D 2020

U.PORTO
FEUP FACULDADE DE ENGENHARIA
UNIVERSIDADE DO PORTO

DEVELOPMENT OF BIOCIDE FORMULATIONS BASED ON PHYTOCHEMICAL PRODUCTS FOR SURFACE DISINFECTION

JOANA FILIPA FERNANDES MALHEIRO

TESE DE DOUTORAMENTO APRESENTADA
À FACULDADE DE ENGENHARIA DA UNIVERSIDADE DO PORTO EM
ENGENHARIA QUÍMICA E BIOLÓGICA

U.PORTO
FEUP FACULDADE DE ENGENHARIA
UNIVERSIDADE DO PORTO

Development of Biocide Formulations Based on Phytochemical Products for Surface Disinfection

Dissertation presented for the Ph.D. degree in the Doctoral Program in Chemical and Biological Engineering at the Faculty of Engineering, University of Porto, Portugal

Joana Filipa Fernandes Malheiro

Supervisor:	Manuel José Vieira Simões
Co-supervisors:	Jean-Yves Maillard
	Maria Fernanda Martins Borges

Porto, 2020

Joana Filipa Fernandes Malheiro
up201409754@fe.up.pt; joana.fernandes.malheiro@gmail.com

This thesis was developed for the Doctoral Program in Chemical and Biological Engineering, at:

Chemical Engineering Department - Laboratory for Process Engineering, Environment, Biotechnology and Energy (LEPABE); Faculty of Engineering of University of Porto; Rua Dr. Roberto Frias, s/n, 4200-465, Porto, Portugal
Under the supervision of Professor Manuel José Vieira Simões (mvs@fe.up.pt)

Cardiff School of Pharmacy and Pharmaceutical Sciences; Cardiff University; Cardiff, Wales CF10 3NB, United Kingdom
Under the supervision of Professor Jean-Yves Maillard (maillardj@cardiff.ac.uk)

Department of Chemistry and Biochemistry – Chemistry Research Centre (CIQ); Faculty of Sciences of University of Porto; Rua do Campo Alegre, 4169-007 Porto, Portugal
Under the supervision of Professor Maria Fernanda Martins Borges (fborges@fc.up.pt)

This work was financially supported by: Base Funding - UIDB/00511/2020 of LEPABE funded by national funds through the FCT/MCTES (PIDDAC); Project Biocide_for_Biofilm - PTDC/BII-BTI/30219/2017 - POCI-01-0145-FEDER-030219, ABFISH – PTDC/ASP-PES/28397/2017 - POCI-01-0145-FEDER-028397 and ALGAVALOR - POCI-01-0247-FEDER-035234, funded by FEDER funds through COMPETE2020 – Programa Operacional Competitividade e Internacionalização (POCI) and by national funds (PIDDAC) through FCT/MCTES. This work was the result of the PhD grant awarded to Joana Malheiro (SFRH/BD/103843/2014) from national funds through FCT – Fundação para a Ciência e Tecnologia.

“A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales.”

Marie Curie

Acknowledgments

The PhD that resulted in this thesis was a journey in which I had the pleasure of meeting and learn with lots of people from different countries and with distinct backgrounds. It would not be possible to enumerate all of them but, to every single person that crossed my path, my deepest and heartfelt Thank you for making me the scientist and the person I am today!!!

To my supervisor Professor Manuel Simões that has helped me with my research since 2013, guiding and pushing me to always do better. In the end, a PhD thesis was achieved! Thank you for all your support, patience and all the advices along this journey. I hope the future may bring new common objectives.

To Professor Jean-Yves Maillard that after a lot of patience I ended up with just Jean-Yves, for everything, especially for making me feel at home in a foreign country! His knowledge and experience have pushed me outside of the box and I couldn't be happier with the result. I am sure the future will bring new projects and collaborations.

To Professor Fernanda Borges for always having new ideas on how to improve the chemical part of the thesis. Her suggestions and knowledge were extremely important for this thesis. Also, from her group, I would like to thank Catarina and Fernando for all the help with part of my work.

To Professor Isabel Couto and her Postdoc Sofia Santos for the great welcoming to Lisbon and to the Institute and for helping me with an important part of my thesis with their expertise.

During this journey I have been in several laboratories and it was always amazing to me how people were friendly and always egger to help. Also, I have found bright people that were always up for a great conversation where new ideas always emerged or problems were solved. I would like to express my gratitude to all the people from Lab007, Jean-Yves lab in Cardiff and all the technicians. Some of these amazing people are, to date, my friends, and they will have forever a special place in my heart. In Porto, I had the pleasure to meet Alexandra, Ana Abreu, Anabela, Andreia, Carla, Hélder, Inês, Luís, Mafalda, Maria João, Marta, Patrícia, Rita Fulgêncio, Rita Santos, Sara Souto, Tiago. In Jean-Yves laboratory I am extremely grateful to Andy, Greg, Hélène, Kate, Lauren, Leo, Magdalena, Maru, Matt, Melissa, Mike and Rebeca. I couldn't have wished for better luck!

I am also very grateful to all my friends in Cardiff, Porto and Braga, for all the memories and moments of joy, party, help, companionship and for understanding the good and the bad days. I just hope I have been as a good friend for them as they had been

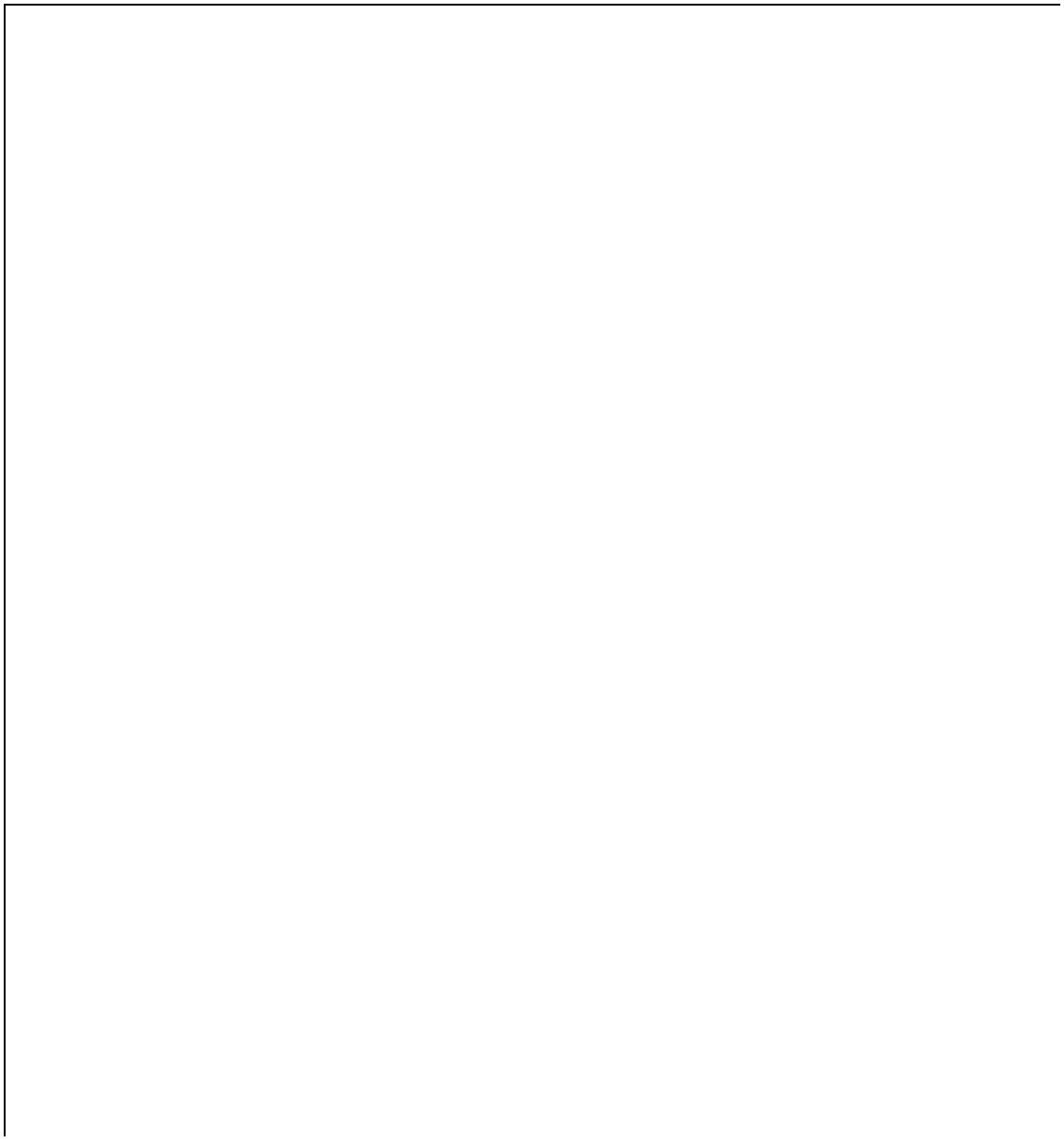
for me. Thank you Adi, Adriana Capela, Adriana Laguez, Alex, Ana Pires, Ana Xavier, Carina, Catarina, Cátia, Daphy, Davide, Eva, Fani, Hanitra, Jessica, Joana, Jonathan, Jorge, Julien, Lauren, Lisa, Lucie, Mafalda, Manuel, Mariana, Mika, Mouna, Nikki, Nuno, Pontinho, Raquel, Ricardo, Roberto, Samy, Sara, Shakil, Steffy, Tomás, Vânia.

At last, my final and most important thank you is to my family that have always been by my side in the good and the worst possible moments, without ever doubting me and reinforcing every time I needed, that I could do this. They were right!!!

"If I have seen further it is by standing on the shoulders of Giants."

Isaac Newton

Abstract

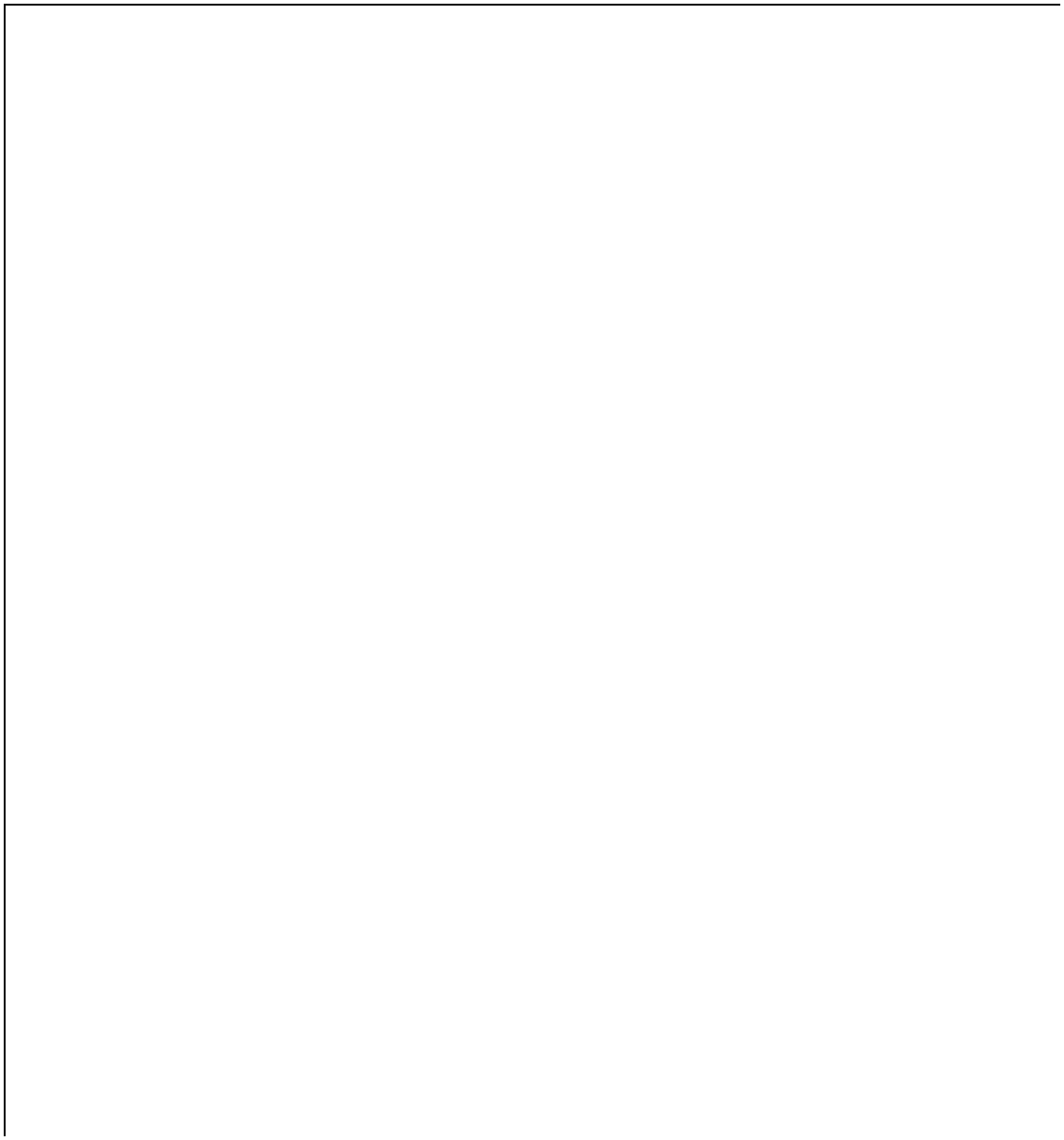


Surface disinfection has been one of the frontline strategies used in different areas such as industry, domestic and healthcare associated facilities to control the spread of microorganisms. However, while some experts support their widespread use others are concerned that their misuse may potentiate selective pressure towards bacteria. This selective pressure ultimately may lead to biocide resistance and possibly cross-resistance to antibiotics. Resistance to commonly used biocides has, in fact, already been reported and some resistance determinants have been identified, such as *qacA/B* and *smr* genes that, among others, are responsible for resistance to quaternary ammonium compounds. Therefore, the development of new and improved biocidal products is imperative to control microbial burden. Phytochemicals, plant secondary metabolites, have been extensively studied due to their promising properties such as antibacterial and antifungal. The main purpose of this thesis project was to evaluate phytochemicals and derivatives profile as biocide potentiators and to develop a biocidal formulation to be impregnated in ready-to-use wipes for surface disinfection. To achieve these goals, the work was initiated by exploring the antibacterial properties of seven structurally related phytochemicals. From the data we concluded that, cinnamaldehyde was able to inhibit bacterial growth at low concentrations while cinnamic acid completely removed adhered bacteria from polystyrene. Considering the overall results, a new selection of sixteen phytochemicals and their derivatives was considered being cinnamic acid used as starting point. Accordingly, three series were tested comprising structural modifications on the carboxyl group (series 1), on the unsaturated side-chain (series 2) or on the benzene ring type/position of the substituents (series 3). The most promising phytochemicals and derivatives belong to the series 1 and 2 as they were able to inhibit bacterial growth and the quorum sensing system of *Chromobacterium violaceum.* However, adhered bacteria removal was inferior to 1 $\log_{10}$ CFU cm^{-2} for the compounds under study. Next, the phytochemicals or their derivatives were tested in combination with some commonly used biocides (cetyltrimethylammonium bromide (CTAB) and lactic acid (LA)) to understand if there is the possibility of potentiation. In fact, potentiation was observed in bacterial growth inhibition when LA was combined with cinnamic, hydrocinnamic, α-methylcinnamic, and α-fluorocinnamic acids and against early sessile bacteria when the majority of the phytochemicals and derivatives were combined with CTAB. Considering that potentiation was achieved it was important to understand if the phytochemicals and derivatives were capable to inhibit efflux of bacteria that overexpress efflux pumps that confer resistance to biocides and antibiotics (NorA, MrsA, TetK, QacA and Smr). The

data showed that cinnamaldehyde was able to promote ethidium bromide accumulation in all the strains tested, except for the strain harbouring *qacA* gene, probably by causing membrane destabilization with consequent disruption of the proton motive force necessary for the efflux. Globally the results obtained have led to the selection of cinnamaldehyde or α-methylhydrocinnamic acid in combination with CTAB as interesting ingredients for the development of a biocidal formulation. The formulation composed by 1 mM cinnamaldehyde, 25 mM EDTA (ethylenediamine tetraacetic acid), 0.5 mM CTAB in phosphate buffer pH 7 and isopropanol (5 % v v^{-1}) was tested in laboratory simulation conditions of surface wiping. The preliminary data showed that a reduction of 4.27 to 4.35 $\log_{10}$ CFU was achieved when the wipe was impregnated with the formulation in comparison with 1.50 to 2.45 $\log_{10}$ CFU of removal just by mechanical action. In addition, the use of the formulation prevented bacterial transfer from the contaminated surface to clean surfaces. Despite these auspicious results, stability of cinnamaldehyde in the formulation decreases with room temperature shelf storage after one month. So, some adjustments in the formulation composition must be done in a near future. Overall, this thesis highlights the potential of phytochemicals and their derivatives as antimicrobial agents as well as their use in combination with commercially available biocides that ultimately can lead to the development of new formulations that surpass the efficacy of their constituents when used individually. The use of combinatorial approach to develop new formulations is also interesting since it reduces the concentration of the biocide which consequently reduces the potential environmental and public health burden of their use.

