after 10 minutes (C10), 20 minutes (C20) and 24h incubation (C24) on each treated surface. The results are means based on data from three replicates and standard deviations are indicated by error bars. Equivalent lowercase letters mean no significant differences between each condition (p>0.05).

Table 3.4.1.3. Antibacterial activity (R) for *Listeria monocytogenes* Scott A.

	PVC	**Glass**	**Stainless steel**
C0	6.22 ± 0.09^{a}	6.67 ± 0.04^{a}	5.00 ± 0.40^{b}
C10	6.22 ± 0.09^{a}	6.67 ± 0.04^{a}	5.00 ± 0.40^{b}
C20	6.22 ± 0.09^{a}	6.67 ± 0.04^{a}	5.00 ± 0.40^{b}
C24h	6.22 ± 0.09^{a}	6.67 ± 0.04^{a}	5.00 ± 0.40^{b}

Recovery of bacteria immediately after inoculation (C0), and after 10 minutes (C10), 20 minutes (C20) and 24h (C24) incubation on each treated surface. The results are means based on data from three replicates and standard deviations are indicated by error bars. Equivalent lowercase letters mean no significant differences between each condition (p>0.05).

As observed for the other pathogens, no significant differences between surfaces were observed for T0 (ρ>0.05), unlike for T24 (ρ<0.05). *Listeria monocytogenes* did not survive within 1 minute of contact time for all three surfaces tested, with reductions of >6.0 log cycles for glass surface, >6.0 log cycles for PVC surface, and >5.0 log cycles for stainless steel surface.

After a 1-minute exposure to the treated surface, all pathogens were completely inhibited, being the antibacterial activity directly influenced by the bacterial growth after 24h. No differences between PVC and glass surfaces were observed regarding antibacterial activity against *L. monocytogenes* and *A. baumannii* (ρ>0.05). Recovery of *E. coli* was significantly different (p<0.05) among all the surfaces. Stainless steel was the antimicrobial coating treated surface that showed lower antibacterial activity against all the pathogens. Although no growth was observed for any of the treated surfaces for any bacteria, the lower R-value of stainless steel is directly influenced by the decreased growth of the pathogen after 24h incubation compared with growth on the other tested surfaces.

Contact time of the antimicrobial agent with the surface is crucial and depends on the active substance of the product. The test product from this study requires a 15-minute contact time with the surface before cleaning to achieve the maximum antimicrobial activity, and manufacturer guidelines should always be followed.

Contact killing time between the treated surface and the pathogen differs for each antimicrobial coating, mainly depending on its composition. QAC act by inhibiting bacterial adherence to the surface through cell lysis, destroying the cell before it attaches to the surface and thus having a short contact killing time, averaging between 3 to 10 minutes depending on the QAC (Fu et al., 2007; Ioannou et al., 2007). Our results are in agreement with published literature, being the contact killing time established at 1 minute after exposure to the treated surface, suggesting rapid and total growth inhibition, in a short time exposure (Ioannou et al., 20107).

Contact killing-based coatings have a clear advantage compared to other types of antimicrobial coatings, mainly their stability on the surface, high and broad-spectrum antimicrobial activity, relatively unlikely to develop antibiotic resistance and durability on surfaces (Jiao et al., 2017).

QACs antibacterial activity against *Escherichia coli* and *Listeria monocytogenes* has been reported by several authors (Murata et al., 2007; Medrano-Félix et al., 2010; Gozzelino et al., 2011; Buffet-Bataillon et al., 2012; Fazlara et al., 2012; Makvandi et al., 2018; Ramzi et al., 2020). However, some authors have also reported that some QACs formulas were ineffective against *E. coli* (Shtyrlin et al., 2016; Ramzi et al., 2020). Regarding *Acinetobacter baumannii*, Ramzi et al. (2020) has reported that different formulations of QACs-based disinfectants have an impact on antibacterial activity (Reichel et al., 2014; Ramzi et al., 2020). Antibacterial activity of QACs coatings is majorly influenced by many factors, such as concentration of the product, formulation, contact time, temperature, surface soiling, and bacterial strain (Albin, 1966; Taylor et al., 1999; Araújo et al., 2013).

QACs are cationic surfactants known for their broad bactericidal activity (Jiao et al., 2017) and they have been proven to have good antibacterial activity, mainly against Gram-positive bacteria and enveloped viruses (McDonnel et al., 2009; Jiao et al., 2017). Nevertheless, they can also be effective towards certain Gram-negative bacteria; Escherichia coli ATCC 25922 was inhibited by the antimicrobial coating tested. Long alkyl chains that penetrate the cell membrane as well as ion exchange between the positive charge of the treated surface and the negative charge of the bacterial membrane and that subsequently result in loss of cell integrity and cell death have been proposed as the mechanisms behind the antimicrobial action of QACs grafted surfaces (Grandin et al., 2012; Lia et al., 2014). QACs immobilized on surfaces have been shown to possess different antimicrobial proprieties than the same QACs in solution, being able to inhibit the growth of *Pseudomonas aeruginosa,* which has been reported to not be susceptible to QACs in solution, hence being theorized that the antimicrobial mechanisms are different for immobilized QACs in surfaces and QACs in solution. However, this hypothesis has still not been deeply studied nor proven in laboratory settings (Lia et al., 2014).

To achieve the most biocidal effects, QAC molecules immobilized on a surface must have charge densities greater than defined thresholds values (Kügler et al., 2005; Murata et al., 2007); otherwise, bactericidal activity will not take place or will be severely impaired.

Multiple factors regarding biocide formulation have been shown to influence its antibacterial effect. The molecular weight of QACs-based disinfectants is the most reported factor because of its direct impact on the efficacy of the compound and its cytotoxicity. Increased length of the N-alkyl chain has been reported to achieve higher antibacterial activity with lower compound cytotoxicity until reaching an established threshold at which too long of a chain will decrease antibacterial activity and thus resulting in a less effective biocide. Optimum chain lengths differ from microorganisms; 12 carbons are recommended for yeasts and filamentous fungi, 14 carbons for Gram-positive bacteria, and 16 carbons for Gram-negative bacteria (Jiao et al., 2017; Makvandi et al.,2018).

Several reports show the high antimicrobial activity of QAC, with their efficacy being proven for a myriad of applications, from clinical settings to industrial and household environments (Gozzelino et al., 2012; Lia et al., 2014).

3.4.2. Antimicrobial coating durability on surfaces

QACs-based surfaces coatings have been reported to be long-lasting disinfection alternatives (Ellingson et al., 2019). Testing the antimicrobial coating durability on PVC, glass, and stainless steel surfaces were assessed by ISO 22196 (2011) over 7 days.

The durability of the compound on day 1 and after 7 days, determined by its antimicrobial activity against *A. baumannii* ESB260, is shown in Figures 3.4.2.1 and 3.4.2.2, respectively.

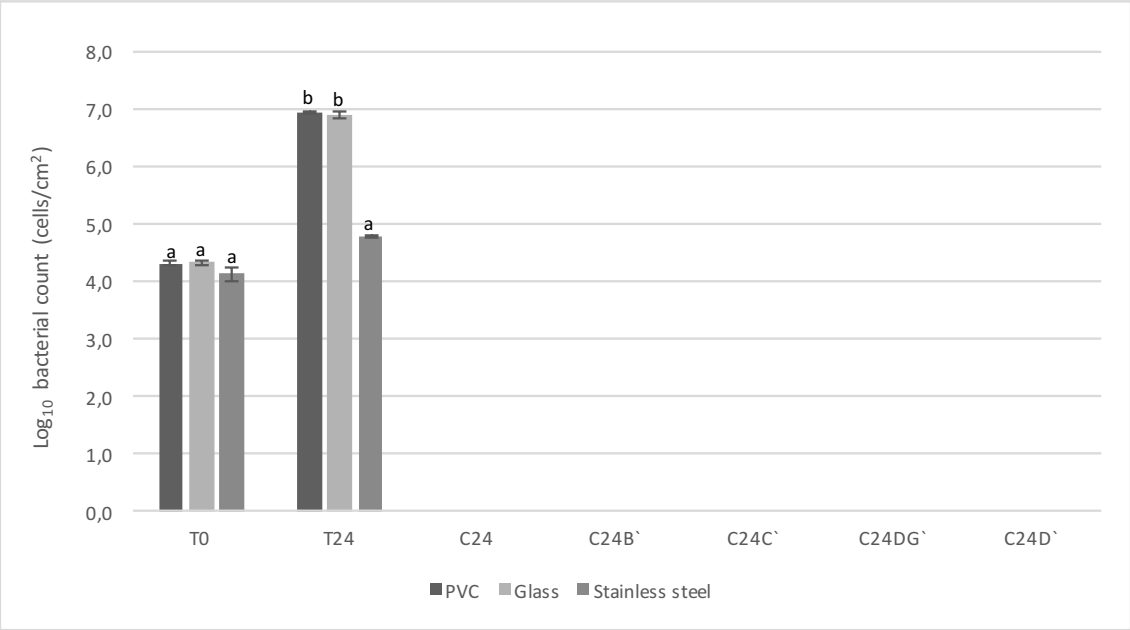

Figure 3.4.2.1. Antimicrobial activity of the compound on day 1 for *A. baumannii* ESB260. Recovery of bacteria immediately after inoculation (T0); after 24h incubation (T24) on untreated surface; after 24h (C24) incubation on treated surface previous to cleaning; 24h incubation (C24L`) on treated surface cleaned with bleach previous to cleaning; 24h incubation (C24C`) on treated surface cleaned with damp cloth previous to cleaning; 24h incubation (C24DG`) on treated surface cleaned with commercial degreaser previous to cleaning and 24h incubation (C24D) on treated surface cleaned with commercial disinfectant previous to cleaning. The results are means based on data from three replicates and standard deviations are indicated by error bars. Equivalent lowercase letters mean no significant differences between each condition ($p>0.05$).