Keywords: Bacteria, biocides, disinfection, European Standards, phytochemicals, resistance, surface, wiping

Resumo

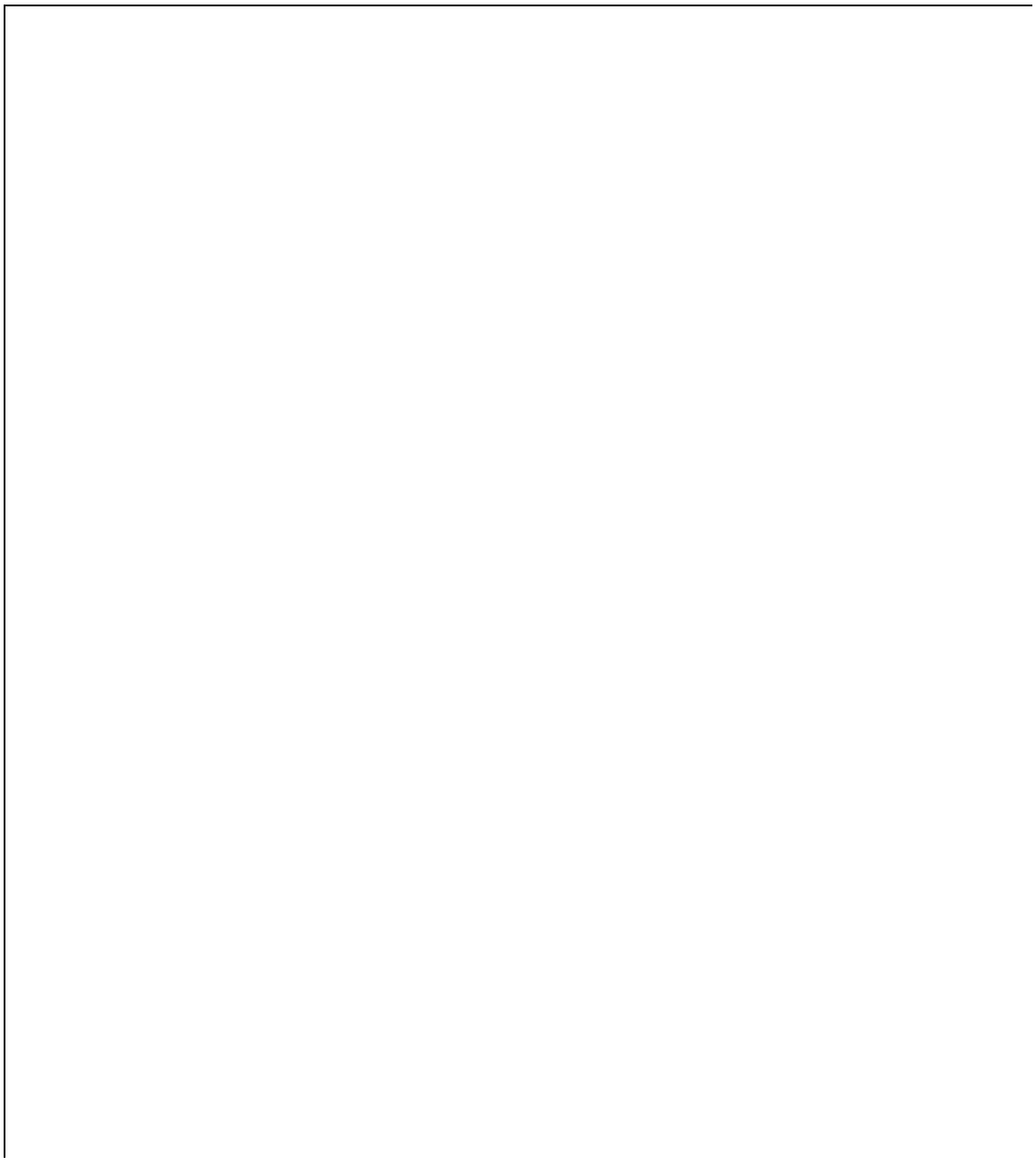


A desinfeção de superfícies tem sido uma das principais estratégias usadas para o controlo de contaminações por microrganismos em diferentes áreas, tais como indústria, edifícios associados à prestação de cuidados de saúde e habitações pessoais. No entanto, não há consenso entre os entendidos da área sobre a utilização dos biocidas, enquanto uns apoiam o seu uso para generalizado, outros referem a possibilidade de que a sua má utilização possa potenciar uma pressão seletiva nas bactérias e, assim, promover o desenvolvimento de resistência aos biocidas ou até mesmo resistência cruzada a antibióticos. A resistência a biocidas de uso comum, tais como os compostos quaternários de amónio, já foi comprovada e, para além disso, alguns genes de resistência foram inclusive identificados, tais como, *qacA/B* e *smr*, que entre outros conferem resistência a estes compostos. É, por isso, necessário o desenvolvimento de novos e melhores produtos biocidas de forma a controlar as contaminações microbianas. Os fitoquímicos, que são metabolitos secundários das plantas, têm sido muito estudados para a sua utilização em diversas áreas devido às suas propriedades antimicrobianas e antifúngicas. A ideia principal para o desenvolvimento desta tese de doutoramento foi avaliar a possibilidade utilização de fitoquímicos e derivados como potenciadores de biocidas, com o objetivo de desenvolver uma formulação biocida que possa, posteriormente, ser impregnada em paninhos para uma desinfeção de superfícies mais eficiente e rápida. De forma a atingir este objetivo, inicialmente foram exploradas as propriedades antibacterianas de sete fitoquímicos estruturalmente relacionados. Um dos compostos, o cinamaldeído, a baixas concentrações inibiu crescimento bacteriano enquanto que o ácido cinámico foi capaz de remover completamente as bactérias aderidas em polistireno. Tendo em consideração estes resultados, foi feita uma nova seleção de desaseis fitoquímicos e derivados com base na estrutura do ácido cinámico. Assim, foram estabelecidas três séries com modificações no grupo carboxílico (série 1), cadeia lateral insaturada (série 2) e anel benzeno (série 3). Os fitoquímicos/derivados mais promissores pertencem às séries 1 e 2, uma vez que para além de inibirem o crescimento bacteriano e também foram eficazes na inibição do sistema de *quorum sensing* da bactéria *Chromobacterium violaceum*. No entanto, a capacidade destes compostos na remoção de bactérias aderidas foi inferior a 1 $\log_{10}$ UFC cm^{-2}. De seguida, os fitoquímicos e os derivados foram testados em combinação com biocidas de uso comum (brometo de cetiltrimetilamônio (CTAB) e ácido lático (LA)) de forma a perceber se são capazes de potenciar a atividade destes biocidas. Esta hipótese foi comprovada, com a combinação do LA com ácido cinámico, ácido hidrocinámico, ácido α-metilcinámico e ácido α-fluorocinámico na inibição de

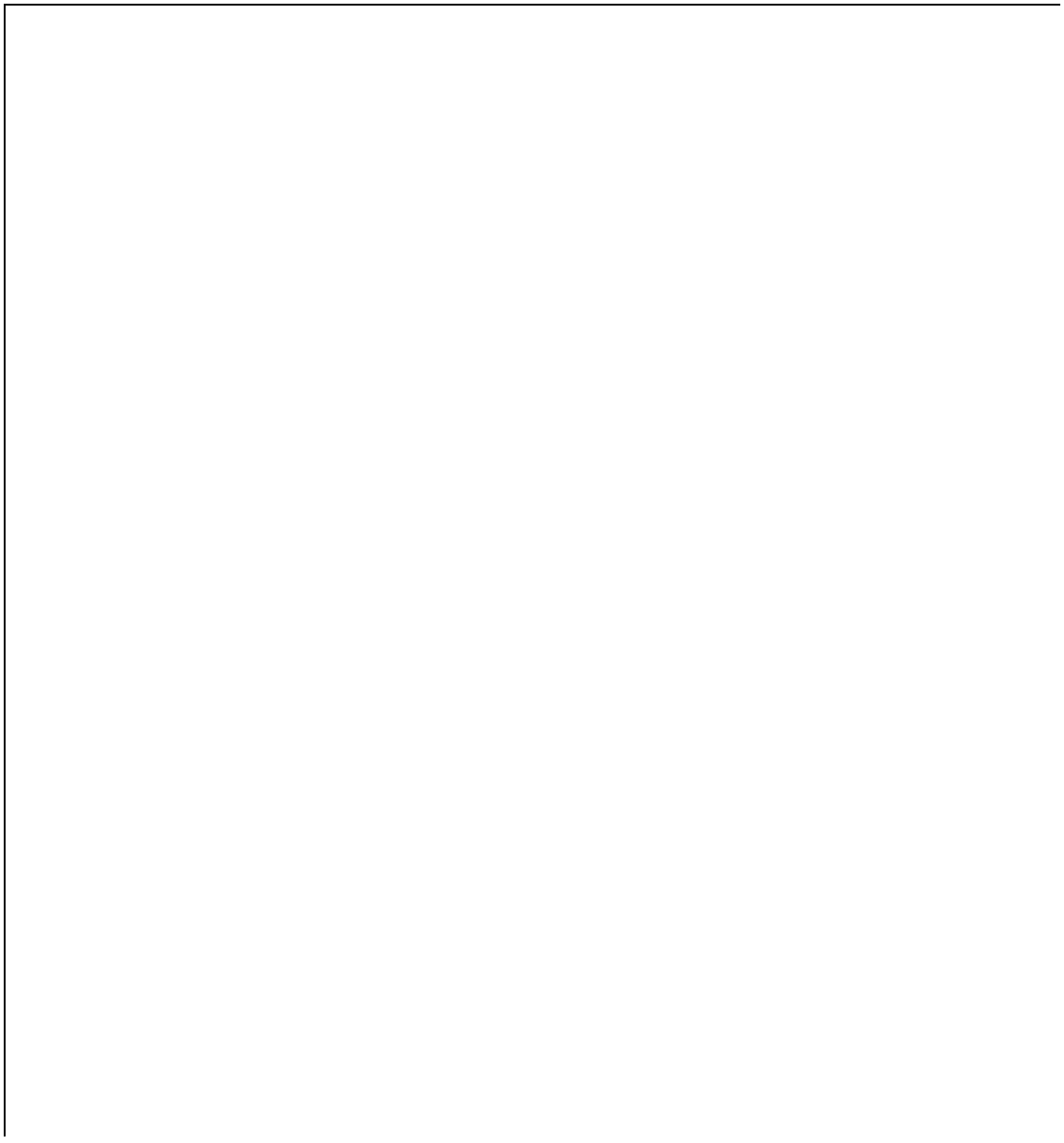
crescimento bacteriano e, em combinação com CTAB e a maioria dos fitoquímicos/derivados testados, contra adesão. Uma vez que se verificou potenciação, o passo seguinte foi o estudo da inibição do efluxo em bactérias. Para isso, foram utilizadas bactérias que sobre-expressam bombas de efluxo específicas mas que estão descritas na resistência bacteriana a biocidas e antibióticos (NorA, MrsA, TetK, QacA and Smr). Neste caso, foi possível observar que o cinamaldeído potenciou a acumulação de brometo de etídio em todas as estirpes testadas, com a exceção da estirpe que possuí o gene *qacA*, possivelmente por destabilização da membrana que, consequentemente, destabilizou a força motriz de protão que é necessária para que o efluxo ocorra. Os resultados obtidos até esta fase foram essenciais para a seleção do cinamaldeído e do ácido α-metilhidrocinámico em combinação com CTAB para prosseguir com o projeto e desenvolver a seguinte formulação: 1 mM cinamaldeído, 25 mM EDTA (ácido etilenodiamino tetra-acético), 0.5 mM CTAB em tampão fosfato pH 7 e isopropanol (5 % v v^{-1}). A última etapa desta tese incluía testar a formulação desenvolvida em limpeza de superfícies simulada em laboratório com recurso aos paninhos. Neste caso, foi conseguida uma redução de 4.27 a 4.35 $\log_{10}$ UFC quando o paninho estava impregnado com a formulação ao invés de 1.50 a 2.45 $\log_{10}$ UFC, quando apenas se analisou a força mecânica da limpeza. Para além disso, o uso da formulação na limpeza preveniu a transferência de bactérias da superfície contaminada para superfícies limpas onde foi utilizado o mesmo paninho. Apesar dos resultados auspiciosos, a estabilidade do cinamaldeído na formulação diminuiu com o armazenamento da formulação à temperatura ambiente e, após um mês, a concentração do fitoquímico é ¼ da inicial. Esta tese evidencia o potencial dos fitoquímicos/derivados como antimicrobianos, bem como, o seu uso em combinação com biocidas comerciais. A combinação de fitoquímicos e derivados com biocidas pode, por sua vez, levar ao desenvolvimento de novas formulações cuja eficácia ultrapassa a eficiência dos compostos quando usados individualmente. A metodologia de combinação de compostos, de forma a potenciar a sua eficácia é, também, importante pois pode resultar numa redução da concentração do biocida em uso. Para além disso, esta redução de contrações também diminui a contaminação ambiental e o impacto na saúde pública inerente do uso destes biocidas.

Palavras-chave: Bactérias, biocidas, desinfeção, fitoquímicos, Normas Europeias, paninhos, resistência, superfícies.

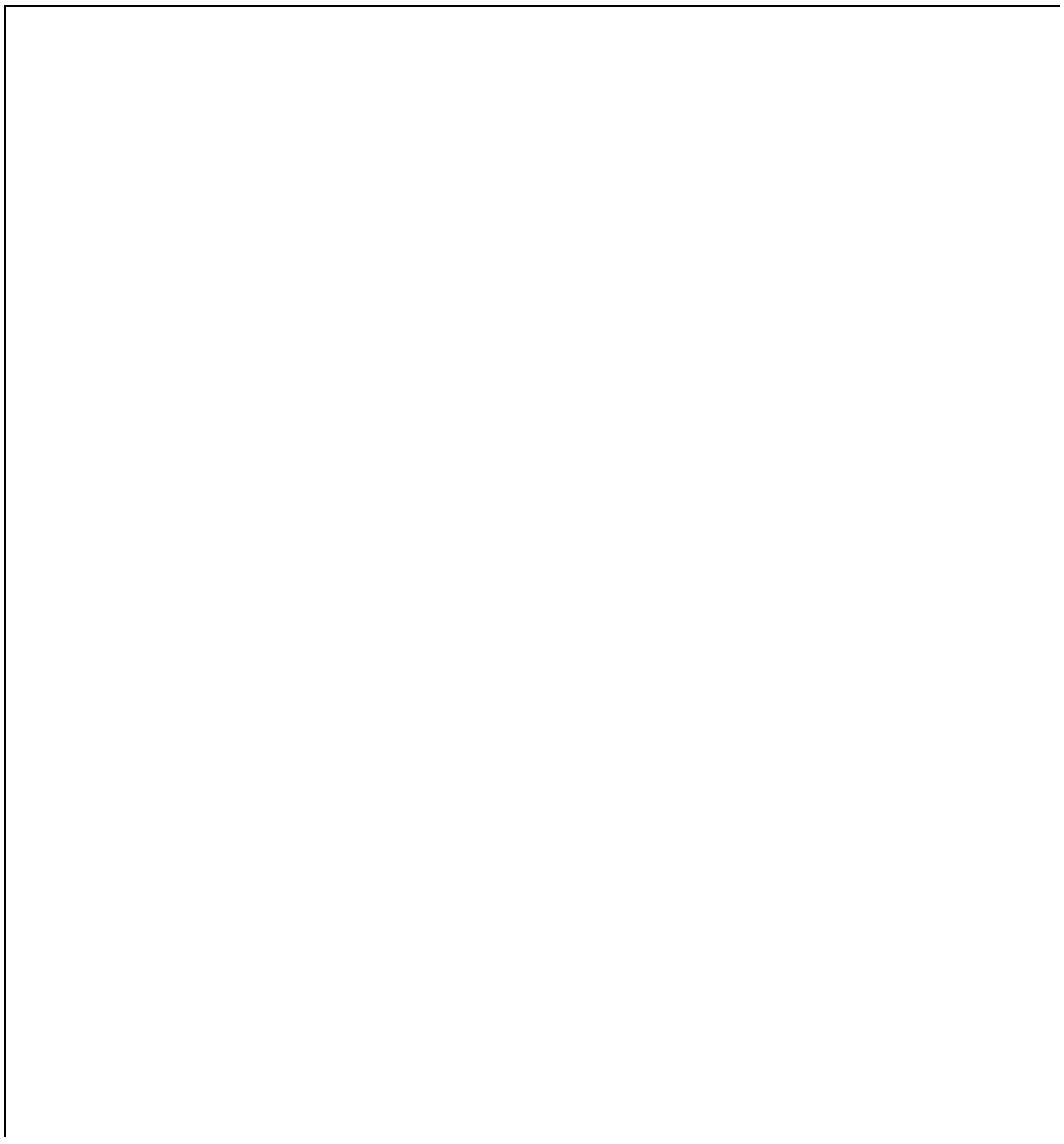
Table of Contents



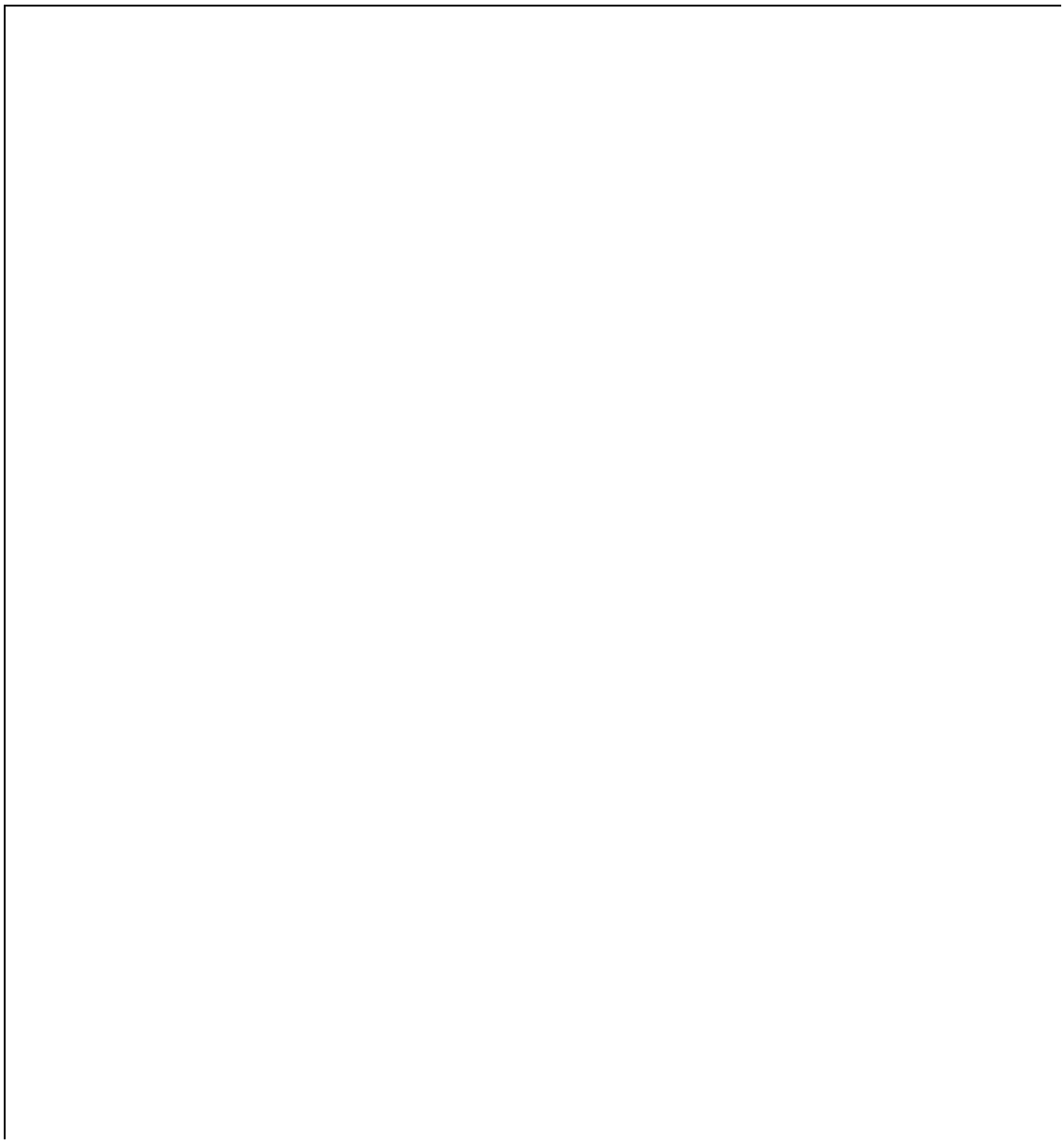
List of Figures



List of Tables



List of Abbreviations



ABC	ATP binding cassette family
ATP	Adenosine triphosphate
BAC	Benzalkonium chloride
BG	Biguanides
BSA	Bovine serum albumin
CA	Citric acid
CEN	European Committee for Standardization
CFU	Colony forming units
CHX	Chlorhexidine
CPC	Cetylpyridinium chloride
CRAs	Chlorine-releasing agents
CTAB	Cetyltrimethylammonium bromide
CTM	Cetrimide
DDAB	Didecyldimethylammonium bromide
DMSO	Dimethyl sulfoxide
DMT	Drug/metabolite transporter superfamily
EB	Ethidium bromide
ECHA	European Chemicals Agency
EDTA	Ethylenediamine tetraacetic acid
FDA	Food and Drug Administration
FEMA	The Flavour and Extract Manufactures' Association of the USA
FICI	Fractional Inhibitory Concentration Index
GRAS	Generally Recognized as Safe
HAI	Healthcare Associated Infections
HP	Hydrogen peroxide
IB	Inner bark
LA	Lactic acid
LogP	Partition coefficient
MATE	Multidrug and toxic compounds extrusion family
MBC	Minimum bactericidal concentration
MDR	Multidrug resistance
MFS	Major facilitator superfamily
MIC	Minimum inhibitory concentration

MW	Molecular weight
NMR	Nuclear Magnetic Resonance
OB	Outer bark
OPA	*o*-phthalaldehyde
PA	Peracetic acid
PACE	Proteobacterial antimicrobial compound efflux family
PCR	Polymerase Chain Reaction
PHMB	Polyhexamethylene biguanide
PHN	Phenolics
PMF	Proton motive force
QAC	Quaternary ammonium compounds
QSI	Quorum sensing inhibition
RND	Resistance nodulation division family
SH	Sodium hypochlorite
SMR	Small multidrug resistance family
TPPCI	Tetraphenylphosphonium chloride
TPSA	Topological polar surface area
TRI	Triclosan

Chapter I. General Information

1. Thesis Outline
2. Relevance and Motivation
3. Objectives

I.1. Thesis outline

This PhD dissertation is divided into nine chapters and each one is divided into several subtopics. In addition, the literature cited is also presented as well as additional information in the Appendix section.

Chapter I is a section about the organization of the thesis, in order to give the reader a better perspective on what to encounter throughout the different sections, the main relevance of this thesis as well as the motivation that lead to its accomplishment. In addition, the objectives that this thesis is meant to achieve are also presented.

Chapter II is the introduction, where all the information relevant for the understanding of this thesis is summarized to allow the readers a better comprehension of the subsequent sections.

In Chapter III all the methodology used to accomplish the results obtained are presented, together with all the chemicals and microorganisms used.

Chapters IV, V, VI, VII and VIII are the sections where all the data obtained during the PhD work plan are presented. Chapter IV includes the initial screening of phytochemicals as antimicrobials. Chapter V includes a new selection of phytochemicals and derivatives, based on the results of the previous section, in order to explore the antimicrobial properties of these chemicals. In Chapter VI, the best candidates were combined with in use biocides to explore the possibility of potentiation. Chapter VII explores the activity of phytochemicals and derivatives as efflux modulators. Finally, Chapter VIII includes the development of a formulation based on the best candidates obtained throughout the previous sections.

Chapter IX includes the final remarks of the work accomplished during the PhD as well as the future work and questions that need to be answered.

All the literature cited in this thesis is compiled in a specific section, References.

The last section, Appendix, it is divided into Supplementary information, where all the results obtained that are not included in the chapters is presented, Publications, that includes all the original papers that were published during the development of the thesis, and finally, Communications in scientific meetings, includes all the conferences where work from this thesis was presented.

I.2. Relevance and motivation

Biocides are used in industry, healthcare settings and for general purposes such as in households. Their indiscriminate use has led to an increased concern for the development of resistant microorganism and cross-resistance to antibiotics (Bock, 2019; Paul *et al.*, 2019; Russell *et al.*, 1999). In addition, the misuse of biocides can also lead to an inefficient disinfection which may contribute to the dissemination of bacteria and consequent contaminations and even outbreaks. In addition, environmental contamination is also a problem that emerges from biocides misuse (Bock, 2019; Fraise, 2002; Paul *et al.*, 2019; Rotter, 2008). The use of improved biocides and decontamination processes is essential to control and prevent the spread of resistant microorganisms since, in the last decades, several cases of increased tolerance to biocides have been documented. Contrarily to antibiotics, biocides are known to act on bacteria by a multitarget mechanism of action, being triclosan the exception (Gilbert *et al.*, 2003a; Heath *et al.*, 1999; McDonnell *et al.*, 1999). Among the different mechanisms that bacteria have that inactivates the biocide or that reduces its concentration inside the cell, bacteria can also adhere to a surface and form biofilms. The fact that a biofilm has different microenvironments and that the bacteria inside have different metabolisms is an additional step that confers resistance to biocides. These facts highlights the need for effective biocides that are able to kill bacteria in a biofilm and also remove the biofilm from the surface (McDonnell *et al.*, 1999; Simões, 2011; Smith *et al.*, 2008).

Plants are a natural and attractive source of antimicrobial products (Gibbons, 2004; Simões *et al.*, 2009). In fact, several plant secondary metabolites (phytochemicals) are being studied for their promising properties besides antimicrobial, such as anticancer, antioxidant, hepatoprotective, antidiabetic, anti-inflammatory, immunosuppressive, antimalarial, insecticidal and antiviral (Altemimi *et al.*, 2017; Ayaz *et al.*, 2019; Barbieri *et al.*, 2017; Gerometta *et al.*, 2020; Hussain *et al.*, 2018; Kumar *et al.*, 2019; Kweyamba *et al.*, 2019; Priya *et al.*, 2018; Sarker *et al.*, 2020; Simões *et al.*, 2009). One example is the case of pine oil that is used for disinfection (Dellanno *et al.*, 2009).

The study of phytochemicals as biocide potentiators has also gained interest due to the promising results reported by several authors, both with antibiotics and biocides (Abreu *et al.*, 2017; Alabdullatif *et al.*, 2017; Wang *et al.*, 2018). Being biocides potentiation auspicious, however, poorly explored.

I.3. Objectives

The purpose of this PhD thesis is to understand the possibility of using phytochemicals for general purpose disinfection, as single products and in combination with biocides as a formulation. To achieve this aim, the following objectives will be accomplished:

- Establishment of the antimicrobial activity of phytochemicals and derivatives (structure as primary criteria), using high throughput measurement of their minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC). These data will be used for the selection of the most promising phytochemicals and derivatives for further investigation;
- Characterization of the bacterial physiological changes induced by the exposure to phytochemicals and derivatives, providing information on their mode of action;
- Evaluation of the possibility of potentiation with established biocides (exemplars of several classes), for which antimicrobial resistance has already been reported. These data will highlight the potential of phytochemicals and derivatives to potentiate the activity of biocides for the development of a formulation with increased efficacy;
- Assessment of the potential of the phytochemicals to control bacterial adhesion both individually and as a mixture containing a commercially available biocide;
- Evaluation of the potential of phytochemicals and derivatives as resistance modifying agents in biocide resistant bacteria that overexpress efflux pumps;
- Development of a method to study impregnated wipes action on surfaces that allows a rapid screening without the need of a specific equipment;
- Evaluation of a formulation to be impregnated in ready-to-use wipes for surface disinfection.

The data obtained during the development of this PhD will result on the publication of papers in scientific journals and a thesis. In addition, these publications will contribute the knowledge on how to minimize the spread of antimicrobial resistant microorganisms by the design of a formulation to be used for surface disinfection.

Chapter II. Introduction

1. Phytochemicals
2. Biocides
3. Bacterial resistance mechanisms to biocides
4. Combinatorial approach to enhance biocidal efficiency
5. Development of a biocidal formulation for surface disinfection

II.1. Phytochemicals

Plants are constantly exposed to a wide range of abiotic and biotic environmental stresses (Borges *et al.*, 2016). Abiotic stresses include nutrient deficiency, salinity, temperature fluctuation, exposure to pesticides and pollutants and also UV radiation, while biotic stresses include the exposure to microorganisms, insects and animals (Suzuki *et al.*, 2014). Their ability to adapt to these adversities has led plants to produce a wide range of secondary metabolites, known as phytochemicals, to be used for defence (Borges *et al.*, 2016).

Until now phytochemicals have been extensively studied and several properties have been reported. In fact, phytochemicals are described, to act as antimicrobials, anticancer, antioxidant, hepatoprotective, antidiabetic, anti-inflammatory, immunosuppressive, antimalarial, insecticidal and antiviral agents (Altemimi *et al.*, 2017; Gerometta *et al.*, 2020; Hussain *et al.*, 2018; Kumar *et al.*, 2019; Kweyamba *et al.*, 2019; Priya *et al.*, 2018; Sarker *et al.*, 2020). However, to date no plant-derived antibiotics have been discovered. On the contrary, reports of antibiotic enhanced activity when combined with phytochemicals are numerous (Abreu *et al.*, 2016a; 2016b; Araujo *et al.*, 2020; Ayaz *et al.*, 2019; Gomes *et al.*, 2019; Hemaiswarya *et al.*, 2008; Ohene-Agyei *et al.*, 2014; Sivasankar *et al.*, 2020).

II.1.1. Phytochemicals classification

Phytochemicals classification can vary depending on the chosen feature, such as origin, biological property or chemical structure (Liu, 2004; Scalbert *et al.*, 2011). Considering the classical chemical classification, some of the most important classes of phytochemicals are alkaloids, carotenoids, organosulfur compounds and polyphenols, being these last the largest class and one of the most studied (Scalbert *et al.*, 2011).

Alkaloids are among the more structurally diverse and therapeutic significant phytochemicals (Roy, 2017). Alkaloids are heterocyclic nitrogenous compounds, and, among other properties, some are known for their antimicrobial activity, such as morphine, codeine and berberine (Barbieri *et al.*, 2017). Berberine, for instance, is able to intercalate DNA interfering with cell division and consequently leading to cell death (Wang *et al.*, 2011).

Carotenoids are responsible for the pigmentation in plants. The distinctive structural element of these type of phytochemicals is a polyene backbone consisting of a series of conjugated double bonds. In fact, this characteristic is important for their pigmentation properties and also for their ability to act as antioxidants, interacting with free radicals and singlet oxygen (Barbieri *et al.*, 2017; Young *et al.*, 2018). One example of an antibacterial carotenoid is fucoxanthin, which is extracted from algae and was able to inhibit growth of *Streptococcus agalactiae*, *Staphylococcus aureus* and *Staphylococcus epidermidis* (Karpiński *et al.*, 2019).

Organosulfur compounds contain one or more sulfur atoms bonded with carbon. These phytochemicals are volatile, extremely unstable and can be rapidly decomposed to form other sulfur-containing compounds. Organosulfur compounds such as allicin, ajoene and isothiocyanates have demonstrated to possess antibacterial activity against both Gram-positive and Gram-negative bacteria (Barbieri *et al.*, 2017). In fact, isothiocyanates (allylisothiocyanate and 2-phenylethy-lisothiocyanate) besides inhibiting bacterial growth they were able to prevent biofilm formation of *Escherichia coli* and *Pseudomonas aeruginosa* (Borges *et al.*, 2014b).

Polyphenolics is one of the biggest groups of phytochemicals and comprise aromatic compounds containing hydroxyl groups that are usually involved in plant protection. Regarding their structure polyphenols can be divided into flavonoids, phenolic acids and other polyphenols, Figure II.1. Flavonoids, in general, are coloured compounds that are part of the fruits and flowers of plants and can be divided into anthocyanidins and anthocyanins, flavan-3-ols, flavanones, flavones, flavonols and isoflavonoids (Zhang *et al.*, 2019). Flavonoids comprise two aromatic rings through a 3 carbon chain (C6-C3-C6) that may or not be part of a third ring (Garrido *et al.*, 2013). These polyphenolics are associated with plants defence to microbial infection and their antimicrobial properties have been reported (Farhadi *et al.*, 2019; Górniak *et al.*, 2019). In fact, their mode of antibacterial action comprise alteration of cytoplasmic membrane function (alteration of permeability and inhibition of porins and efflux pumps), inhibition of energy metabolism and nucleic acid synthesis and reduction in cell attachment and biofilm formation (Bello *et al.*, 2016; Farhadi *et al.*, 2019). Phenolic acids can be divided in two groups, cinnamic acids comprising nine carbon atoms (C6-C3) and benzoic acids with seven carbon atoms (C6-C1), being cinnamic acids one of the major classes of phenolic compounds found in nature (Garrido *et al.*, 2013; Kumar *et al.*, 2019).The antimicrobial activity of phenolic acids has already been extensively reported for both Gram-positive and Gram-negative

bacteria and there are some studies that correlate their structure activity relationship (Alves *et al.*, 2013; Andrade *et al.*, 2015; Barbieri *et al.*, 2017; Bouarab-Chibane *et al.*, 2019; Guzman, 2014; Kumar *et al.*, 2019; Sharma, 2011). However, their activity is generally weaker when compared to flavonoids.