On day 1 no significant differences ($p>0.05$) between untreated surfaces were observed for bacterial recovery immediately after inoculation (T0), but after 24h incubation (T24), significant differences ($\rho<0.05$) were observed for stainless steel surface. Since fresh coating was applied on that day, no growth was observed for any of the treated surfaces (C24, C24B, C24C, C24DG, and C24D), as shown in Figure 4.2.2.

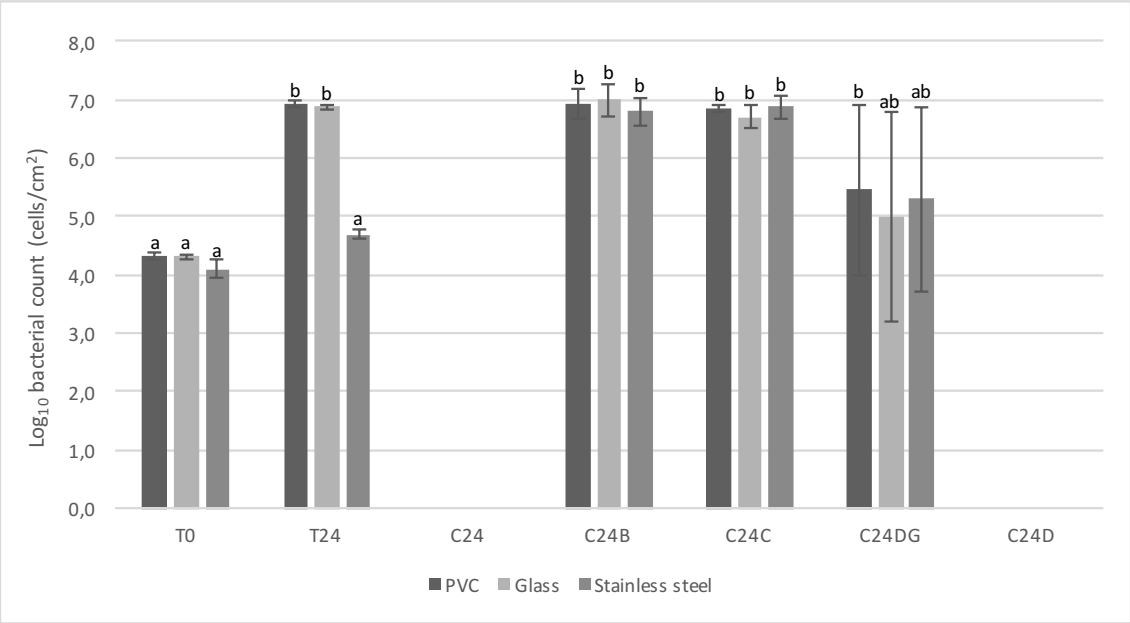

Figure 3.4.2.2. Antimicrobial activity of the compound on day 7 for *A. baumannii* ESB260. Recovery of bacteria immediately after inoculation (T0); after 24h incubation (T24) on untreated surface; after 24h incubation on treated surface; 24h incubation (C24L) on treated surface cleaned with bleach; 24h incubation (C24C) on treated surface cleaned with damp cloth; 24h incubation (C24DG) on treated surface cleaned with commercial degreaser and 24h incubation (C24D) on treated surface cleaned with commercial disinfectant. The results are means based on data from three replicates and standard deviations are indicated by error bars. Equivalent lowercase letters mean no significant differences between each condition ($p>0.05$).

After 7 days, for treated surfaces cleaned with a wet cloth (C24C), bleach (C24B), and commercial degreaser (C24DG), no antimicrobial activity against *A. baumannii* ESB260 was observed (Figure 4.2.2). However, the growth of *A. baumannii* ESB260 was inhibited for the treated surfaces cleaned with the commercial disinfectant (C24D) as well as freshly treated surfaces (C24) no growth was observed. For each treatment, no significant differences ($\rho>0.05$) on antimicrobial activity were found between each surface (Figure 3.4.2.2).

In Figures 3.4.2.3 and 3.4.2.4 are shown the durability of the compound against *E. coli* ATCC 25922, on day 1 and after 7 days, respectively.

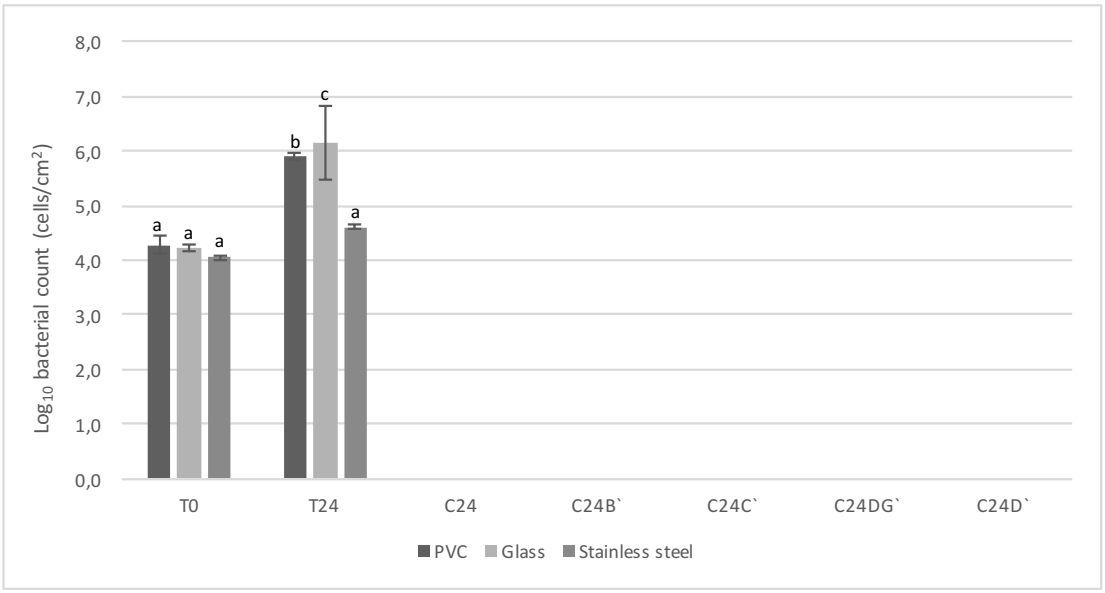

Figure 3.4.2.3. Antimicrobial activity of the compound on day 1 for *E. coli* ATCC 25922. Recovery of bacteria immediately after inoculation (T0); after 24h incubation (T24) on untreated surface; after 24h (C24) incubation on treated surface previous to cleaning; 24h incubation (C24L`) on treated surface cleaned with bleach previous to cleaning; 24h incubation (C24C`) on treated surface cleaned with damp cloth previous to cleaning; 24h incubation (C24DG`) on treated surface cleaned with commercial degreaser previous to cleaning and 24h incubation (C24D) on treated surface cleaned with commercial disinfectant previous to cleaning. The results are means based on data from three replicates and standard deviations are indicated by error bars. Equivalent lowercase letters mean no significant differences between each condition (p>0.05).

No significant differences (ρ>0.05) in antimicrobial activity were found on day 1 between untreated surfaces immediately after inoculation (T0), unlike after 24h inoculation (T24), where the antimicrobial activity found in all three surfaces was significantly different (ρ<0.05). As expected, no growth of *E. coli* ATCC 25922 was observed for any treated surfaces (C24, C24B, C24C, C24DG, and C24D).