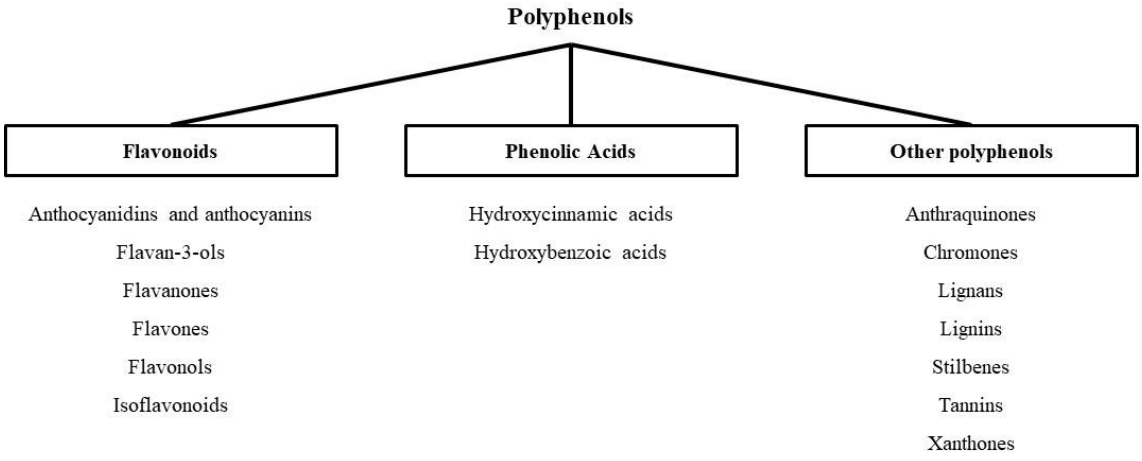


Figure II.1. Chemical classification of polyphenols (Martinez *et al.*, 2017).

II.1.2. Cinnamaldehyde and cinnamic acid

Cinnamaldehyde and cinnamic acid are two phytochemicals that are widely studied since they possess interesting properties that can be useful in different areas such as health and food production.

Cinnamaldehyde is a phytochemicals that occurs naturally in plants of the genus *Cinnamon*, such as in cinnamon oil of *Cinnamomum cassia* leaves and twigs (Poole *et al.*, 1994). This phytochemical it's a liquid characterised by its pale yellow colour with a warm, sweet, spicy odour and pungent taste reminiscent of cinnamon (Gowder, 2014; Si *et al.*, 2006). Cinnamaldehyde, when exposed to air can be converted into cinnamic acid (Eilerman, 2014). This phytochemical acute toxicity (LD50) ranges from 0.6 to 2 g Kg^{-1} in various species and its oral toxicity (LD50) is between 2.2 to more than 3.4 g Kg^{-1} (Adams *et al.*, 2004). However, cinnamaldehyde can cause allergic contact dermatitis and stomatitis (Gowder, 2014). FEMA (The Flavour and Extract Manufactures' Association of the USA) has given this phytochemical GRAS (Generally Recognized as Safe) status and is approved by the FDA (Food and Drug Administration of United States) (Adams *et al.*, 2004; Smith *et al.*, 2003). It is already approved to be used in air care products, perfumes and fragrances, polishes and waxes, washing and cleaning products, cosmetics

and personal care products, pharmaceuticals and biocides in the European Union (Commission Implementing Regulation (EU) No 872/2012, 2012). In the US, cinnamaldehyde was approved to be used in different applications such as flavouring agent (maximum concentration of 48.46 mM in fruits and juices, 26.5 mM in baked goods, in 16.6 mM in breakfast cereals, 15.1 mM in baby food and desserts and 8.3 mM in chewing gum), as fragrance (cosmetics, soaps, detergents) and as medicine (stomachic, antipyretic, antiallergic, tonic in traditional Chinese medicines) (Gowder, 2014).

The interest on cinnamaldehyde is due to its properties such as anti-inflammatory, antimicrobial, antifungal, antioxidant, anticancer and protection of cardiovascular system (Di Pasqua *et al.*, 2006; Doyle *et al.*, 2019; Gill *et al.*, 2004; Holley *et al.*, 2005; Khorasani *et al.*, 2017; Rao *et al.*, 2014; Wagle *et al.*, 2019; Wang *et al.*, 2018).

Cinnamic acid is a phytochemical necessary for lignin formation in plants. This chemical is a white crystalline solid with a low intensity sweet honeylike aroma and has been identified as the main constituent of diverse botanical extracts of Benzoin (*Styrax benzoin*), Peru Balsam (*Myroxylon pereirae*), Styrax (*Liquidamber orientalis*) and Tolu Balsam (*Myroxylon balsamum*) (Eilerman, 2014). This phytochemical has oral toxicity (LD50) between 3.4 to more than 5 g Kg^{-1} (Adams *et al.*, 2004). Cinnamic acid is a flavour ingredient that as well as cinnamaldehyde has also been approved to be used in flavour and fragrance compounds, by the FDA and FEMA/GRAS guidelines (Cohen *et al.*, 2018). However, cinnamic acid causes irritation to skin, eyes, when inhaled and ingested (Eilerman, 2014).

The biological interest in cinnamic acid is attributed to its properties such as anti-inflammatory, antioxidant**,** anticancer, antimicrobial, neuroprotective, anti-hypertensive**,** anti-hyperlipidemic and diabetes control (Adisakwattana, 2017; Alam *et al.*, 2016; Anantharaju *et al.*, 2016; Anwar *et al.*, 2018; Chan *et al.*, 2013; de Cassia da Silveira e Sá *et al.*, 2014; Peperidou *et al.*, 2017; Pontiki *et al.*, 2014; Prorok *et al.*, 2019; Yilmaz *et al.*, 2018).

II.2. Biocides

Biocides haven been used for centuries essentially as preservative agents in water (copper pipes), for food preservation and wound cleaning (vinegar and honey) (Wand, 2017). In terms of clinical use, biocides were introduced in the nineteenth century for the antiseptic surgery. Iodine was being used to disinfect wounds and carbolic acid (phenol) for wound dressings, while for general disinfection the preference was water with chlorine and formaldehyde. In the twentieth century chlorine-releasing agents and quaternary ammonium compounds (QACs) were introduced. By the end of the century the panoply of available biocides increased and other chemicals such as aldehydes, amphoteric surfactants, biguanides, bisphenols, chlorine-releasing agents, iodine-releasing agents and peroxygens have been introduced (Maillard, 2005; Russell, 2002; Wand, 2017).

Biocides' fields of application include industry, healthcare facilities, households and general cleaning. In healthcare environment, biocides can be used for surface, water and equipment disinfection but also for sterilizing medical devices (Ribeiro *et al.*, 2018).

The use of biocides for disinfection and antisepsis purposes involves high concentrations of the chemicals, exceeding their MBC, in order to achieve a rapidly a killing effect. When high concentrations are used it is believed that the biocide interacts with multiple targets which possibly makes the emergence of resistance less probable (Maillard, 2002). Their use implies a careful choice that balances the benefit of controlling infection and the potential risk (resistance, toxicity and environmental pollution) (Gilbert *et al.*, 2003b; Maillard, 2005; Rutala *et al.*, 2004). To ensure biocide effectiveness it is important to know the biocide's chemical activity and limitations, train the end users and comply with the manufacturer´s guidelines for use and storage (Maillard, 2005).

II.2.1. Biocides classification

Biocides can be divided into several classes such as organic acids, biguanides, peroxygens, alcohols, aldehydes, phenolics, halogen-releasing agents and cationic antimicrobial agents (QAC) (Hirshfield *et al.*, 2003).

II.2.1.1. Organic acids

Organic acids, like acetic, propionic, lactic (LA) and sorbic acid, are weak acids that are usually found in fruits and vegetables. As organic acids, they have a pH around 5 or less which difficult bacterial growth, making them appealing as food preservatives (Hirshfield *et al.*, 2003).

Bacterial growth inhibition by weak acids is related with their lipid permeability as their mode of action, represented in Figure II.2, is based on their protonation state that depends on the pKa of the acidic group and the environment pH (Tan *et al.*, 2015). The uncharged acid is lipid permeable and diffuses into the cytoplasm of the bacteria in order to achieve concentration equilibrium between the cytoplasm and the exterior. The dissociation of the weak acid inside the cytoplasm leads to the accumulation of the anion (A^-) and protons (H^+) that consequently increases the osmolarity of the cytoplasm and influences the activity of enzymatic reactions that may lead to cell death (Hirshfield *et al.*, 2003).

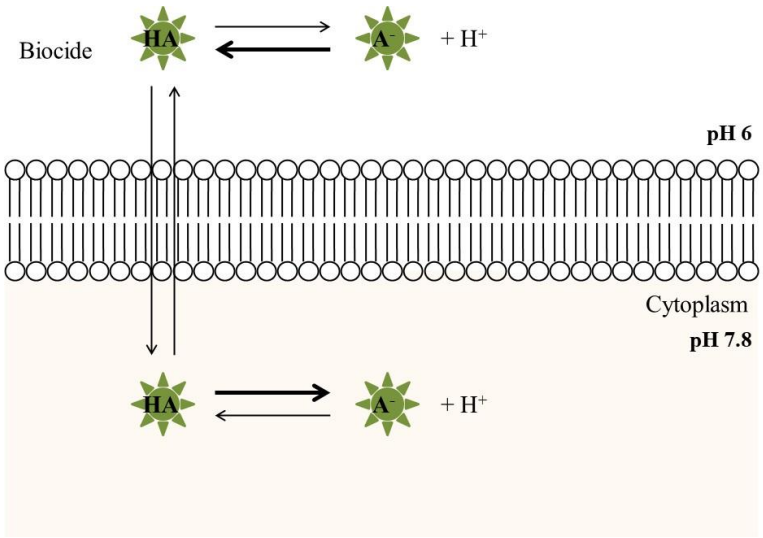


Figure II.2. Schematic representation of the generalized mode of action of weak organic acids. HA represents the uncharged form of the weak acid that passes the bacterial membrane to equilibrate the external and internal concentration of acid. Weak acids can dissociate into the anionic form of the acid (A^-) with the release of a proton (H^+) into the external environment. This reaction depends on the pKa of the acid and the environment pH, however, when the cytoplasm is alkaline the preferential reaction is highlighted by thicker arrows. H^+ accumulation can reduce the internal pH while the accumulation of A^- has osmotic effects, consequently H^+ and A^- accumulation lead to metabolic perturbations. This Figure was adapted from Hirshfield *et al.* (2003).

II.2.1.2. Biguanides

Biguanides are derivatives of guanidine, that occurs naturally in vegetables such as turnips and cereals. Chemically they are strong bases that are usually supplied as a salt, soluble in water and alcohol (Fisher, 2003). Biguanides mode of action, Figure II.3, is based on their association with anionic sites of the microbial surface, in particular with the acidic moieties of phospholipids and proteins (Wand, 2017). Next, they form a bridge-like structure displacing Mg^{2+} and Ca^{2+}, which results in a reduction of membrane fluidity and osmoregulation, and a consequent decrease of membrane enzymatic activity. At higher concentrations of the biocide, this effect is amplified, and the membrane can assume a liquid crystalline state that ultimately leads to the leakage of cellular contents. Biguanides optimum activity is between pH 3 and 9, below which the activity is supressed and above this pH the biocide precipitates (Fisher, 2003).

Chlorhexidine (CHX) and polyhexamethylene biguanide (PHMB) are the most commonly used biguanides. CHX and PHMB action is concentration dependent, being bacteriostatic at low concentrations and bactericidal at high concentrations. Positively charged CHX, as described for biguanides, interacts with adjacent phospholipids contrarily to PHMB that is able to interact with more distant phospholipid heads. Both lead to generalized cellular leakage (Gilbert *et al.*, 2005; Wand, 2017). In addition, CHX has been reported to have a residual efficacy when applied to the skin or to root canal (María Ferrer-Luque *et al.*, 2014; Sogawa *et al.*, 2010).

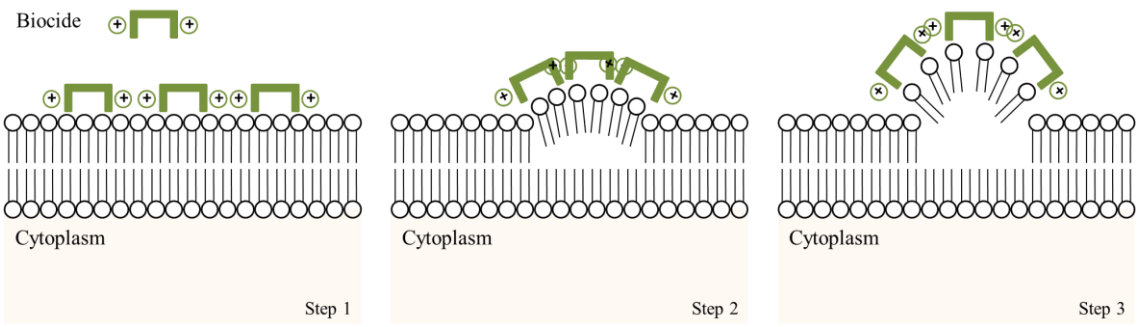


Figure II.3. Schematic representation of biguanides mode of action. The positively charged biocide binds to the negatively charged sites of the bacterial membrane destabilizing it and ultimately promoting leakage of cell components. Figure adapted from Gilbert *et al.* (2005).

II.2.1.3. Peroxygens

Peroxygens include hydrogen peroxide (HP) and peracetic acid (PA). Their degradation produces water and oxygen that are completely harmless (Al-Adham *et al.*, 2013; Eissa *et al.*, 2014; Fisher, 2003).

HP (H_2O_2) mode of action includes the production of hydroxyl free radicals that react with membrane lipids, DNA and proteins. However, HP can be inactivated by bacterial enzymes - catalases (Finnegan *et al.*, 2010; Wand, 2017). PA, as other oxidizing agents that generates hydroxyl radicals, is able to denature proteins, disrupt the bacterial wall affecting permeability and oxidizes sulphydryl and sulphur bonds of enzymes and proteins (Finnegan *et al.*, 2010; Wand, 2017).

II.2.1.4. Alcohols

Alcohols are organic compounds that contain one or more hydroxyl groups attached to a carbon chain. According to their structure alcohols can be divided into aliphatic alcohols (such as ethanol and isopropanol) and aromatic alcohols (such as benzyl alcohol and phenylethanol). Aliphatic alcohols are mainly used as antiseptics and disinfectants however, they can also be used as preservatives. On the other hand, aromatic alcohols are essentially used as preservatives (Al-Adham *et al.*, 2013). The mechanism of action of this type of biocides is thought to consist of membrane disruption as well as protein denaturation. Their activity is also increased in the presence of a certain percentages of water that makes the process of protein denaturation quicker (McDonnell *et al.*, 1999). Low concentrations of alcohols can be used as preservatives or to potentiate the activity of other biocides, such as CHX (McDonnell *et al.*, 1999; Wand, 2017)

In the case of ethanol, it induces a rapid release of intracellular components and disruption of the membrane by interacting with the hydrocarbon component of the phospholipid bilayer (Al-Adham *et al.*, 2013).

II.2.1.5. Aldehydes

Aldehydes are organic compounds that possesses a carbonyl group at the end of a carbon chain. Aldehydes that are considered important biocides are glutaraldehyde, formaldehyde and *ortho*-phthalaldehyde (OPA). Aldehydes mechanism of action comprises the reaction of various chemical groups associated with proteins and nucleic acids, resulting in the subsequent cross-linking of diverse macromolecules (Al-Adham *et al.*, 2013; McDonnell *et al.*, 1999). For instance, glutaraldehyde strongly associates with

the outer layers of the cell wall in particular with unprotonated amines causing the cross-linking of amino groups within proteins and consequently the transport into the cell is inhibited (Al-Adham *et al.*, 2013; Wand, 2017). Its reaction is pH dependent, increasing considerably between pH 4 to 9. Formaldehyde, as glutaraldehyde, is also sporicidal being able to penetrate the outer layers of the spore. This chemical is also able to inactivate bacteria by reacting with proteins amino and sulphydryl groups as well as of ring nitrogen atoms of purine bases (Wand, 2017). OPA binds to membrane receptors by cross-linkage influencing membrane functions which allows the chemical to enter by increasing permeabilization. It can also interact with reactive molecules such as RNA and DNA affecting microbial growth (Al-Adham *et al.*, 2013).

II.2.1.6. Phenolics

Phenol or phenol derivatives have one of the hydrogen atoms of the ring replaced by a functional group (alkyl, benzyl, halogen, phenyl) which in most of the cases results in improved biocide activity in comparison with phenol. At high concentrations phenolics penetrate and disrupt the cell wall and precipitate proteins. Low concentrations, however, induce a progressive leakage of intracellular constituents and inactivation of enzymes (Karsa, 2007).

Bisphenols are a group of chemical compounds with two hydrozyphenyl functions, being triclosan (TRI), a polychlorophenoxyphenol, one example of these compounds (Al-Adham *et al.*, 2013). TRI targets the fatty acid synthesis and, by competitive inhibition, inhibits the enzyme enoyl reductase FabI (Heath *et al.*, 1999). However, this process is slow, and the high antimicrobial activity of high concentrations of TRI hypothesize an additional mechanism of action. In addition, it has been shown that membrane integrity is compromised by the insertion of TRI into the cell membrane (Guillen *et al.*, 2004).

II.2.1.7. Halogen-Releasing Agents

Halogen-releasing agents include chlorine-releasing agents (sodium hypochlorite (SH), chlorine dioxide and sodium dichloroisocyanurate) and iodophors. Free chlorine mechanism of action is not fully understood, however, it has been reported the oxidation of sulphydryl enzymes and amino acids, chlorination of amino acids rings, inhibition of proteins synthesis, decrease on the uptake of nutrients and oxygen, oxidation of respiratory chain components and decrease on the production of ATP, as well as alteration

of DNA synthesis (Dukan *et al.*, 1996; Gray *et al.*, 2013; Mizozoe *et al.*, 2019; Virto *et al.*, 2005). Low pH increases activity of the type of biocides while the presence of organic matter can decrease its effectiveness (Wand, 2017). One important aspect on the use of biocides, such as chlorine based ones, is to consider the production of by-products upon their use (Jin *et al.*, 2015).

Iodophors are a combination of iodine and a solubilizing agent or carrier that can work as an iodine reservoir. Similarly, to chlorine, iodine mechanism of action includes rapid penetration into bacteria and reacts with cysteine and methionine amino acids, nucleotides and fatty acids, inhibiting their activity and synthesis (Al-Adham *et al.*, 2013). In terms of disinfection, iodophors are usually used as antiseptics (Wand, 2017).

II.2.1.8. Quaternary ammonium compounds

QACs are amphoteric surfactants widely used, such as benzalkonium chloride (BAC), cetylpyridinium chloride (CPC), cetyltrimethylammonium bromide (CTAB), didecyldimethylammonium bromide (DDAB) and tetraphenylphosphonium chloride (TPPCI), whose action is specific for certain species of bacteria depending on their hydrophobicity that is related to chain length of the n-alkyl chain. QACs with a C16 hydrophobic tail length are more active against Gram-negative in comparison with QACs with a shorter-chain, possibly due to a higher affinity of the C16 chain with the fatty acid portion of lipid A (Report, 1997; Wand, 2017). QACs activity is also concentration dependent, where low concentrations cause cellular leakage of potassium and hydrogen ions and loss of the ability for osmoregulation since the biocide binds to the anionic site on the surface of bacterial membrane (Figure II.4). At higher concentrations of QACs, bacteria are killed as a consequence of the solubilization of the cellular membrane and ultimately leads to a fast leakage of cell components (Buffet-Bataillon *et al.*, 2012; Ioannou *et al.*, 2007).

The charged form of CTAB (CTA^+) interacts with the negative charged bacteria by nonselective electrostatic interactions. CTAB can interfere with bacterial growth at low concentrations (Jin *et al.*, 2015). CTAB can generate superoxide and hydrogen peroxide and inhibit the regulatory gene *soxS* function and decreases MnSOD (manganese superoxide dismutase) activity leading to cell death (Nakata *et al.*, 2011).

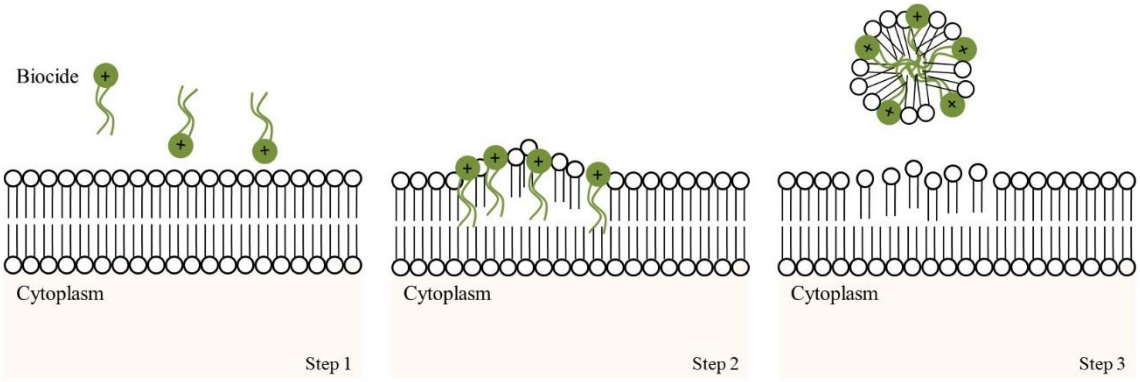


Figure II.4. Schematic representation of QAC mode of action. The positively charged biocide binds to the acidic phospholipids of the bacterial membrane. This destabilization leads to decrease in fluidity of the bilayers with consequent creation of hydrophilic voids in the membrane which ultimately promotes leakage of cell components. Figure adapted from Gilbert *et al.* (2005).

II.2.2. Factors that affect biocide efficacy

Biocides effectiveness is dependent on several factors, therefore, when choosing a biocide for a certain purpose, they need to be taken into consideration.

Biocides are commonly used as the final product or as an ingredient of a formulation. Their concentration is one important aspect for their antimicrobial activity, since it must be a balance of the efficacy (amount of microorganisms that it can destroy) and toxicity, both human and environmental (Maillard, 2005; Ribeiro *et al.*, 2018). Besides concentration, several other factors inherent to the biocide can contribute to decrease in their efficiency such as organic load (biocides inactivation), exposure time (manufactures guidelines should be followed since a shorter contact time can result in microbial survival), pH (affects biocides ionization and stability and microbial growth), temperature (some biocides can be inactivated by higher temperatures) and the formulation constituents (antagonistic effects could inactivate the efficacy of the biocide) (Maillard, 2005; Ribeiro *et al.*, 2018; Russell, 2004). In addition, biocides efficacy can also vary due to factors inherent to the microorganism, such as microbial contamination, type of microorganisms (intrinsic properties result in different levels of resistance) and concentration as well as presence of a biofilm (Maillard, 2005; Ribeiro *et al.*, 2018; Russell, 2004).

II.3. Bacterial resistance mechanisms to biocides

It is believed that the development of bacterial resistance or tolerance to biocides, is more difficult when compared with resistance to antibiotics since biocides are used in higher concentrations and also, their mechanisms of action comprise a multi target approach (Maillard, 2002).

Bacteria have different resistance mechanisms to biocides, in fact, they can decrease their concentration inside the cell (impermeability, degradation and modification of biocides and efflux pumps) as well as affect the targets (target modification and metabolism modification). Among this distinction the most important mechanisms of resistance are membrane impermeability, biocide efflux, enzymatic degradation and modification of the target site, represented in Figure II.5 (Ribeiro *et al.*, 2018). The presence of a biofilm is also an important factor that can affect biocides effectiveness (Smith *et al.*, 2008).

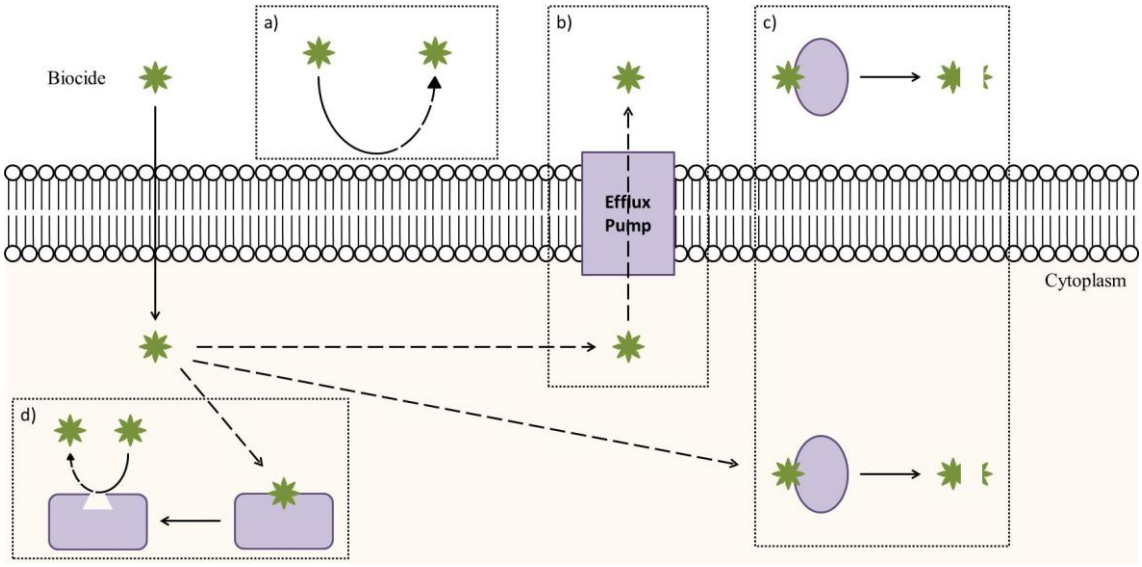


Figure II.5. Representation of the different mechanisms of resistance of the bacteria to biocides: a) membrane impermeability, b) biocide efflux, c) enzymatic degradation and d) modification of the target site. Figure adapted from Borges *et al.* (2016) and Wand (2017).

These mechanisms can be categorized as intrinsic to the bacteria or acquired (Borges *et al.*, 2016). Intrinsic resistance is defined as the innate ability of a bacterial species to resist the action of a biocide due to its inherent structural or functional characteristics. Several factors contribute to this resistance, such as impermeability of the cell wall witch difficult the access to inside the bacteria, production of enzymes that inactivate the biocide, the presence of active exporters that extrude the biocide and lack of affinity to the target site (Ribeiro *et al.*, 2018). Among these factors, production of specific enzymes and lack of affinity to the target site are the less common mechanisms of resistance towards biocides. Acquired resistance to biocides, however, is observed when bacteria develop increased tolerance, which can happen by mutation of existing genes, expression of silent genes or even acquisition of new genes by horizontal gene transfer on extrachromosomal elements (plasmids and transposons) (Bello *et al.*, 2016; Borges *et al.*, 2016). Contrarily to antibiotic resistance, biocide tolerance is rarely associated with gene acquisition.

In Figure II.6 it is possible to see the differences in the susceptibility of microorganisms to biocides. The mechanism of bacterial resistance is also different depending on the biocide to which the microorganism is resistant, Table II.1.

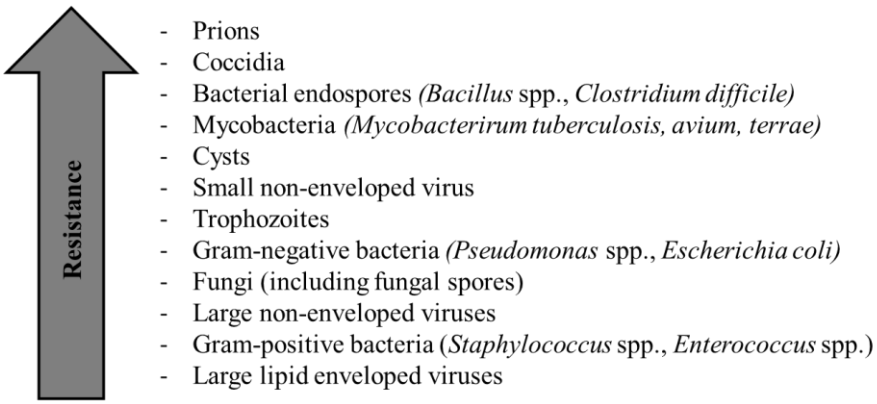


Figure II.6. Classification of microbial susceptibility to biocides (Maillard, 2005; Maillard, 2002; Russell, 1997).

Table II.1. Mechanisms of resistance that were identified on the bacterium that confers resistance to the most commonly used biocides

Biocide	Bacterium	Mechanism of resistance	Reference
Organic acids	Acetic acid bacteria *(such as Acetobacter and Komagataeibacter strains)*	Modification of membrane composition Enzymatic degradation Overexpression of efflux pumps Metabolism modification	Yang *et al.* (2019)
Biguanides	*K. pneumoniae, Pseudomonas stutzeri*	Modification of membrane composition Overexpression of efflux pumps Metabolism modification	Allen *et al.* (2006); Brooks *et al.* (2004); Fang *et al.* (2002); Kishk *et al.* (2014); Tattawasart *et al.* (2000)
Alcohol	*Enterococcus faecium, Rhodococcus erythropolis, Clostridium thermocellum, Bacillus subtilis, Bacillus psychrosaccharolyticus, Bacillus pallidus*	Modification of membrane composition Enzymatic degradation Overexpression of efflux pumps Metabolism modification on the carbohydrate uptake	Pidot *et al.* (2018); Torres *et al.* (2011)
Peroxygens	*Acetobacterium wieringae, Actinomyces naeslundii, Clostridium* sp., *Chromatium vinosum, E. coli*, bacteria from genus *Bacteroides, Porphyromonas, Desulfovibrio, Desulfotomaculum, Methanobrevibacter, Chromatium*	Enzymatic degradation	Brioukhanov *et al.* (2004)
Aldehydes	*E. coli, Mycobacterium chelonae*	Modification of cell wall polysaccharides Enzymatic degradation	Kummerle *et al.* (1996); Manzoor *et al.* (1999)
Phenolics	*A. baumannii, E. coli, P. aeruginosa, S. aureus, Salmonella Enteritidis, S. Typhimurium, Salmonella enterica serotype Virchow*	Inhibition of fatty acid synthase system Overexpression of efflux pumps Target site modification	Braoudaki *et al.* (2005); Chen *et al.* (2009); Heath *et al.* (1999); Huang *et al.* (2016); Webber *et al.* (2008); Yao *et al.* (2016); Zhu *et al.* (2010)
Halogen-Releasing agents	Gram-positive spore-forming bacilli, actinomycetes, and some micrococci	Membrane hydrophobicity Biofilm formation	Ding *et al.* (2019); Li *et al.* (2013); Ridgway *et al.* (1982)
QAC	*Burkholderia cepacia, E. coli, E. faecalis, Listeria monocytogenes, P. aeruginosa, S. aureus, S. intermedius, S. marcescens*	Membrane permeability modification Degradation of biocide Overexpression of efflux pumps	Ahn *et al.* (2016); Bjorland *et al.* (2003); Bjorland *et al.* (2001); Braoudaki *et al.* (2005); Chen *et al.* (2003); Guerin-Mechin *et al.* (2000); He *et al.* (2004); Hegstad *et al.* (2010)

II.3.1. Mechanisms of resistance that decrease biocides concentration inside the cell

II.3.1.1. Membrane Impermeability

Membrane impermeability is directly related to the cell envelop that surrounds bacteria which is a selective barrier that protect cells against compounds in the extracellular environment while allowing the entry of essential nutrients to the bacteria (Borges *et al.*, 2016; Fernandez *et al.*, 2012).

Generally, Gram-positive bacteria are highly susceptible to several biocides since their cell wall is essentially constituted by peptidoglycan and teichoic acid that is not an effective barrier (Araya-Cloutier *et al.*, 2018; Wand, 2017). However, spore-forming bacteria and mycobacteria have different sensitivities towards biocides when compared to most Gram-positive bacteria. Spores from *Clostridium* and *Bacillus* species make these bacteria extremely resistant to biocides. In fact, the main structural characteristics that provide this protection are the spore coat and the cortex that protect the target site (Russell, 1990; Young *et al.*, 2003;2004). Mycobacteria resistance is due to the complexity of their cell wall. In fact, it has a high concentration of high molecular weight lipids while the inner region has peptidoglycan linked to another polysaccharide polymer (arabinogalactan) (Lambert, 2002). Also, it also has a tick waxy coat that functions as permeability barrier, due to mycolic acids anchored to this structure (Brennan *et al.*, 1995).

Gram-negative bacteria in addition of having the cytoplasmic membrane have an outer membrane that consists of an inner layer of phospholipids and an outer layer of lipopolysaccharides (Fernandez *et al.*, 2012). Bacteria can decrease biocides concentration within the cell by the modification of cell surface composition and loss of entry channels, such as porins (Vila *et al.*, 2007).

II.3.2.2. Degradation and modification of biocides

Degradation and modification of biocides is based on the modification and/or destruction of the active component of the biocide, which can be achieved by enzymatic hydrolysis, chemical group transfer or redox processes (Blair *et al.*, 2015; Borges *et al.*, 2016). Catalases, superoxide dismutase, and alkyl hydroxyperoxidases are examples of enzymes that are able to inhibit biocides (Brioukhanov *et al.*, 2004; Maillard, 2005; Park *et al.*, 2008).

II.3.2.3. Efflux pumps

Efflux pumps are transport proteins located in the cytoplasmic membrane and are found both in Gram-negative and Gram-positive bacteria as well as in eukaryotic organisms. These pumps are able to remove toxic compounds, such as biocides and antibiotics, out of the bacterium without compromising the structure of the compound (Fernandez *et al.*, 2012). In addition, these pumps can be specific for one substrate or transport structurally dissimilar substances, being the second ones more common in multidrug resistance (MDR) bacteria (Borges *et al.*, 2016).

Efflux pumps are associated with both bacteria intrinsic and acquired resistance. In fact, the expression of genes that regulate this type of pumps can be induced by antimicrobials and can also be modulated by environmental conditions. The main mechanisms of MDR, include: amplification and mutation of genes that encode for efflux pumps, changing the expression level or activity; mutations in specific or global regulatory genes that result in overexpression of efflux pump genes; intercellular transfer of resistance genes on plasmids or transposons (Costa *et al.*, 2013a; Costa *et al.*, 2013b; Putman *et al.*, 2000).

Bacteria efflux systems (Figure II.7) that are able to extrude antimicrobials can be classified as: multidrug and toxic compounds extrusion family (MATE), major facilitator superfamily (MFS), small multidrug resistance family (SMR; part of drug/metabolite transporter (DMT) superfamily), resistance nodulation division family (RND), ATP (adenosine triphosphate) binding cassette family (ABC) and the more recently discovered, by Hassan *et al.* (2015), the proteobacterial antimicrobial compound efflux family (PACE) (Paulsen *et al.*, 1993; Poole, 2007; Putman *et al.*, 2000). MATE, MFS, SMR, RND and PACE efflux pumps are drug-proton antiporters, as they use the proton motive force (PMF) to extrude antimicrobials (Poole, 2002;2007; Vila *et al.*, 2007). PMF includes a chemical proton gradient (ΔpH, inside alkaline) and an electrical potential (ΔΨ, inside negative) that is needed to catalyse drug extrusion (Mitchell *et al.*, 1999; Ng *et al.*, 1994; Putman *et al.*, 2000). ABC efflux pumps are ATP-driven and, therefore, use ATP as energy source to extrude antimicrobials from the cytoplasm to the extracellular environment (Mitchell *et al.*, 1999; Ng *et al.*, 1994; Putman *et al.*, 2000). This type of efflux pumps are rarely involved in acquisition of resistance to antimicrobials in gram-negative bacteria (Vila *et al.*, 2007).