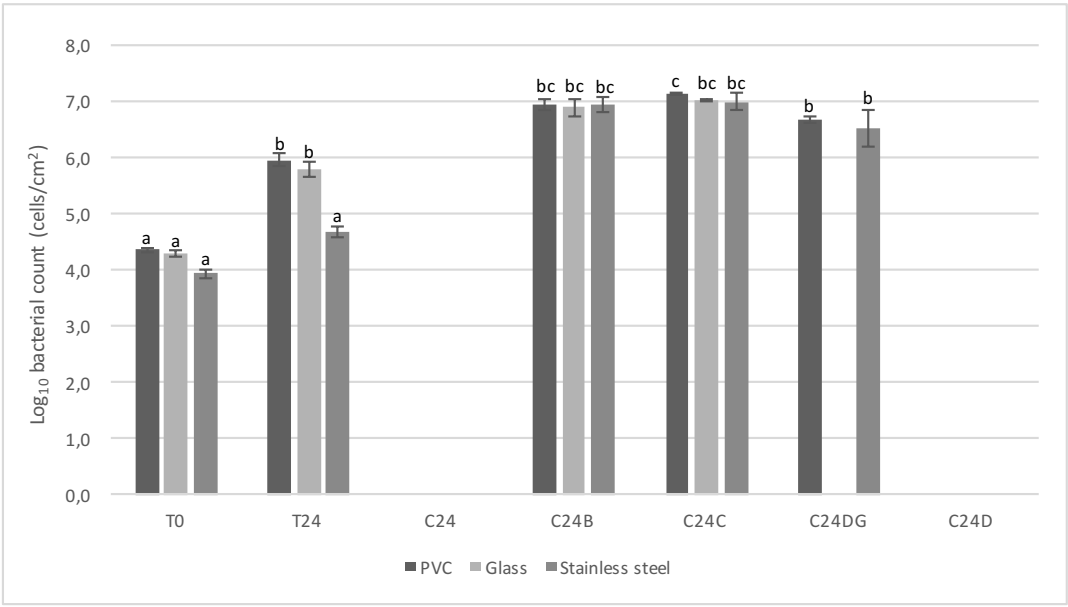

Figure 3.4.2.4. Antimicrobial activity of the compound on day 7 for E. coli ATCC 25922. Recovery of bacteria immediately after inoculation (T0); after 24h incubation (T24) on untreated surface; after 24h incubation on treated surface; 24h incubation (C24L) on treated surface cleaned with bleach; 24h incubation (C24C) on treated surface cleaned with damp cloth; 24h incubation (C24DG) on treated surface cleaned with commercial degreaser and 24h incubation (C24D) on treated surface cleaned with commercial disinfectant. The results are means based on data from three replicates and standard deviations are indicated by error bars. Equivalent lowercase letters mean no significant differences between each condition (p>0.05).

On day 7, no significant differences in antimicrobial activity were found between surfaces for T0 (ρ>0.05) and T24 (ρ>0.05). Regarding treated surfaces, no growth of *E. coli* ATCC 25922 was observed in surfaces freshly treated (C24) and cleaned with the commercial disinfectant (C24D). In addition, among surfaces treated with the commercial degreaser (C24DG), only in glass surface was no growth observed (ρ<0.05).

The antimicrobial coating durability for *L. monocytogenes* Scott A is shown in Figure 3.4.2.5 (on day 1) and Figure 3.4.2.6 (on day 7).

On day 1, no significant differences (ρ>0.05) in antimicrobial activity were observed between untreated surfaces for T0 and T24; *L. monocytogenes* Scott A was able to grow in every tested surface. On the contrary, no growth of *L. monocytogenes* Scott A was observed for any treated surfaces (C24, C24B, C24C, C24DG, and C24D).

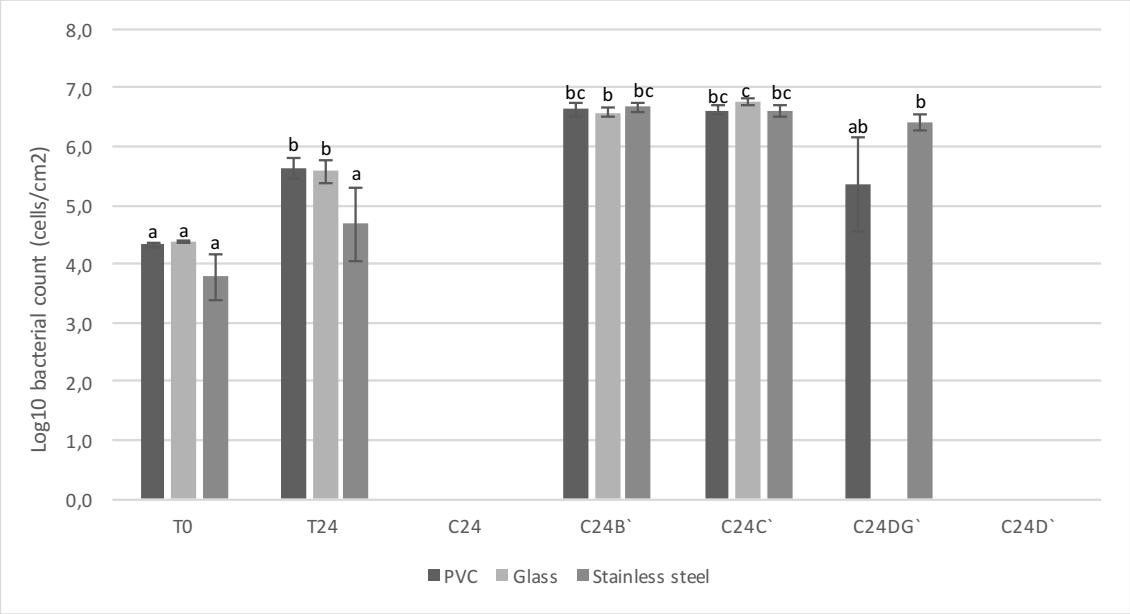

Figure 3.4.2.5. Antimicrobial activity of the compound on day 1 for *L. monocytogenes* Scott A. Recovery of bacteria immediately after inoculation (T0); after 24h incubation (T24) on untreated surface; after 24h (C24) incubation on treated surface previous to cleaning; 24h incubation (C24L`) on treated surface cleaned with bleach previous to cleaning; 24h incubation (C24C`) on treated surface cleaned with damp cloth previous to cleaning; 24h incubation (C24DG`) on treated surface cleaned with commercial degreaser previous to cleaning and 24h incubation (C24D) on treated surface cleaned with commercial disinfectant previous to cleaning. The results are means based on data from three replicates and standard deviations are indicated by error bars. Equivalent lowercase letters mean no significant differences between each condition (p>0.05).

On day 1, no significant differences (ρ>0.05) in antimicrobial activity were observed between untreated surfaces for T0 and T24; *L. monocytogenes* Scott A was able to grow in every tested surface. On the contrary, no growth of *L. monocytogenes* Scott A was observed for any treated surfaces (C24, C24B, C24C, C24DG, and C24D).

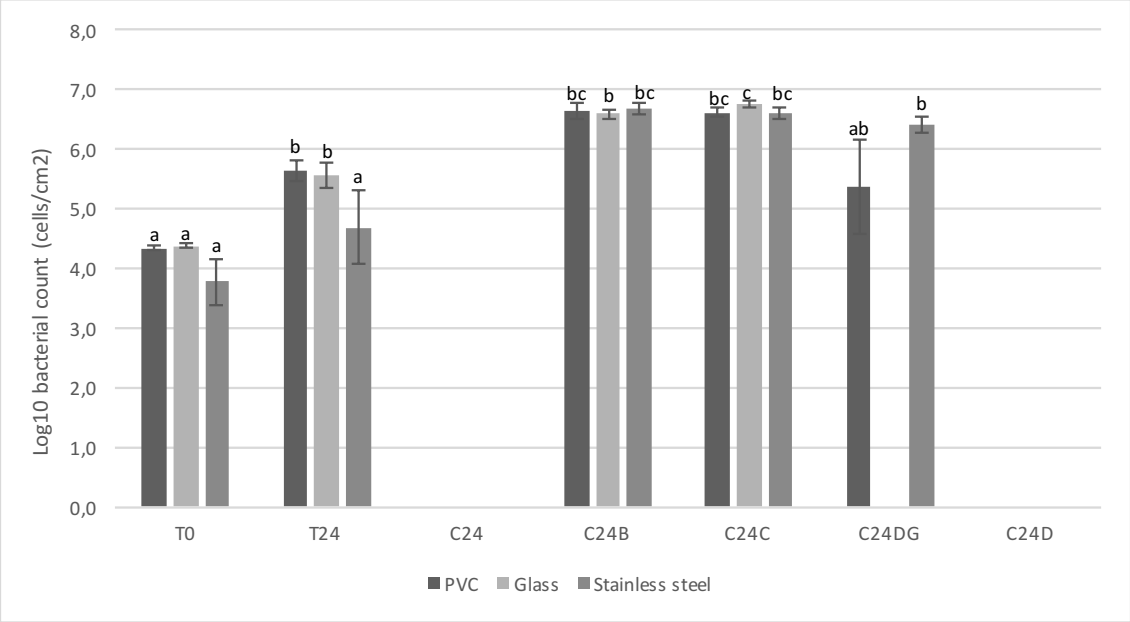

Figure 3.4.2.6. Antimicrobial activity of the compound on day 7 for *L. monocytogenes* Scott A. Recovery of bacteria immediately after inoculation (T0); after 24h incubation (T24) on untreated surface; after 24h incubation on treated surface; 24h incubation (C24L) on treated surface cleaned with bleach; 24h incubation (C24C) on treated surface cleaned with damp cloth; 24h incubation (C24DG) on treated surface cleaned with commercial degreaser and 24h incubation (C24D) on treated surface cleaned with commercial disinfectant. The results are means based on data from three replicates and standard deviations are indicated by error bars. Equivalent lowercase letters mean no significant differences between each condition ($p>0.05$).