Efflux pumps transport can be antimicrobial specific (e.g. Tet pump, Mef exporter) or compatible to a range of chemically distinct antimicrobials (e.g. NorA pump).

Typically, genes encoding for antimicrobial specific pumps occur on mobile genetic elements such as transposons, integrons or plasmids, that when acquired confer resistance to the microorganism (Butaye *et al.*, 2003). On the other hand, multidrug efflux pumps are usually encoded by endogenous chromosomal genes where its expression contributes for bacterial intrinsic resistance or acquired in case of mutation (Poole, 2005;2007). In Table II.2 the most important efflux pumps of each family that confer resistance to biocides are presented.

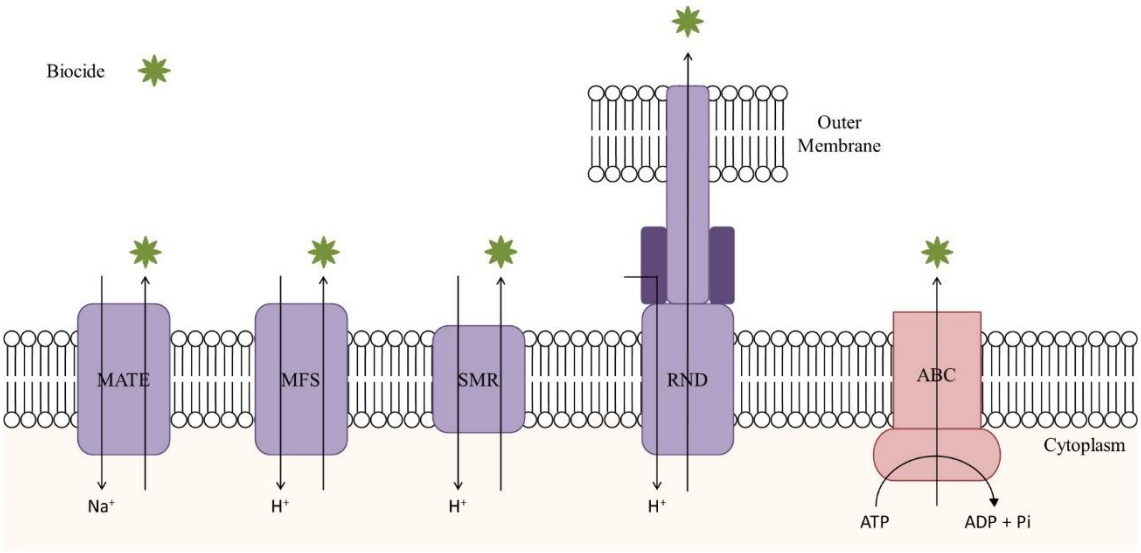


Figure II.7. Representation of the five families of multidrug resistance efflux pumps: multidrug and toxic compounds extrusion family (MATE), major facilitator superfamily (MFS), small multidrug resistance family (SMR), resistance nodulation division family (RND), proteobacterial antimicrobial compound efflux (PACE) and ATP (adenosine triphosphate) binding cassette family (ABC). Figure adapted from Putman *et al.* (2000).

Table II.2. Most important efflux pumps of each family found in microorganisms that confer resistance to biocides

Family	Efflux pump	Subtract specificity *	Bacterium	Reference
MATE	abeM (chromosomic)	TRI	*Acinetobacter baumannii*	Su *et al.* (2005)
	emmdR (chromosomic)	BAC	*Enterobacter cloacae*	Slipski *et al.* (2018)
	mepA (chromosomic)	CHX, QAC	*S. aureus*	Costa *et al.* (2013c)
	norM (chromosomic)	BAC	*Neisseria gonorrhoeae, Neisseria meningitidis, E. coli*	Poole (2007); Slipski *et al.* (2018)
	pmpM (chromosomic)	QAC, TRI, TPPCI, CHX	*P. aeruginosa*	He *et al.* (2004)
	vmrA (chromosomic)	TPPCI	Vibrio spp.	Chen *et al.* (2002)
MFS	emeA	QAC	*Enterococcus faecalis*	Rizzotti *et al.* (2016)
	emrB	CTAB	*Salmonella enterica subsp. Enterica* Typhimurium	Nishino *et al.* (2001); Slipski *et al.* (2018)
	emrD (chromosomic)	BAC	*E. coli*	Slipski *et al.* (2018)
	kpnGH	BAC, CHX, TRI	*Klebsiella pneumoniae*	Srinivasan *et al.* (2014)
	lmrS (chromosomic)	CTAB	*S. aureus*	Floyd *et al.* (2010)
	mdfA(chromosomic)	BAC	*E. coli*	Slipski *et al.* (2018)
	mdtM	QAC	*E. coli*	Kampf (2018)
	mdeA (chromosomic)	CHX, QAC	*S. aureus*	Costa *et al.* (2013c); Fernández Fuentes *et al.* (2014)
	norA (chromosomic)	CHX, CTM, QAC	*S. aureus*	Costa *et al.* (2013c)
	norB (chromosomic)	CHX, CTM, QAC	*S. aureus*	Costa *et al.* (2013c)
	norC (chromosomic)	CHX, CTM, CPC	*Pantoea ananatis*	Fernández Fuentes *et al.* (2014)
	qacA (plasmidic)	BG, QAC	*E. faecalis, S. aureus*	Costa *et al.* (2013c)

***** BAC – benzalkonium chloride, BG – biguanides, CHX – chlorhexidine, CPC - Cetylpyridinium chloride, CTAB - cetyltrimethylammonium bromide, CTM – cetrimide (a mixture of QACs), DDAB - didecyldimethylammonium bromide, PHN – phenolics, QAC – quaternary ammonium compounds, TPPCI – tetraphenylphosphonium chloride, TRI – triclosan.

Table II.2. Continue

Family	Efflux pump	Subtract specificity *	Bacterium	Reference
MFS	qacB (plasmidic)	QAC	*E. faecalis, S. aureus*	Costa *et al.* (2013c)
	smfY	BAC	*Serratia marcesens*	Slipski *et al.* (2018)
	smvA	CHX	*K. pneumoniae*	Slipski *et al.* (2018)
SMR	abeS (chromosomic)	CHX, BAC	*A. baumannii*	Srinivasan *et al.* (2009)
	cepA (plasmidic)	CHX	*K. pneumoniae*	Slipski *et al.* (2018)
	emrE	BAC, CHX	*E. coli, S. marcesens*	Nishino *et al.* (2001); Slipski *et al.* (2018)
	kpnEF	BAC, CHX, TRI	*K. pneumoniae*	Srinivasan *et al.* (2013)
	qacE (plasmidic	QAC	Widespread in Gram-negative	Fernández Fuentes *et al.* (2014)
	qacEΔ1 (plasmidic)	QAC	Widespread in Gram-negative, *S. aureus, E. faecalis*	Poole (2007)
	qacF (plasmidic)	QAC	*Enterobacter* spp., *P. aeruginosa*	Slipski *et al.* (2018)
	qacG (plasmidic)	QAC	*P. aeruginosa, S. aureus*	Costa *et al.* (2013c)
	qacH (plasmidic)	QAC	*S. aureus*	Costa *et al.* (2013c)
	qacJ (plasmidic)	QAC	*S. aureus*	Costa *et al.* (2013c)
	smr (plasmidic)	QAC	*S. aureus*	Costa *et al.* (2013c)
	sugE	CTAB, CTM	*E. coli*	Slipski *et al.* (2018)
RND	acrAB-TolC (chromosomic)	QAC, TRI	*E. coli*, *Salmonella enterica* serotype Typhimurium	Fernández Fuentes *et al.* (2014); Nishino *et al.* (2001); Poole (2007)
	acrEF-TolC	BAC	*E. coli*	Nishino *et al.* (2001)
	adeABC (chromosomic)	BAC, CHX	*A. baumannii*	Rajamohan *et al.* (2010)
	adeIJK	TRI	*A. baumannii*	Rajamohan *et al.* (2010)

* BAC – benzalkonium chloride, BG – biguanides, CHX – chlorhexidine, CPC - Cetylpyridinium chloride, CTAB - cetyltrimethylammonium bromide, CTM – cetrimide (a mixture of QACs), DDAB - didecyldimethylammonium bromide, PHN – phenolics, QAC – quaternary ammonium compounds, TPPCI – tetraphenylphosphonium chloride, TRI – triclosan.

Table II.2. Continue

Family	Efflux pump	Subtract specificity *	Bacterium	Reference
RND	cmeABC	TRI	*Campylobacter jejuni*	Mavri *et al.* (2012)
	cmeDEF	TRI	*C. jejuni*	Mavri *et al.* (2012)
	mexAB-OprM (chromosomic)	PHN, TRI	*P. aeruginosa, Pseudomonas azelaica*	Poole (2005)
	mexCD-OprJ (chromosomic)	BAC, CHX, TRI	*P. aeruginosa*	Poole (2005;2007)
	mexEF-OprN (chromosomic)	TRI	*P. aeruginosa*	Poole (2005)
	mexJK-OpmH	TRI	*P. aeruginosa*	Poole (2005)
	oqxAB (plasmidic)	BAC, CHX, CTM, TRI	*E. coli*	Hansen *et al.* (2007)
	sdeXY	TRI	*S. marcescens*	Poole (2005)
	smeDEF	TRI	*Stenotrophomonas maltophilia*	Poole (2005)
	triABC-OpmH (chromosomic)	TRI	*P. aeruginosa*	Ntreh *et al.* (2016)
	yhiUV-TolC	BAC	*E. coli*	Poole (2005)
PACE	aceI	CHX	*A. baumannii, E. coli, K. pneumoniae*	Slipski *et al.* (2018)
ABC	efrAB	CHX, TRI, QAC	*E. faecalis, S. aureus, Bacillus* spp.	Fernández Fuentes *et al.* (2014)
?	sepA (chromosomic)	BAC, CHX	*S. aureus*	Narui *et al.* (2002)

* BAC – benzalkonium chloride, BG – biguanides, CHX – chlorhexidine, CPC - Cetylpyridinium chloride, CTAB - cetyltrimethylammonium bromide, CTM – cetrimide (a mixture of QACs), DDAB - didecyldimethylammonium bromide, PHN – phenolics, QAC – quaternary ammonium compounds, TPPCI – tetraphenylphosphonium chloride, TRI – triclosan.

II.3.2. Mechanisms that affect biocides targets and bacteria metabolism

II.3.2.1. Target modification

Target modification consists on the alteration of the natural site of binding of the biocide to the cell (Costa *et al.*, 2013c). This modification can be the result of a spontaneous chromosomal mutation or homologous recombination with exogenous DNA that phenotypically results in a different target and, therefore, with different affinity for the biocide (Blair *et al.*, 2015). Considering biocide resistance mechanism, target modification is the less common since the majority of the biocides have multiple targets in their mechanism of action (Wand, 2017).

II.3.3.2. Metabolism modification

Several authors have reported an increased resistance to biocides in cells that are in a biofilm when compared to the planktonic state. Several mechanisms have been proposed to explain this increased resistance, such as restricted biocide penetration, reduced cell growth rate and metabolism, alterations in quorum sensing and induction of specific phenotypes associated with biofilms (usually known as persister cells) (Bridier *et al.*, 2011; Mah *et al.*, 2001; Patel, 2005; Stewart, 2015; Szomolay *et al.*, 2005). In addition, the rate of mutation and horizontal gene transfer is higher in bacteria that are growing in a biofilm which makes more likely the acquisition of resistance genes (Molin *et al.*, 2003).

A biofilm generally includes different species of bacteria or even multiple microorganisms that are adhered to a surface surrounded by an exopolysaccharide matrix. Biofilm diversity as well as cell density are important aspects that contribute to its higher resistance (Leriche *et al.*, 2003; Stewart, 2015). The biofilm matrix is another factor that explains biofilms resistance, since it can act as a diffusional barrier by diminish the concentration of biocide that reaches the cell or even by neutralizing some biocides. In addition, the nutrient and oxygen gradient that is created by the matrix is considered a microenvironment and it is also responsible for the reduction of cell metabolism (persister cells) (Mah *et al.*, 2001; Patel, 2005). These cells are characterized by their slow metabolism and high tolerance to antimicrobials without developing resistance (Lewis, 2008; Simões *et al.*, 2011).

II.3.3. Biocide resistance and cross-resistance to antibiotics

Bacterial resistance to biocides has been widely studied and documented *in vitro* as it is possible to observe from Table II.1, where some examples of bacterial resistance to biocides from different classes is depicted.

The increase use of biocides has led to an increasing concern, and speculation, on if the selective pressure that causes the development of resistance towards biocides can also lead to cross resistance to antibiotics (Cieplik *et al.*, 2019; Maillard, 2007). Considering that the mechanisms of a bacterium that confer biocide resistance, can also promote resistance to resist to antibiotics, may lead to assume the existence of a cross-resistance (Bock, 2019; Cieplik *et al.*, 2019). In fact, several biocide resistance genes are usually carried in plasmids, which also contain antibiotics resistance genes, supporting the possibility of biocide acquired resistance leading to antibiotic resistance (Bello *et al.*, 2016; Wand, 2017).

Studies that prove clinical cases of resistance to biocides that has also led to cross-resistance to antibiotics are scarce if not inexistent. In fact, these lack of data is a consequence of no consense on the methodologies that must be used as well as by the fact that cross-resistance can also occur by the development of resistance to antibiotics resistance that ultimately leads to biocide resistance (Bock, 2019). Nonetheless, some groups have explored the *in vitro* development of resistance to biocides and the consequent increase in antibiotic tolerance while others have showed that clinical isolates had increased resistance to biocides and antibiotics (Donaghy *et al.*, 2019; Henly *et al.*, 2019; Karmakar *et al.*, 2019; Oniciuc *et al.*, 2019; Paul *et al.*, 2019). The study performed by Schwaiger *et al.* (2014) has explored both hypothesis and observed that *E. faecalis* and *E. faecium* isolated from blood and feces of hospitalized humans, feces of outpatients and livestock, from food and the *in vitro* adapted strain, had an increased resistance to a QAC as well as high-level-aminoglycoside and aminopenicillin resistance. Also, Shepherd *et al.* (2018) has adapted clinical isolates to octenidine which resulted in increased resistance to chlorhexidine as well as to some of the antibiotics tested. On the contrary, Roedel *et al.* (2019) and Shirmohammadlou *et al.* (2018), had not found any correlation between biocide resistance and increased tolerance to antibiotics.

II.4. Combinatorial approach to enhance biocide efficiency

The fight against multidrug-resistant microorganisms has demanded a wide search for new antimicrobials as well as new strategies in order to overcome or, at least control, this problem. The search of new antimicrobials, for instance, has the advantage of including new mechanisms of action that will not face previously selected resistance determinants (Fischbach, 2011; Putman *et al.*, 2000). On the other hand, a combinatorial approach can be chosen, where already known antimicrobials can be rescued and combined with other chemicals, such as biocides. The main goal of this strategy is to obtain synergy, which means that their combined activity is higher than their individual ones (Fischbach, 2011; Pieren *et al.*, 2012). This approach has the advantage of avoiding the need of new molecules approval to be used for disinfection, since the idea is to combine antimicrobials that are already accepted under European Regulation, and are, therefore, commercially available biocides.

Combinatorial approach has already been extensively studied for clinical therapy with antibiotics, even by the combination of multiple antibiotics (Amison *et al.*, 2020; Basri *et al.*, 2012; Dundar *et al.*, 2010; Lee *et al.*, 2003; Leite *et al.*, 2015; Wang *et al.*, 2020). For instance, Ejim *et al.* (2011) demonstrated the synergistic activity of loperamide with cephalosporins and polymyxin B against MDR clinical strains of *P. aeruginosa*. Some combinations with antibiotics are, in fact, already approved for medical use, such as the use of clavulanic acid, sulbactam, tazobactam, avibactam and vaborbactam as β-lactamase inhibitors. Clavulanic acid, sulbactam, tazobactam were FDA (Food and Drug Administration) approved between 1984 and 1993 while tazobactam was accepted in 2014, avibactam in 2015 and vaborbactam in 2017 (Tehrani *et al.*, 2018).

Phytochemicals and plant derived products are among the most explored chemicals used to evaluate synergistic effects with antibiotics (Araujo *et al.*, 2020; Ayaz *et al.*, 2019; Kępa *et al.*, 2018; Langeveld *et al.*, 2014; Nayim *et al.*, 2018). The combination of biocides with plant derived products, despite auspicious, has been poorly explored. The few studies done so far are presented in Table II.3.