The same results were found for untreated surfaces on day 7, with the growth of *L. monocytogenes* Scott A observed for T0 and T24 (Figure 3.4.2.6). Also, no significant differences were found ($\rho>0.05$) between all the surfaces for both controls. As noted for *E. coli* ATCC 25922, no growth of *L. monocytogenes* Scott A was observed in surfaces freshly treated (C24) and cleaned with the commercial disinfectant (C24D), and only for glass surface treated with the commercial degreaser (C24DG).

Results for the durability of the tested antimicrobial coating showed that no antimicrobial activity was observed after 7 days for surfaces cleaning with typical household and clinical disinfectants, C24B; C24C; C24DG and C24D. This finding goes against several reports of the long-term antimicrobial activity of QACs-based coatings. However, a reason for this may be that certain disinfectants may disturb the coating layer and inactivate it or physical removal of the coating layer. In a study with viruses by Butot et al. (2021), the authors have shown that the antiviral activity of QAC-based coating was removed after only one round of cleaning. The authors proposed that the coating sprayed on the surface was probably removed by wiping off during the cleaning process. They also stated that the antiviral activity of the coating was lost after cleaning

with 70% (v/v) ethanol, which instates an interesting future study on the effect that ethanol and ethanol-based disinfectants may have on our test antimicrobial coating (Butot et al., 2021). Another reason may be that too low of a concentration of QACs is used and if any compound leaching occurs, loss of antimicrobial activity also occurs. Test antimicrobial coating concentration is ten times lower than the concentration of other QACs-based commercial disinfectants included in this study, which may be the reason behind why no growth was observed for the treated surfaces cleaned with the commercial QAC-based disinfectant. Since concentration affects the antimicrobial activity of the compound, this should be an essential factor when formulating the coating (Albin, 1966).

For *L. monocytogenes* Scott A and *E. coli* ATCC 25922 no growth was observed at day 7 for the glass surface. The fact that only *A. baumannii* was able to growth may be related to its adept ability to form biofilms, which may have played a role in the survival and bacterial growth on the surface (Colquhoun et al., 2020).

QACs proprieties such as high stability, proven broad-spectrum antimicrobial activity, low corrosion, and long life span have paved the way to its use for long term disinfection processes by the medium of surface coatings (Gozzelino et al., 2011; Swartjes et al., 2015; Jiao et al., 2017). Due to the contact-killing mechanism, the coating layer can remain intact and active against bacteria on the treated surface. The contact-killing method is based on surface-immobilized QAC-molecules that disrupt the cell membranes and may lead to cell death (Asri et al., 2014; Ellingson et al., 2019). The primary mechanism believed to be behind the antimicrobial activity of contact-active surfaces is the spacer effect in which a surface grafted biocide is able to enter the bacterial cell wall and disrupt the membrane phospholipids due to their positive charge, and thus cause bacterial death (Adlhart et al., 2018). Surface grafted biocides are usually present in higher concentrations than those used in solution disinfectants, and thus the ability of antimicrobial activity towards microorganisms that may not be susceptible to them in solution, providing for a different overall mechanism between QACs immobilized on surfaces, antimicrobial coating, and QACs in solution (Siedenbiedel et al., 2012; Swartjes et al., 2015).

Ellingson et al. (2019), showed promising results for the use of QACs-based coating in clinical settings. The durability of the coating while retaining antimicrobial

efficacy was shown to be up to 15 weeks for multiple surfaces, from bedframes to medical equipment to high-touch surfaces such as doors and furniture in two hospitals. Since surface charge and hydrophobicity of the surfaces are affected, with enhanced adhesion of microorganisms, aerosolizing and touch transfer contamination probability will decrease. With significant decreases in CFU levels observed in both hospitals and significant healthcare-associated infection reduction, the use of antimicrobial coatings is proven to be a step into a new era of disinfection. However, the impact of QACs-based coatings needs to be evaluated for long-term exposures as well as antimicrobial stewardship that should be taken in place to assure efficacy and safe end-use (Ellingson et al., 2019).

The antimicrobial efficacy of coatings for food contact surfaces has also been observed. Lee et al. (2020) showed that spray-coated polymerized QACs were effective towards Gram-positive and Gram-negative bacteria, having for the latter only been observed moderate antimicrobial activity. Furthermore, the antimicrobial coating showed high durability on the surface and was shown to remain active for 20 wash and rinse cycles in food contact surfaces in food processing industries (Lee et al., 2020). This finding is important due to the fact that food spoilage and subsequent foodborne diseases that may follow are mainly from Gram-negative bacteria and thus the necessity for improved QACs-based coatings that will have the same level of antimicrobial activity towards both Gram-negative and Gram-positive bacteria. The addition of chelating agents or weak acids has been proposed by the author to achieve better microbial reduction for Gram-negative pathogens due to their role in destroying or disintegrating the outer membrane (Lee et al., 2020).

QACs-based coatings have shown promising results for the hindrance of both nosocomial and foodborne infections in clinical and food industry settings. With the high efficacy killing for both Gram-positive and Gram-negative pathogens, our test product would benefit from achieving long-term antimicrobial activity.

3.4.3. Standard Limitations

ISO 22196 (2011) is one of the most used antimicrobial efficacy tests in Europe based on the Japanese Standard JIS Z 2801 and has been classified as proof of principle,

tier 1 test by OECD (OECD, 2008). However, several limitations have been reported in regards to the experimental process as well as results report (Molling et al., 2014; Ojeil, 2014; Campos et al., 2016; Wiegand et al., 2018). One of the main shortcomings of this standard is that testing is carried out in non-real-life scenarios and under optimal artificial conditions. Thus, an overestimation of the efficacy of the antimicrobial agent may be caused by test parameters such as high incubation temperature, high relative humidity, bacterial concentration, and contact time that do not reflect what happens in clinical or food processing settings making it difficult to extrapolate any results (Molling et al., 2014). It has been reported that this standard is recommended for use only to measure the maximum antimicrobial efficacy of the antimicrobial coating since treated surfaces may react differently for lower test temperatures and lower test relative humidity affecting the antimicrobial activity and thus leading to incorrect findings (Molling et al., 2014; Campos et al., 2016).

Several studies have shown modified versions of the standard, thus invalidating the universality of the protocol and thus not fully validating the results that may come from it (Wiegand et al., 2018). Future antimicrobial efficacy testing should consider a less time-consuming approach than ISO 22196 (2011), comparable parameters that will help result' extrapolation and a universal bulletproof protocol representative of real-life environments (Molling et al., 2014; Campos et al., 2016). Wiegand et al. (2018) reported that results from round-robin tests had shown significant differences between laboratories when testing according to ISO 22196 (2011). Therefore, all protocol modifications should be recorded, and the result report should follow a well-defined system (Wiegand et al., 2018).

ISO 22196 (2011) is, however, a good and reliable tier 1 test to determine the antimicrobial activity of treated surfaces under artificial conditions, with tier 2 testing that should emulate real-life conditions and use and evaluate product characteristics such as durability and maintenance of total antimicrobial activity with wear (OECD, 2008; Ojeil, 2014).

3.5. EN1276:2019 Evaluation of bactericidal activity of chemical disinfectants

To satisfy regulatory requirements, all antimicrobial agents must undergo antimicrobial efficacy testing. Tests should consider several parameters such as type of water used, type of microorganism, contact time, high or low organic matter content, temperature and, most importantly, should guarantee the inclusion of parameters found in real-life settings. Standardized protocols such as BS EN 1276, suspension tests, are necessary so that antimicrobial efficacy claims can be supported by a recognized test as well as communication of results being reliable and homogeneous throughout Europe (Taylor et al., 1999; Araújo et al., 2013).