Table II.3. Biocide and phytochemical combinations tested and published online until May 2020

Biocide	Phytochemical	Microorganism	Action	Area of application	Observations	Reference
Organic acids						
Citric acid	Extract of: *Acer saccharum var. saccharum*	*Aspergillus niger* *Fusarium subglutinans* *Trichoderma viride*	Fungicidal	Wood Industry	Inner bark (IB) extract contained: 4-hydroxy-4-methyl-2-pentanone, palmitic acid, linoleic acid, caffeine, p-hydroxy benzoic acid. Outer bark (OB) extract contained: 1,2-benzenedicarboxylic acid, bis(2-ethylhexyl) ester, (Z,E)-9,12-tetradecadien-1-ol, cis-tetrahydro-6-methoxy-2Hpyran-3-ol, p-hydroxy benzoic acid, gallic acid, salicylic acid. IB 0.25% + OB 0.25% + CA (citric acid) 0.25% produced the highest antifungal effects against growth of *T. viride* with an inhibition percentage of 10.37 %, IB 0.5% + CA 0.5% (16.66%) with *F. subglutinans*, while CA 0.5% and OB 0.25%, inhibited 27.77% and 23.70% with *A. niger*, respectively.	Salem *et al.* (2019)
Citric acid Lactic acid	Essential oils of: Basil Cinnamon, Citronella, Clove, Lavender, Lemon, Orange Tea tree	*Candida albicans* *E. coli* *Methicillin-resistant Staphylococcus aureus* (MRSA) *P. aeruginosa* *S. aureus*	Bactericidal, fungicidal	Households and healthcare settings	All the essential oils were synergic with 1% of citric acid against *S. aureus* (2.3-6.2 log reduction). Basil, cinnamon, and citronella oil showed superior synergistic activity against all the bacteria tested (4.3-6.5 log reduction). A cinnamon oil/citric acid combination was highly effective against *Candida albicans (*4.5-5.4 log reduction). Their antimicrobial activity as soap was much higher (5.09-6.10 log reduction) than that of soap containing triclosan (0.27-0.75 log reduction).	Baiju *et al.* (2007)

Table II.5. Continue

Biocide	Phytochemical	Microorganism	Action	Area of application	Observations	Reference
Malic acid Lactic acid	Extract of grape seed	*E. coli*	Bactericidal	Food Industry	Malic acid/lactic acid and Malic acid/lactic acid/Extract inhibited *E. coli* on spinach (by 4.0 and 2.7 log CFU g^{-1}, respectively) and iceberg lettuce (2.5 and 2.8 log CFU g^{-1}, respectively). The antimicrobial action was improved over the days of storage and the colour of the produce was not compromised.	Ganesh *et al.* (2012)
Biguanide						
Chlorhexidine-gluconate	Extracts of: *Artemisia herba-alba* *Lavandula multifida*, *Origanum marjoram* *Rosmarinus officinalis* *Thymus capitatus*	*Staphylococcus epidermidis*	Bactericidal	Medical	Most abundant oxygenated terpenoid compounds: linalool in *L. multifida*, thymol in *T. capitatus*, camphor in *R. officinalis* and *A. herbaalba*, and 4-terpineol in *O. marjoram* *L. multifida* synergistically enhanced the anti-biofilm activity of chlorhexidine-gluconate	Alabdullatif *et al.* (2017)
	Extract of *Salvia officinalis* Manool (terpene) Salvigenin (flavonoid) Viridiflorol (terpene)	*Porphyromonas gingivalis*	Bactericidal	Medical	The combination was additive.	Mendes *et al.* (2020)

Table II.5. Continue

Biocide	Phytochemical	Microorganism	Action	Area of application	Observations	Reference
Chlorhexidine Polyhexamethylene biguanide	Baicalein (flavone) Baicalin (flavone glycoside) Oroxylin A (flavone) Wogoniside (flavone)	*Acanthamoeba castellani* *Acanthamoeba polyphaga*	Amoebicidal	Medical	Effectiveness of the solutions alone did not exceed 0.27 log reduction, but addition of combined baicalein and oroxylin A resulted in 0.92 and 0.64 log reductions of *A. castellani* and *A. polyphaga*, respectively.	Cho *et al.* (2016)
Polyaminopropyl biguanide	Cinnamon oil	*Coagulase-negative Staphylococci (CoNS)* *Enterococcus spp.* *Klebsiella pneumoniae* *Moraxella spp.* *Pseudomonas spp.* *S. aureus* *Streptococcus pneumoniae* *A. baumannii* *E. coli*	Bactericidal	Medical	Oil composition: cinnamic aldehydes, eugenol, cinnamyl acetate, linalool, benzyl benzoate, cymene. Formulation: hydroxyalkylphosphonate, poloxamine, polyaminopropyl biguanide (0.0001%), boric acid, disodium edetate, sodium borate, and sodium chloride. Time kill assay revealed that combination of cinnamon oil and the disinfectant successfully eradicated the tested microorganisms at all tested concentrations within 2 h contact time except for 0.312% concentration (3 h) versus 24 h for multipurpose contact lens disinfectant solution alone.	Bassyouni *et al.* (2016)
Peroxygens						
Hydrogen peroxide	Carvacrol (terpene) Thymol (terpene) β-resorcylic acid (dihydroxybenzoic acid)	*L. monocytogenes*	Bactericidal	Food Industry	The combinations decreased *L. monocytogenes* to undetectable levels by 5 min at 55, 65 °C, and 10 min at 25 °C and has also reduced transfer from cantaloupe surface to interior.	Upadhyay *et al.* (2014)

Table II.5. Continue

Biocide	Phytochemical	Microorganism	Action	Area of application	Observations	Reference
Peracetic acid	Essential oils of: *Lippia sidoides* *Pimenta pseudochariophyllus* *Thymus vulgaris*	*S. aureus*	Bactericidal	Food industry	*L. sidoides* oil contained: thymol, pcymene, β-caryophyllene, γ-terpinene. *P. pseudochariophyllus* oil contained: chavibetol, 1,8-cineole, eugenol, βpinene, p-cymene, α-pinene. *T. vulgaris* oil contained: thymol, p-cymene, γ-terpinene, carvacrol, linalool. Increased efficiency against biofilms.	Vázquez-Sánchez *et al.* (2018)
	Extract of *Quillaja saponaria*	*Asaia bogorensis* *Asaia lannensis*	Bactericidal	Beverage industry	The combination MIC was 4-8 times less than the individual value of the biocide. A synergistic effect was also observed against biofilms	Antolak *et al.* (2018)
	Extracts of: *Copaifera duckei* *C. oblongifolia* *C. reticulata*	*E. coli* *S. aureus* *S. epidermidis*	Bactericidal	Medical	*Copaifera* oleoresins and disinfectants did not act synergistically at any of the combinations tested.	Vieira *et al.* (2018)
Alcohols						
Ethanol	Extract of *Pulicaria undulata*	MRSA	Bactericidal	Medical	The combination increased the inhibition zone against MRSA.	Abed *et al.* (2019)

Table II.5. Continue

Biocide	Phytochemical	Microorganism	Action	Area of application	Observations	Reference
Halogen-releasing agents						
Povidone–iodine	Extract of cinnamon bark Cinnamic acid (phenolic acid)	*B. subtilis* *C. albicans* *E. coli* *E. faecalis* *K. pneumoniae* *P. aeruginosa* *P. mirabilis* *S. aureus* *S. pneumoniae* *Streptococcus pyogenes*	Bactericidal Fungicidal	Medical	Addition of povidone-iodine into cinnamic acid nanoparticles increases the bacterial growth control but does not make a difference for cinnamon bark extract. The compounds lose their antifungal activity and their activity towards *E. faecalis* when impregnated on sutures.	Edis *et al.* (2020)
Sodium hypochlorite	Extracts of: *Copaifera duckei* *C. oblongifolia* *C. reticulata*	*E. coli* *S. aureus* *S. epidermidis*	Bactericidal	Medical	*Copaifera* oleoresins and disinfectants did not act synergistically at any of the tested combinations.	Vieira *et al.* (2018)
QACs						
Benzalkonium chloride Cetrimide	Tiliroside (flavonoid)	*S. aureus*	Bactericidal	Medical	Tiliroside decreased up to 16 times the MIC.	Falco-Silva *et al.* (2009)
Dimethyldiethyl ammonium bromide	Extract of: *Quillaja saponaria*	*A. bogorensis* *A. lannensis*	Bactericidal	Beverage industry	The combination MIC was half in comparison with the MIC of the biocide. A synergistic effect was also observed against biofilms	Antolak *et al.* (2018)

II.5. Development of biocidal formulations for surface wiping

II.5.1. Disinfection

Biocides are used in difference areas, from households to industry and healthcare settings (Oniciuc *et al.*, 2019). In this context, they can be used for different applications such as sterilization of devices, disinfection of surfaces, skin antiseptics and water disinfection (Maillard, 2005).

In order to ensure a proper disinfection, official guidelines have been developed to aid on the categorization of situations, settings, surfaces and instruments, Table II.4 (Rutala *et al.*, 2008, updated 2019; World Health Organization and Pan American Health Organization, 2016). While in some surfaces the use of biocides is extremely important due to the risk of spreading healthcare associated infections (HAI) others may simply require cleaning, since they are rarely contaminated. Medical articles always require thorough cleaning with detergents and biocides (Maillard, 2005).

Table II.4. Categories of risk to patient and equipment in medical facilities (Rutala *et al.*, 2008, updated 2019; World Health Organization and Pan American Health Organization, 2016)

Item Category	Policies	Examples
Critical	High risk of infection to patients if contaminated Should be purchased as sterile or be sterilized	Surgical instruments, cardiac and urinary catheters, implants, and ultrasound probes used in sterile body cavities
Semi-critical	Contact with mucous membranes or nonintact skin Should be free from all microorganisms; however, small numbers of bacterial spores are permissible High-level disinfection using chemical disinfectants	Respiratory therapy and anaesthesia equipment, some endoscopes, laryngoscope blades, esophageal manometry probes, cystoscopes, anorectal manometry catheters, and diaphragm fitting rings
Non-critical	Contact with intact skin but not mucous membranes Noncritical items are divided into noncritical patient care items and noncritical environmental surfaces. The second ones can be a source of secondary transmission. Cleaning and decontamination with low level disinfectants	Non-critical patient-care items: bedpans, blood pressure cuffs, crutches and computers Non-critical environmental surfaces: bed rails, some food utensils, bedside tables, patient furniture and floors.

II.5.2. Surface disinfection

The effective use of biocides to prevent surface contamination and the consequent food spoilage or even infection of patients with HAI, is part of a multibarrier strategy. Regarding the food industry, due to consumers habits that include consumption of raw vegetables and undercooking to retain the natural taste and preserve heat-labile nutrients, the risk of foodborne transmission has increased in the recent years (Bintsis, 2018; Galié *et al.*, 2018). In fact, a study conducted for 2 years revealed a decrease in *Listeria* spp. in environmental samples (drains, non-food contact surfaces, employee contact surfaces and food contact surfaces) from 26.1% to 19.5% after implementing intervention strategies (Lappi *et al.*, 2004). Healthcare facilities have also reported patient's infection microorganisms on the surrounding surfaces. In a Hospital in Bangladesh where surface disinfection routine is poor, *K. pneumoniae* was detected on both respiratory swabs (32%, 33/104 patients) and on surfaces near patients positive for this organism (97%, 32/33 patients) (Hassan *et al.*, 2019).

Surfaces can get contaminated by microorganisms by contact with shoes, wheels, objects, produce and even spills (Bhatta *et al.*, 2018; Bintsis, 2018; Lavilla Lerma *et al.*, 2013; Russotto *et al.*, 2015; Todd *et al.*, 2009). Another source of surface contamination is the contamination of biocides solutions with resistant microorganisms as well as the use of the same solution to mop several surfaces. In this case, the increasingly dirty water can have a high microbial load if manufactures recommendations were not followed (Ferreira *et al.*, 2014; Galié *et al.*, 2018; Lavilla Lerma *et al.*, 2013).

The frequency of disinfection and the choice of biocide for surface disinfection should always be in accordance with the severity of the consequences if a contamination occurs (Rutala *et al.*, 2008, updated 2019; Tuladhar *et al.*, 2012). The number of reports highlighting the importance of surface disinfection is high, however, the opinion on the use of biocide formulations for noncritical surface disinfection are contradictory and, some report it as unnecessary (Bhatta *et al.*, 2018; Fraise, 2002;2013; Lei *et al.*, 2017; Russotto *et al.*, 2015; Rutala *et al.*, 2004). In Hospitals, the intensive use of biocides has also led to mixed opinions, while some are in favour of their use throughout hospitals, other alert that biocides should be used with restrictions (Maillard, 2005; Maillard, 2007). Therefore, in hospital settings, 3 levels of disinfection were stablished (high, intermediate and low) that takes into consideration the risk of microbial survival and transmission to patients (Rutala *et al.*, 2004; 2008, updated 2019).

II.5.2.1. Wipes

One approach that has gained a lot of interest and, in fact, is already being used in some healthcare facilities are the microfibers ready-to-use wipes. The main purpose of their use is to be able to remove contamination, both dirt and microbial, from surfaces (Gold *et al.*, 2013; Wesgate *et al.*, 2019). Additionally, some wipe products can provide antimicrobial activity by being impregnated with a biocide, however, when choosing a wipe several aspects must be taken into consideration to ensure efficacy, such as contact time, type of surface and contamination present (Song *et al.*, 2019; Tyan *et al.*, 2019).

Wipes can be categorised into two categories: detergent and disinfectant wipes. Detergent wipes are used for the cleaning of surfaces where dirt and microorganisms will be retained by the wipe and therefore will be removed from the surface. In consequence, the microorganism that prevail on the surfaces remain inactivated but available to be transferred to other surfaces and patients by the wipes or hands of staff (Ramm *et al.*, 2015). Disinfectant wipes can also have detergent on their composition. However, if no detergent is added, the surface needs to be properly cleaned before using the disinfectant wipe to avoid inactivation of the disinfectant. In this case, the wipes efficacy is dependent on several factors such as a) the ability of the wipe to remove visible dirt from surfaces, if the wipes are used in multiple surfaces transfer of microorganisms is a potential risk, b) the possibility of the wipe to leave a layer of liquid disinfectant after wiping and c) the disinfectant efficiency. This last factor is related to the activity of the biocide during the wiping and after the wiped surface dries. In this case, after drying all the activity should stop and no effect should be observed on future contaminations by microorganisms (Song *et al.*, 2019).

The most common disinfectants that are used to develop wipe formulations are alcohols, QACs, chlorine-based biocides and peroxygens. The efficacy of these chemicals can be compromised by the exposure times since, in a realistic scenario, it takes seconds, which for the majority of the standards contact time, may not be mandatory to test (Gold *et al.*, 2013; Panousi *et al.*, 2009; Song *et al.*, 2019; Tyan *et al.*, 2019; Wesgate *et al.*, 2019).

II.5.2.2. European Regulation

General biocide products and disinfectants are categorized in terms of area where they can be used: human hygiene biocidal products, private area and public health area disinfectants and other biocidal products, veterinary hygiene biocidal products, food and feed area disinfectants or drinking water disinfectants (Hopkins, 2013).

In 1998, the European Union has published a Regulation (Dir.98/8/EC, 2012) to control the biocides that are placed in circulation in the European Union which has been replaced by the Regulation (EU) 528/2012.

According to the Regulation (EU) 528/2012 a biocidal product is:

> "… any substance or mixture, in the form in which it is supplied to the user, consisting of, containing or generating one or more active substances, with the intention of destroying, deterring, rendering harmless, preventing the action of, or otherwise exerting a controlling effect on, any harmful organism by any means other than mere physical or mechanical action.
> … any substance or mixture, generated from substances or mixtures which do not themselves fall under the first indent, to be used with the intention of destroying, deterring, rendering harmless, preventing the action of, or otherwise exerting a controlling effect on, any harmful organism by any means other than mere physical or mechanical action."

Biocidal Products Regulation is responsible to stablish rules to control the availability on the market of these products as well as ensuring high level protection of human and animal health and the environment safety. In fact, Biocidal Products Regulation publish rules to establish the list of approved active substances, authorise the supply and use of the biocidal products and supply of "articles treated" with biocidal products (EC, 2020). In addition, the responsibility for administering the legislation falls on the European Chemicals Agency (ECHA) (Hopkins, 2013). Therefore, all biocidal products are subjected to approval which comprises testing in accordance with European standards.

The formation of European Committee for Standardization (CEN) of the Technical Committee CEN/TC 216 for chemical disinfectants and antiseptics, in the 1990`s, was the first step to the rationalisation of disinfection testing in Europe (Humphreys, 2011; Reybrouck, 1998). Next it was developed the disinfection standard tests, that are organised within a structured framework that considers their field of application such as medical, veterinary, food, industrial, domestic and institutional areas

(CEN, 2018). These tests are hierarchically applied in order to replicate a progressive increase in complexity and realism. **Phase 1** corresponds to suspension tests to assess the basic activity without regard of a specific area of application. **Phase 2**, step 1, is based also on suspension tests but under conditions that represents a practical use for a specific area of application, while step 2, are additional tests to simulate practical conditions (hand wash, hand rub, surface test). **Phase 3** corresponds to the field tests performed in realistic conditions (Humphreys, 2011; Reybrouck, 1998).

II.5.2.3. Mandatory Standard Tests

When considering the development of a formulation the aim is to achieve some important criteria such as fast-acting (< 5 min), ability to be effective with high levels of microbial contamination and organic contamination, be compatible with different materials (surfaces and equipment) and be safe to use. However, depending on the application field, some of these aspects can be adapted or even ignored (CEN, 2018; Humphreys, 2011).