The choice of antimicrobial agent should be carefully thought and focus its relevancy in terms of target microorganism, toxicological safety, surfaces used, and environmental characteristics. Since the fact that QACs-based disinfectants can be affected by the presence of organic matter, interfering substances, by chemical and/or ionic interactions, choosing the right one is crucial to assess the real antimicrobial activity of the compound (Taylor et al., 1999; Araújo et al., 2013). In this study, experimental conditions were carried out at obligatory temperature (20℃), contact time (5 min), and interfering substance (bovine albumin) in order to mimic the disinfection process in real life under dirty conditions. Obligatory contact time has been set at 5 minutes due to being the maximum acceptable time to which antimicrobial activity must be observed and to keep the validity of the test results (ECHA, 2016). Contact time to achieve disinfection varies with the substance used; however, it has been reported that the effectiveness of the antimicrobial agent may be enhanced by longer contact time. Economic issues have also been reported to be one of the reasons (Taylor et al., 1999). Temperature is set at 20 ℃ considered environmental temperature, due to the fact that disinfectants may be inhibited at low temperatures and degraded and weakened at high temperatures (CDC., 2008; Jang et al., 2014). Interfering substances are used to mimic real-life disinfection processes under dirty conditions. The presence of organic load may hinder disinfection, a reason why thorough cleaning should be performed prior to the disinfection process. Several studies have reported that QACs-based disinfectants have their antimicrobial efficacy mildly impaired by

interfering substances such as bovine albumin as well as hard water (Simões et al., 2006; Araújo et al., 2013).

Bactericidal activity of the commercial coating was determined by the dilution-neutralization method (EN1276: 2019) using the conditions mentioned above. According to EN1276 (2019), "test product shall be deemed to pass the standard if at least a 5 log reduction is observed within obligatory contact time and temperature and chosen interfering substance".

In Table 3.5.1.1 are presented the bactericidal concentrations of QACs-based coating against *A. baumannii* ESB260. At 22 ℃ and a 5-minute contact time, antimicrobial coating at 3.12% (v/v) allowed a reduction of more than 5.0 log cycles under dirty conditions.

Table 3.5.1.1. Decimal log reduction for *A. baumannii* ESB260.

Product concentration (V/V %)	Log R
25.00	>6.0 ± 0.0 (P)
12.50	>6.0 ± 0.0 (P)
6.25	5.7 ± 0.2 (P)
3.12	5.5 ± 0.3 (P)
1.56	4.0 ± 0.0 (F)
0.78	<1. 0 ± 0.0 (F)
0.39	<1.0 ± 0.0 (F)
0.19	<1. 0 ± 0.0 (F)
0.09	<1.0 ± 0.0 (F)
0.05	<1. 0 ± 0.0 (F)

Log R – decimal log reduction. P – Valid test with at least 5 log reduction in viable counts. F – less than 5 log reduction of viable counts.

Bactericidal concentrations of a QACs-based coating against *E. coli* ATCC 25922 are presented in Table 3.5.1.2. Under dirty conditions, a reduction of more than 5.0 log cycles was observed for antimicrobial coating at 3.12% (v/v).

Table 3.5.1.2. Decimal log reduction for *E. coli* ATCC 25922.

Product concentration (V/V %)	Log R
25.00	>6.0 ± 0.0 (P)
12.50	>6.0 ± 0.0 (P)
6.25	5.9 ± 0.4 (P)
3.12	5.4 ± 0.2 (P)
1.56	3.6 ± 0.6 (F)
0.78	<1. 0 ± 0.0 (F)
0.39	<1.0 ± 0.0 (F)
0.19	<1. 0 ± 0.0 (F)
0.09	<1.0 ± 0.0 (F)
0.05	<1. 0 ± 0.0 (F)

Log R – decimal log reduction. P – Valid test with at least 5 log reduction in viable counts. F – less than 5 log reduction of viable counts.

In Table 3.5.1.3 are presented the bactericidal concentrations of QACs-based coating against *L. monocytogenes* Scott A. Apparently, this species was more sensitive than the others since, under dirty conditions, a lower concentration of the antimicrobial coating (1.56% v/v) was enough to reduce *L. monocytogenes* Scott A in more than 5 log cycles.

Table 3.5.1.3. Decimal log reduction for *L. monocytogenes* Scott A.

Product concentration (V/V %)	Log R
25.00	>6.0 ± 0.0 (P)
12.50	>6.0 ± 0.0 (P)
6.25	>6.0 ± 0.0 (P)
3.12	>6.0 ± 0.0 (P)
1.56	5.4 ± 0.5 (P)
0.78	4.1 ± 0.3 (F)
0.39	<1. 0 ± 0.0 (F)
0.19	<1.0 ± 0.0 (F)
0.09	<1. 0 ± 0.0 (F)
0.05	<1.0 ± 0.0 (F)

Log R – decimal log reduction. P – Valid test with at least 5 log reduction in viable counts. F – less than 5 log reduction of viable counts.

Gram-negative bacteria have been reported as being more resistant than Gram-positive (Langsrud et al., 2003). Although the antimicrobial coating was proven effective against all the pathogens tested, this ISO standard helped to show that Gram-negative

bacteria *E. coli* ATCC 25922 and *A. baumannii* ESB260 were less susceptible to QACs-based coating than the Gram-positive pathogen *L. monocytogenes* Scott A. Previous studies have also shown this difference (Shtyrlin et al., 2016).

Several pathogens with decreased susceptibility to disinfectants have been isolated from clinical and food processing settings, showing increased minimum inhibitory concentrations against tested disinfectants. This ISO standard based on cell suspension confers a suitable protocol for the search and monitoring of potential resistance to disinfectants.

3.6. Cytotoxicity of antimicrobial test compound and commercial cleaning products on HaCat and mouse fibroblast cells

Mouse fibroblast cells and HaCat cells were used to test the cytotoxic potential of the antimicrobial QACs-based coating. Fibroblasts are widely used and an established model to assess the potential cytotoxicity of compounds towards skin due to their crucial role in wound healing processes (Cavallaro et al., 2015). HaCat cells are used as a model for the epidermal skin layer and used to assess the skins irritation potential of a particular compound (Korting et al., 1994; Boyce et al., 1995; Ward et al., 1998).

MTT assay has been regarded as the “gold standard” of cytotoxicity assays due to its feasibility, simplicity of protocol, sensitivity, and reproducibility (van Tonder et al., 2015; Aslantürk, 2017).

In Figure 3.6.1. is represented the metabolic inhibition of HaCat cells, by MTT assay, after exposure to different concentrations of antimicrobial QACs-based coating.

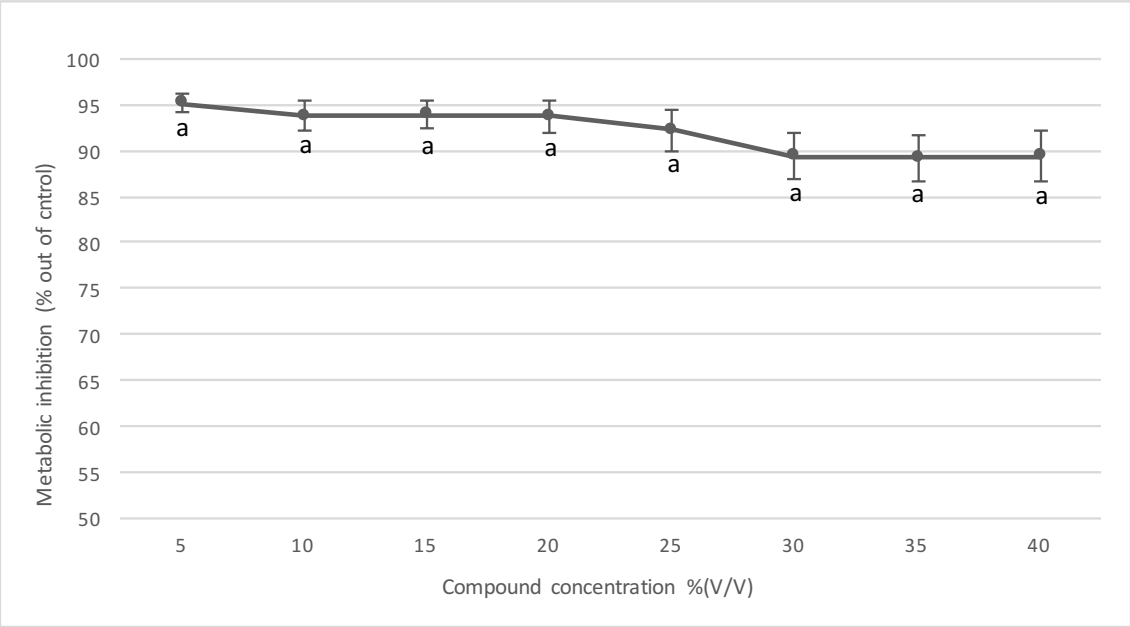

Figure 3.6.1. Metabolic inhibition of HaCat cells exposed to different concentrations of antimicrobial coating. The results are means based on data from two replicates and standard deviations are indicated by error bars. Different letters indicate values significantly different (ANOVA test followed by Tukey HSD post-hoc, $p<0.05$).

Cell viability of HaCat cells decreased when exposed for 24h to different concentrations of the antimicrobial coating compound. When compared to the positive control (cells in the standard growth medium), exposed HaCat cells showed a metabolic inhibition averaging 92%, asserting the compound´s cytotoxicity, based on the premise of cytotoxicity being set when metabolic inhibition is equal or over 70% when in comparison with the positive control (ISO 10993-5: 2009). No significant differences ($\rho>0.05$) were observed between the eight concentrations tested (Figure 3.6.1).

Metabolic inhibition of mouse fibroblast cells after exposure to different concentrations of antimicrobial QACs-based coating is shown in Figure 3.6.2.

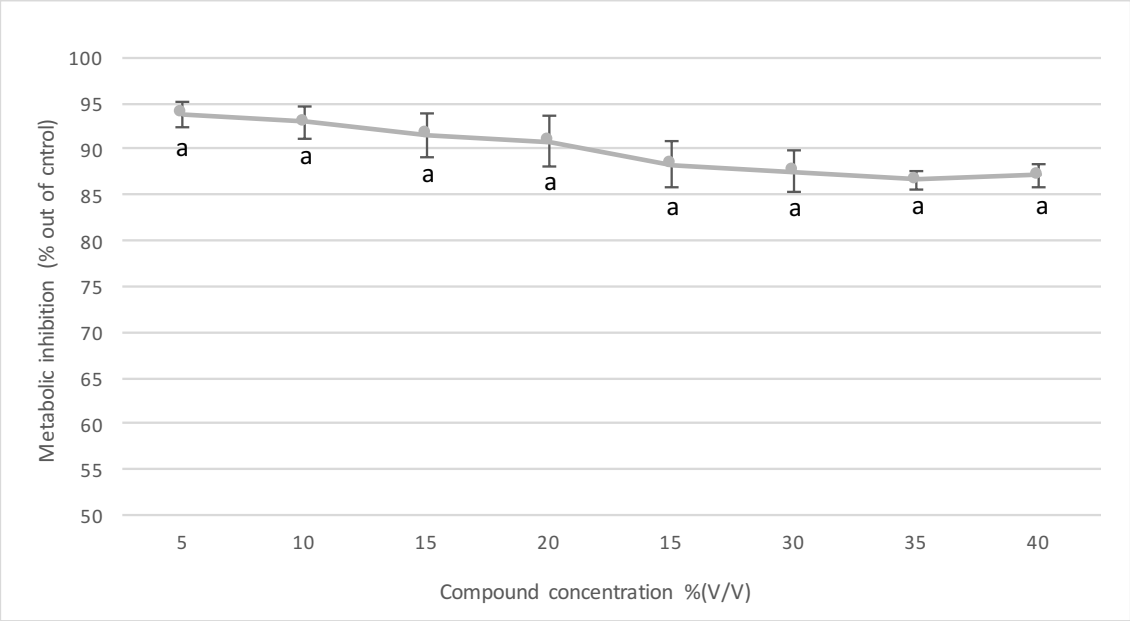

Figure 3.6.2. Metabolic inhibition of mouse fibroblast cells exposed to different concentrations of antimicrobial coating. The results are means based on data from two replicates and standard deviations are indicated by error bars. Different letters indicate values significantly different (ANOVA test followed by Tukey HSD post-hoc, $p<0.05$).

When compared to the positive control, the exposed mouse fibroblast cells showed a sharp decrease in cell viability when exposed to different concentrations of the antimicrobial coating compound, with an average metabolic inhibition of 90%. In addition, the results follow the same pattern as on HaCat cells, with the averaging metabolic inhibition being higher at lower concentrations of the compound.

Although commonly used disinfectants have been shown to be cytotoxic when in direct contact with the skin, quaternary ammonium compounds-based disinfectants are commonly recognized for their low toxicity degree, having been determined that some QAC disinfectants such as benzalkonium chloride (BAC) and didecyldimethylammonium chloride (DDAC) pose little risk to humans by risk assessments conducted by the US EPA in 2006 (Hrubec et al., 2020). However, several studies have shown that chronic exposure is known to cause contact dermatitis and other pathologies such as asthma, ocular inflammation, and hypersensitivity. In addition, irritant and cytotoxic effects of these compounds on human cells/tissues such as keratinocytes, cornea, and respiratory mucosa have been shown previously (Hrubec et al. 2020).

At therapeutic concentrations, QAC-based antiseptics have been proven to be cytotoxic to human keratinocytes and fibroblasts (Damour et al., 1992). Results obtained in this study showed that at concentrations between 5% (v/v) and 40% (v/v), the antimicrobial agent was cytotoxic to HaCat and mouse fibroblast cells (Figures 7.1 and 7.2). It was also possible to conclude that higher metabolic inhibition was achieved at lower concentrations of the antimicrobial coating. Previous studies have reported that low concentrations after short exposure times of QAC can induce apoptosis in mammal cells (Inácio et al., 2013; Debbasch et al., 2001). This phenomenon may be explained by mitochondrial fragmentation caused by the inhibition of mitochondrial respiration through inhibition of NADH-ubiquinone oxidoreductase (Complex I) followed by decreased cellular energy charge, which may induce cellular apoptosis (Inácio et al., 2013). Metabolic inhibition values were calculated through absorbance values (at 570 nm) of the MTT solution as previously described in the methods. Lower absorbance values, and therefore higher metabolic inhibition percentage, at lower concentrations, may be a result of mitochondria fragmentation resulting in cellular debris interference, causing a particle-induced artefact in which particles generate formazan adding to the assay absorbance (Holder et al. 2013). On the other hand, it can be a result of chemical interference, i.e., chemicals that are present in the antimicrobial coating composition, that may result in increased background absorbance; chemicals are mostly reducing compounds that lead to a non-enzymatic reduction of the MTT to formazan (Riss, 2014). Therefore, a lower concentration showing higher absorbance background may not directly reflect the real formazan reduction of the viable cells and thus leading to an overestimation of the cytotoxicity of the compound at the lower concentrations tested.

However, recent literature data has shown surprising results which reveal that in some experimental systems and with many test compounds, MTT reduction appears to be an inadequate test, yielding false results, either activating MTT−reducing dehydrogenases which interfere with mitochondrial dehydrogenase activity and therefore lead to an overestimation or inhibiting mitochondrial dehydrogenases and leading to an underestimation of the assay results, which are a source of misinterpretation. Recent research has also disputed that mitochondria are the sole source of MTT reductions, having been proven that NADH, responsible for most of MTT reduction, not only is associated with the mitochondria but also with the cytoplasm and

associated with membranes in the endosome/lysosome compartment as well as the plasma membrane. MTT reduction at the plasma membrane may account for observations of formazan crystals occurring outside of cells, leading to an overestimate of cytotoxicity (Riss., 2014; Aszczyszyn et al., 2008; van Tonder et al., 2015).

Future research should focus on MTT assay method validation by comparison with results from ATP assay, which has been proven to be the most sensitive, fastest and less prone to artefacts.

QACs have also been shown to be more damaging towards prokaryotic than eukaryotic cells, being the latter still affected by the mechanisms of action of the compound. Finding a balance between the main/desired effect (antimicrobial activity) and the side effect (cytotoxicity) is vital and should be taken into consideration in the development of all new antimicrobial agents, not only allowing for a safer and more efficient design but also the knowledge on the concentration of the compound necessary for each particular use. All these traits are dependent and can be controlled by the molecular structure of the QACs, mainly on the length of the alkyl chain (Loontjens, 2013; Nagamune et al., 2000). Different studies have been shown that long-chain alkyl QACs have the highest antimicrobial activity, and so it has been established that antibacterial activity and drug toxicity are influenced by the length of alkyl chain of these agents (Xie et al. 2017); the higher the antibacterial activity, the higher the hydrophobicity and the higher the cytotoxicity (Zhang et al., 2015).

The cytotoxic effect of different compounds was also tested to determine if commonly used disinfectants had similar effects: two commercial disinfectants, one degreaser, bleach, and ethanol. The cytotoxicity of each compound on HaCat and mouse fibroblast cells is represented by their metabolic inhibition in Figures 3.6.3 and 3.6.4, respectively.

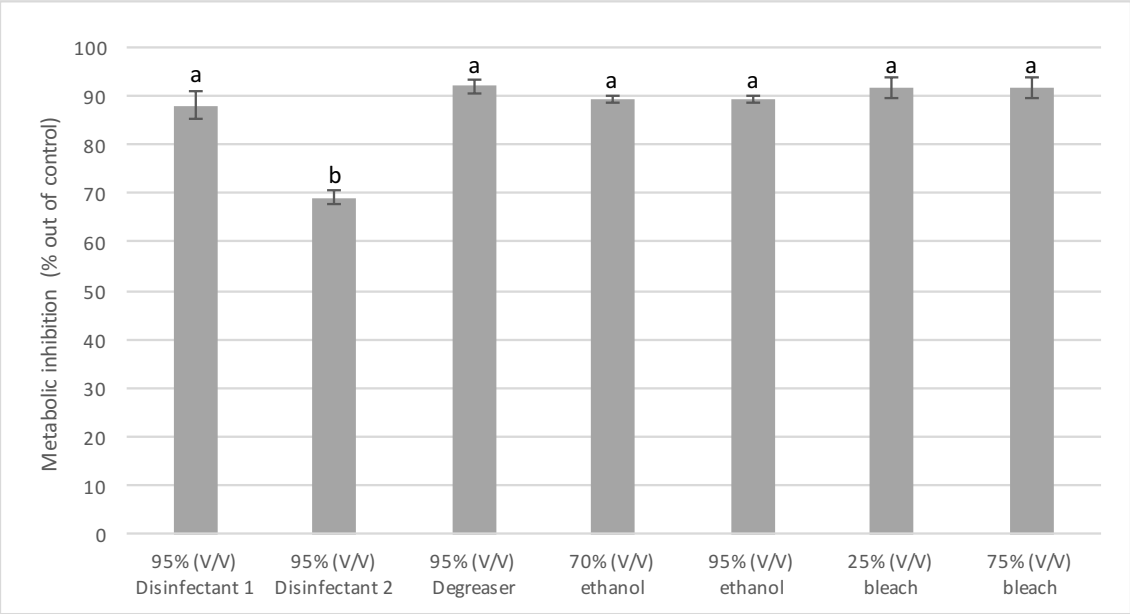

Figure 3.6.3. Metabolic inhibition of HaCat cells exposed to different household disinfectants and degreaser. The results are means based on data from two replicates and standard deviations are indicated by error bars. Different letters indicate values significantly different (ANOVA test followed by Tukey HSD post-hoc, $p<0.05$).

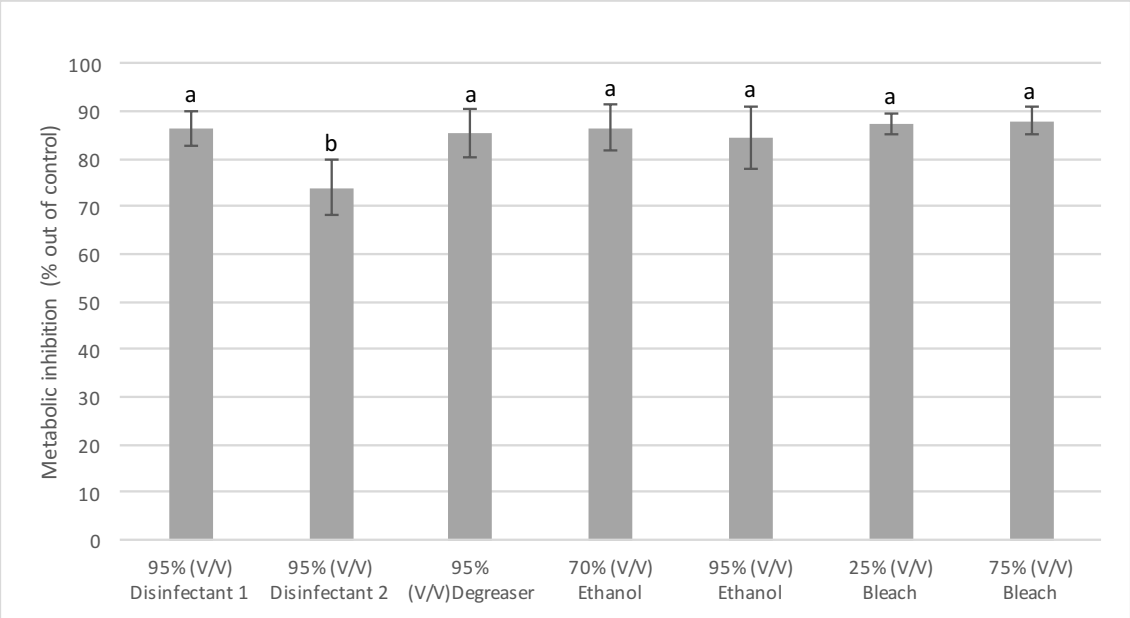

Figure 3.6.4. Metabolic inhibition of mouse fibroblast cells exposed to different household disinfectants and degreaser. The results are means based on data from two replicates and standard deviations are indicated by error bars. Different letters indicate values significantly different (ANOVA test followed by Tukey HSD post-hoc, $p<0.05$).

For HaCat cells, significant decrease ($\rho>0.05$) in cell viability was observed when compared to the positive control. Average metabolic inhibitions of 88%, 80%, and 91% were found for disinfectant 1, disinfectant 2, and the degreaser, respectively. Mouse fibroblast cells showed similar results, with average metabolic inhibitions of 87%

(disinfectant 1), 74% (disinfectant 2), and 86% (degreaser). The two concentrations of bleach and alcohol tested also showed similar cytotoxic effects on both cell lines.

This comparison allows determining that our test compound has an identical cytotoxic profile to commercially relevant and broadly used disinfectants in household surfaces as well as food contact surfaces and hospital surfaces.

Although QACs have been considered safer than other chemical disinfectants such as chlorine-based (for example, chlorhexidine) and glutaraldehyde-based disinfectants, the development of enhanced QACs is still fundamental, not only for the improvement of its original antimicrobial purpose but also for ensuring that these compounds are safer for handling (Nagamune et al., 2000).

Ethanol and bleach are everyday household essentials and are widely used to disinfect surfaces. Regarding the cytotoxicity of ethanol, studies have shown that cytotoxicity increases at higher concentrations and with exposure time. The study of Tapani et al. (1996) showed that exposure to 30-40% (v/v) ethanol resulted in total cell death after 15 seconds, while exposure to 15-20% (v/v) ethanol resulted in metabolic inhibition after 5 min. Concentrations as low as 5% (v/v) have shown to be cytotoxic and compromised cell viability (Nguyen et al., 2020). The results obtained in this study are in line with the published data since they showed that the tested concentrations of 70% (v/v) and 96% (v/v) ethanol were equally cytotoxic to HaCat and mouse fibroblast cells, averaging 90% metabolic inhibition when compared to the positive control. Cells treated with ethanol have shown organelle damage and apoptotic bodies, signalling apoptosis in cultured cells, the main death factor for ethanol-treated cells (Neuman et al., 2002). Bleach, being sodium hypochlorite (NaOCl) its main component, has been shown to fully inhibit fibroblast survival at concentrations greater than 0.05% (v/v) (Hidalgo et al., 2002). At 0.5% (v/v) concentration, a 3-minute treatment showed complete cell death, with the same effect as 99% (v/v) ethanol exposure. Tissue dissolution is the primary mechanism of action behind cell destruction; organic dissolution action can be observed in the saponification reaction when sodium hypochlorite degrades lipids and fatty acids resulting in the formation of soap and glycerol (Estrela et al., 2002). Dose-dependent mitochondrial dysfunction is also an important factor in the cytotoxicity of bleach (Hidalgo et al., 2000). Results obtained in this study showed metabolic inhibition averaging 90% both for HaCat and mouse fibroblast cells when exposed to

concentrations of 25% (v/v) and 75% (v/v) bleach with no significant differences (ρ>0.05) between cells. Interestingly, water has also been shown to reduce the number of viable cells by 40% (Missotten et al., 2008). This may be explained by the requirement of cells for certain growth conditions such as nutrients and growth factors and due to the fact that water will almost instantly kill cells due to osmotic effects namely hypotonic cell lysis (Yao et al., 2017; Xu et al., 2020;. Exposure to water may act as an experiment control to ensure that results with cells exposed to antimicrobial coating are not overestimated.

In brief, cytotoxicity of the test antimicrobial surface coating was observed for the two cell lines tested, proving the compounds' irritant nature towards the skin. Although being this finding coherent with the published literature (Lin et al., 1996; Shutty et al., 2017; Yamamoto et al., 2019), it was also found that commonly used cleaning products available on the market also show cytotoxic traits when cells are directly exposed to them. Effects of the test antimicrobial coating on cytokine gene expression would be an interesting future complement to the cytotoxicity assay to better predict skin irritation by quaternary ammonium compounds. Skin irritation firstly induces immunological events, such as inflammation resulting in skin cell death if the adverse effect is particularly severe (Yamamoto et al., 2018). Therefore, to predict the skin irritation by antiseptics, both a cytotoxicity assay (giving information on the final stage of adverse effects of antiseptics as an index) and a more suitable assay (as another index) must be performed for more accurate evaluation.

Although QACs have been considered safer than other chemical disinfectants such as chlorine-based (for example, chlorhexidine) and glutaraldehyde-based disinfectants, the development of enhanced QACs is still fundamental, not only for the improvement of its original antimicrobial purpose but also for ensuring that these compounds are safer for handling (Nagamune et al., 2000).

Trace concentrations of antimicrobial coating on food items

Even though that a myriad of antimicrobial agents that are commonly used can migrate from antimicrobial treated surfaces to food matrices, cytotoxicity assays are

highly important to be carried out in order to establish valuable guidelines about the use of antimicrobial surface coating for food contact surfaces (Dominguez et al., 2019).

MTT assay on Human colorectal adenocarcinoma (Caco-2) cells was used to evaluate the cytotoxicity of vestigial concentrations of the test product, and the results obtained are presented in Figure 3.6.5.

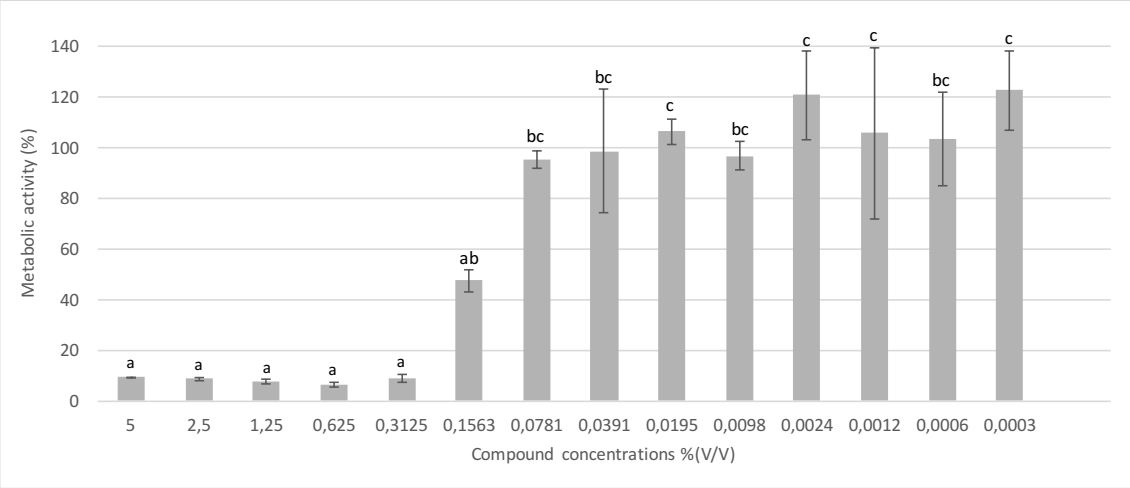

Figure 3.6.5. Metabolic activity of Caco-2 cells exposed to trace concentrations of the coating. The results are means based on data from two replicates and standard deviations are indicated by error bars. Different letters indicate values significantly different (ANOVA test followed by Tukey HSD post-hoc, $p<0.05$).

It is possible to observe that concentrations of the antimicrobial coating greater than 0.3125% (v/v) were cytotoxic to Caco-2 cells. Compared with the positive control, cells proliferation occurred at lower concentrations, between 0.0024% (v/v) and 0.0003% (v/v).

Antimicrobial compound migration to food products is considered contamination as well as a failure within the coating composition due to the premise that the active coating should remain fully attached to its substrate, i.e., the food contact surface that has been treated with the antimicrobial agent. The probability of food contamination by antimicrobial migration and subsequently lowered durability on the surface are still two reasons why AMCs are still a rare find within commercial applications (Yemmireddy et al., 2017; Dominguez et al., 2019).

Trace amounts of cleaning products in food depend on the amount of product required to be used in surface disinfection, the effectiveness of removal methods after treatment, and the ratio between the surface area to volume of the food in contact with

the surface. High ratios such as porous surfaces and/or creviced and cracked have the most potential to transfer trace amounts of disinfectants (GFSI, 2019). Migration of the antimicrobial coating on the food contact surface to food products can occur if, after disinfection, a residue of the antimicrobial agent used is not adequately removed from the treated surface (Bruijnsvoort et al., 2004).

The statutory maximum residue level (MRLs) of 0.1 mg/kg for two of the most used quaternary ammonium compounds, DDAC and BAC, was voted through at the EU Standing Committee on The Food Chain and Animal Health (Residues) in 2014. Toxicology reports for disinfectants used on food contact surfaces are required to assess better which antimicrobial agent may be used for differing situations. Monitoring, approval and maximum residue levels may differ from country to country (Dominguez et al., 2019).

At low concentrations of the QAC-based coating tested, cell proliferation occurs (Figure 3.6.5). These results can be explained by the hormesis effect in which low doses of a particular compound have a beneficial effect. In this case, the stimulation effect of low concentrations of the antimicrobial coating induces cell proliferation, whereas higher concentrations induce a toxic effect on the cells. Hormesis is considered an adaptive process following homeostasis disruption caused by overcompensation for mild stress (Kouda et al., 2010; Mattson et al., 2009; Zhang et al., 2015). In addition, trace amounts of the test compound were shown to be non-cytotoxic toward Caco-2 cells, with cells showing proliferation when exposed to the lowest concentrations tested.

4. Conclusion

The primary purpose of this study was to characterize a novel QAC-based coating. Initial results of this study showed an antimicrobial activity of this coating against a wide range of Gram-positive and Gram-negative bacteria, which was crucial for the selection of the pathogens used to further testing. The growth of *A. baumannii* ESB260, *E. coli* ATCC 25922, and *L. monocytogenes* Scott A, three relevant pathogens, was totally inhibited within 1-minute of contact time on coated surfaces of glass, PVC and stainless steel. Along with the quick contact killing time, it also showed minimum inhibitory concentrations; 1.56% (v/v) for *A. baumannii* and *E. coli*, and 0.78% (v/v) for *Listeria monocytogenes*, in solution. In parallel, temperature, pH, and antibiotic susceptibility profiles were assessed for each pathogen. The characterization of these pathogens was due to the intention of studying the potential acquisition of resistance (to each parameter) after prolonged contact with the antimicrobial coating. Unfortunately, these tests were not performed in time for the scope of the thesis but will be performed in the near future. The durability of the antimicrobial coating on relevant surfaces was shown to be less than expected. After 7 days, no antimicrobial activity was observed against all the pathogens on the coated surfaces cleaned with a wet cloth, bleach, and a commercial degreaser, except for *E. coli* and *L. monocytogenes* on glass surfaces treated with the commercial degreaser. This may be due to product-specific formulation and concentrations of active ingredient use, which does not allow the coating to remain active after surface cleaning or which is physically removed, as well as different adhesion to different surfaces. Furthermore, concentrations as low as 5% (v/v) were cytotoxic towards both human keratinocyte cells and mouse fibroblast cells, despite no vestigial concentrations of antimicrobial coating (≤0.3125% v/v) were cytotoxic to human colorectal adenocarcinoma cells. We believe that future cytokine production studies are more accurate in investigating the mechanisms behind skin and mucosa irritation by QACs-based antimicrobials since the same cytotoxicity levels of commercially available disinfectants were found.

Although promising by a significant reduction of surfaces contamination, the studied novel antimicrobial coating should be further evaluated. First, the employment of standard protocols that mimic real-life conditions would better validate its antimicrobial efficacy. Then, determining its effective durability is also a critical point that deserves attention in future studies.

5. Future studies

Despite the extensive study developed with the novel antimicrobial coating, there is still much interesting and necessary work to be done, such as: i) further antimicrobial activity standard testing to evaluate the validity of the outcome of the modified ISO 22196:2011; Ii) to testing if prolonged contact with the antimicrobial coating may be related to the potential development of antimicrobial resistance; and iii) to evaluate temperature and pH susceptibility profile testing as well as antibiotic susceptibility profiles for bacteria exposed long-term to the coating. Finally, evaluating the evolution of the minimum inhibitory concentration for QACs by EN 1276:2019 should be the focus for future studies regarding this technology.

6. References

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