The principle of using standard test methods is to be able to present data that is accurate, reproducible and to be as close to realistic conditions as possible, and ultimately to be commercialized if auspicious results are achieved (Humphreys, 2011).

Disinfection testing usually involves three categories, suspension testing, carrier tests and surface testing. Suspension tests are a simple quantitative approach that is based on mixing a test suspension with a specific volume of the test disinfectant for a specific contact time and temperature, in this case interfering substance can be added as representative of soiling. The main advantage of this test is to ensure a proper mixing between the test product and the suspension which ensures a good reproducibility of the test. Carrier test involves the contamination of surfaces by submersion that, after drying, are submersed in the test biocide for a specific contact time and temperature, after which the biocide is neutralized, the surviving microorganisms are counted, and reduction is calculated. The main advantage of this test is the number of surfaces that can be used per test, which increases the sensitivity. In the case of surface tests, it includes the contamination of a surface with a specific volume of a test suspension that after drying is putted under contact with a specific volume of the test disinfectant. After biocide neutralization the surviving microorganisms are counted and reduction is calculated (CEN, 2018; Humphreys, 2011).

The EN 14885:2015 is, in fact, the European Standard that specifies the laboratory methods that should be used for testing the activity of chemical disinfectants and antiseptics that will support claims of specific properties depending on their intended application. Considering this European Standard, it is possible to claim biocidal activity of vegetative bacteria (including mycobacteria and *Legionella* spp.), bacterial spores, yeasts, fungal spores and viruses (including bacteriophages) for products to be used in human medicine, veterinary area and in food, industrial, domestic and institutional areas.

The European Standards needed to be used in order to claim Phase 2 bactericidal activity of a certain biocidal product for surface disinfection in the food, industrial, domestic and institutional areas or medical area are listed in Table II.5. Additional testes were also included to englobe wiping tests, since to date their availability is scarce (CEN, 2018).

Table II.5. Standard testes used to evaluate bactericidal activity of a product in suspension, in a surface and for in-wipe purposes to be used in food, industrial, domestic, institutional and medical areas in accordance with EN 144885:2015 (CEN, 2009;2015b;a;2018;2019). Only the obligatory test conditions are presented.

Current available test	Microorganisms	Contact time, Temperature, Interfering Substances	Principle	Possible claims
Suspension test	(phase 2 step 1)			
EN 1276 *Food, industrial, domestic and institutional areas*	*P. aeruginosa* *E. coli* *S. aureus* *E. hirae*	5 min; 20 °C; CC: 0.3 g L^{-1} BSA DC: 3 g L^{-1} BSA	A sample of the product as delivered or diluted is added to the test suspension of bacteria in a solution of an interfering substance. The mixture is maintained at the appropriate contact time and temperature until an aliquot is taken and immediately neutralized by a validated method. The number of surviving bacteria is determined for each sample and the logarithmic reduction is calculated.	The product passes the test if it reduces at least 5 $\log_{10}$ within 5 min at 20 °C in the presence of interfering substance.
EN 13727+A2 *Medical area*	*P. aeruginosa* *S. aureus* *E. hirae*	Until 5 min for surfaces in contact with patients or medical staff or until 60 min for other surfaces 4 - 30 °C CC: 0.3 g L^{-1} BSA DC: 3 g L^{-1} BSA and 3 mL L^{-1} sheep erythrocytes	A sample of the product as delivered or diluted is added to the test suspension of bacteria in a solution of an interfering substance. The mixture is maintained at the appropriate contact time and temperature until an aliquot is taken and immediately neutralized by a validated method. The numbers of surviving bacteria are determined for each sample and the logarithmic reduction is calculated.	The product passes the test if it reduces at least 5 $\log_{10}$ within 5 min and between 4-30 °C in the presence of interfering substance.

CC – Clean Conditions; DC – Dirty Conditions; BSA – Bovine albumin

Table II.5. Continue

Current available test	Microorganisms	Contact time, Temperature, Interfering Substances	Principle	Possible claims
Surface test	(phase 2 step 2)			
EN 13697 *Food, industrial, domestic and institutional areas*	*E. hirae* *E. coli* *P. aeruginosa* *S. aureus*	5 min; 20 °C; CC: 0.3 g L^{-1} BSA DC: 3 g L^{-1} BSA	A bacterial suspension in a solution of interfering substance is inoculated onto a surface of stainless steel and allowed to dry. A sample of the product to be tested is applied uniformly to cover the dried film. The surface is maintained at the appropriate contact time and temperature. The surface is transferred to a validated neutralization medium and the number of surviving bacteria is determined. Another surface treated with hard water in place of the disinfectant is used as control and the bacteria recovered attributed to the product is calculated by difference.	The product passes the test if it reduces at least 4 $\log_{10}$ reduction within 5 min at 20 °C (or between 4-30 °C in medical area) in the presence of interfering substance of a contaminated disc.
Medical area		Until 5 min for surfaces in contact with patients or medical staff or until 60 min for other surfaces 4 - 30 °C CC: 0.3 g L^{-1} BSA DC: 3 g L^{-1} BSA and 3 mL L^{-1} sheep erythrocytes		

CC – Clean Conditions; DC – Dirty Conditions; BSA – Bovine albumin

Table II.5. Continue

Current available test	Microorganisms	Contact time, Temperature, Interfering Substances	Principle	Possible claims
Wipe test				
EN 16615 *Four-field test*	*S. aureus* *E. hirae* *P. aeruginosa.*	Recommended by the manufacturer with a maximum of 60 min. The minimum is 1 min. between 4 °C and 30 °C CC: 0.3 g L^{-1} BSA DC: 3 g L^{-1} BSA and 3 mL L^{-1} sheep erythrocytes	A test surface is market with 4 squares in a row of 5 × 5 cm that are considered the test fields. Test field 1 is inoculated with a bacterial test suspension in a solution of interfering substances and is allowed to dry. A wipe is soaked with the product as delivered or diluted and wrapped around a block. The test surface is wiped across the market fields, starting on the test field 1, turning after the test field 4 and wiped back to test surface 1. A control must also be performed by using water instead of the product. Contact time, temperature and interfering substances should be in accordance with the manufacturer. The test organisms are recovered with moistened cotton swabs which are putted in a tube containing broth and neutralizer and the bacteria are recovered by shaking. The surviving bacteria on each field is determined and the reduction is calculated in comparison with the drying control and the results from the product. Another test where the product is replaced by water should be performed as a control of the spread of the microorganisms though the 4 fields. This European Standard includes also "ready-to-use wipes" that are impregnated with a biocidal solution. In addition, it also applies to areas and situations where disinfection is medically indicated and may occur in the workplace and in the home. It may also include services such as laundries and kitchens supplying products directly for the patients.	The product passes the test if a 5 $\log_{10}$ reduction for *S. aureus*, *E. hirae*, *P. aeruginosa* on test field 1; and an average of equal or less than 50 cfu on test fields 2 to 4 for each test organism.

CC – Clean Conditions; DC – Dirty Conditions; BSA – Bovine albumin

Table II.5. Continue

Current available test	Microorganisms	Contact time, Temperature, Interfering Substances	Principle	Possible claims
Non-standard wipe test				
E2967 − 15 *Wiperator* (ASTM, 2016)	*S. aureus*, *A. baumannii*	10 s, however it can be increased in 5s increments to a maximum of 45s Room temperature: 22 °C CC: 0.3 g L^{-1} BSA DC: 3 g L^{-1} BSA	The Wiperator is designed to simulate the orbital action of wiping with pre-soaked or "ready-to-use" wipes. The pressure, the duration of wiping and the number of wiping strokes can be previously set for greater precision and reproducibility. With Wiperator, transference of microorganisms can also be tested by wiping a clean surface with a used wipe. The surfaces used are stainless steel discs to represent non-porous environmental surfaces. The disc is inoculated with a bacterial test suspension in a solution of interfering substances and is allowed to dry. The disc is placed on the carrier platform of the Wiperator. A 4 cm^2 wipe is cut and mounted on the boss to be placed on the spindle. The platform is then raised to contact the wipe and activate wiping. To test microbial transference a clean disc is used right after wiping the inoculated disc. The discs are transferred to a validated neutralization medium and the number of surviving bacteria is determined. Another surface treated with hard water in place of the product is used as control and the bacteria recovered attributed to the product is calculated by difference.	The product reduces X $\log_{10}$ within a certain time at room temperature in the presence of interfering substance of a contaminated disc.

CC – Clean Conditions; DC – Dirty Conditions; BSA – Bovine albumin

Table II.5. Continue

Current available test	Microorganisms	Contact time, Temperature, Interfering Substances	Principle	Possible claims
Three-step protocol (Williams *et al.*, 2007)	Methicillin-resistant *S. aureus* (MRSA) Methicillin susceptible (MSSA) *S. aureus*	10 s Room temperature: 22 °C CC: 0.3 g L^{-1} BSA DC: 3 g L^{-1} BSA	The methodology allows to access the wiping efficiency of the product or "ready-to-use" wipe (step 1), bacterial transference to clean surfaces by a used wipe (step 2) and the antibacterial activity of the product the wipe is soaked on (step 3). Step 1: The disc is inoculated with a bacterial test suspension in a solution of interfering substances and is allowed to dry. The disc is fixed on a petri dish. A wipe (4 cm diameter) is cut and mounted on the steel rod that is putted in contact with the disc and the rod is mechanically rotated against the surfaces for 10 s at 60 rpm, exerting a weight of 100 ± 5 g. The discs are transferred to a validated neutralization medium and the number of surviving bacteria is determined. Step 2: Bacterial transfer from wipes was assessed by eight consecutive mechanical pressure transfers (100 ± 5 g) to neutraliser plates. Growth was evaluated qualitatively. Step 3: Measurement of antimicrobial activity by direct inoculation of the wipes with a bacterial suspension in a solution of interfering substances for 10 s and the wipe was immediately neutralised. A control test with hard water in place of the product is used in every step and the bacteria recovered attributed to the product is calculated by difference.	The product reduces X $\log_{10}$ within a certain time at room temperature in the presence of interfering substance of a contaminated disc.

CC – Clean Conditions; DC – Dirty Conditions; BSA – Bovine albumin

Chapter III. Materials and Methods

1. Materials and conditions
2. Methods

III.1. Materials and conditions

III.1.1 Chemicals and solutions

All the biocides as well as the phytochemicals and their derivatives used in this study are presented on Table III.1 and Table III.2, where the correspondent brand, CAS number and price are discriminated. Ethidium bromide (EB) was purchased as powder from Sigma (Madrid, Spain) and a stock solution of 10 g l^{-1} (deionized ultrapure water) was used to prepare all the dilutions). Lysostaphin, thioridazine, verapamil, reserpine and bovine serum albumin (BSA) were also purchased from Sigma. Ethylenediamine tetraacetic acid disodium salt (EDTA) was acquired from Panreac. Solutions of thioridazine and verapamil were prepared in deionized water and reserpine in dimethyl sulfoxide (DMSO). BSA and EDTA solutions were prepared with sterile distilled water. High-performance liquid chromatography (HPLC) quality water by Mili-Q water purification system was used. Acetonitrile was supplied by Carlo Erba (Val de Reuil Cedex, France). Lecithin, polysorbate 80, thiosulphate, saponin, isopropanol and DMSO were obtained from VWR chemicals. L-histidin was purchased from Merck. All reagents were of analytical grade. Phytochemicals and derivatives solutions were prepared using DMSO. HP, SH, CTAB, PA and LA were prepared using sterile distilled water whereas CHX, TRI and OPA were prepared using absolute ethanol. The biocide and phytochemical neutralization step was performed in Chapter IV using dilution to sub-inhibitory concentrations according to (Johnston *et al.*, 2002). Neutralization steps in Chapters V, VI, VIII was performed using the universal neutralizer (lecithin 3g L^{-1}, polysorbate 80 30 g L^{-1}, thiosulphate 5 g L^{-1}, L-histidine 1 g L^{-1}, saponin 30 g L^{-1} in 1% phosphate buffer 0.25 M pH 7.2) for 10 min (CEN, 2009).

Table III.1. Technical information of the biocides used in this study. [a] The price per 1 g of product corresponds to the price of the chemicals when purchased by the research group in 2014/2015

Biocide	Brand	CAS number	Price 1 g [a](€)
Cetyltrimethylammonium bromide (CTAB)	Acros Organics	57-09-0	0.26
Chlorhexidine (CHX)	Sigma Aldrich	55-56-1	88.80
Hydrogen peroxide (HP)	Merck	7722-84-1	0.07
Lactic acid (LA)	Fluka	50-21-5	0,14
o-Phthalaldehyde (OPA)	Sigma Aldrich	643-79-8	18.40
Peracetic acid (PA)	Sigma Aldrich	79-21-0	5.94
Sodium hypochlorite (SH)	Acros Organics	7681-52-9	0.03
Triclosan (TRI)	Sigma Aldrich	3380-34-5	3.94

Table III.2. Technical information of the phytochemicals and derivatives used in this study. [a] The price per 1 g of product corresponds to the price of the chemicals when purchased by the research group in 2014/2015

Phytochemical/derivative	Brand	CAS number	Price 1 g [a](€)
3,4-(Methylenedioxy)cinnamic acid	Alfa Aesar	2373-80-0	38.67
4-(Dimethylamino)cinnamic acid	Acros Organics	1552-96-1	46.30
4-Chlorocinnamic acid	Alfa Aesar	1615-02-7	46.92
4-Methoxycinnamic acid	Alfa Aesar	943-89-5	15.38
4-Nitrocinnamic acid	Merck	882-06-4	40.4
Allyl cinnamate	Sigma Aldrich	1866-31-5	0.49
Caffeic acid	Sigma Aldrich	331-39-5	3.80
Cinnamaldehyde	Sigma Aldrich	14371-10-9	0.05
Cinnamamide	Alfa Aesar	621-79-4	7.22
Cinnamic acid	Merck	140-10-3	4.86
Cinnamyl alcohol	Acros Organics	104-54-1	0.15
Coumaric acid	Sigma Aldrich	501-98-4	3.84
Eugenol	Sigma Aldrich	97-53-0	0.23
Ferulic acid	Sigma Aldrich	537-98-4	3.78
Hydrocinnamic acid	Acros Organics	501-52-0	0.31
Methyl trans-cinnamate	Merck	1754-62-7	0.12
Phenylacetone	Acros Organics	103-79-7	0.21
trans-4-(Trifluoromethyl)cinnamic acid	Alfa Aesar	16642-92-5	89.25
Tyrosol	Sigma Aldrich	501-94-0	14.94
α-Fluorocinnamic acid	Sigma Aldrich	350-90-3	91.90
α-Methylcinnamic acid	Acros Organics	1199-77-5	2.64
α-Methylhydrocinnamic acid	Acros Organics	1009-67-2	12.02

III.1.2 Microorganisms and culture conditions

Test suspensions of *E. coli* CECT 434, *E. coli* NCTC 10418, *S. aureus* CECT 976, *S. aureus* NCTC 10788, *S. aureus* SA1199b, *S. aureus* RN4220:pUL5054, *S. aureus* XU212, *S. aureus* SM39, *S. aureus* SM52, *S. aureus* ATCC 25923and *S. aureus* ATCC 25923_EB and *E. hirae* NCTC 13383 were used in this study. The bacteria used in the study were obtained from overnight cultures in 100 mL flasks with 25 mL of Mueller-Hinton broth (MHB; Merck, Germany) (Chapter IV) or MHB prepared in phosphate buffer (0.02 M pH 7; PB pH7) (Chapter V, VI, VII, VIII) incubated at 30 °C and under 150 rpm agitation on an orbital shaker (25 mm of orbital radius Agitorb 200ICP, *Appendix A.1.*). *E. coli* CECT 434 and *S. aureus* CECT 976 were selected as they are reference strains for the methods used as guidelines from the Clinical & Laboratory Standards Institute (CLSI, 2012). *S. aureus* NCTC 10788, *E. coli* NCTC 10418 and *E. hirae* NCTC 13383 were selected for this study taking into consideration the model bacteria used on "Chemical disinfectants and antiseptics"(CEN, 2009). *S. aureus* NCTC 10788, *S. aureus*

SA1199b, *S. aureus* RN4220:pUL5054, *S. aureus* XU212, *S. aureus* SM39, *S. aureus* SM52, *S. aureus* ATCC 25923and *S. aureus* ATCC 25923_EB and were selected as they overexpress specific efflux pumps, essential for efflux pumps assays (Chapter VII). *S. aureus* SM39, *S. aureus* SM52, *S. aureus* ATCC 25923 and *S. aureus* ATCC 25923_EB belong to the Grupo de Micobactérias, Unidade de Microbiologia Médica, Instituto de Higiene e Medicina Tropical (IHMT/UNL).

III. 2. Methods

III.2.1 Antibacterial susceptibility testing

The MIC of each biocide, phytochemical or derivative was determined by the microdilution method according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2012). Bacteria from an overnight culture (≈16 hours) were adjusted to a density of 10^7 colony forming units (CFU) per mL with fresh culture medium. Wide ranges of concentrations were selected in order to ensure that MIC/MBC could be determined without compromising solubility (phytochemicals and derivatives were tested in a range of concentrations from 1 to 25 mM with exception for 4-nitrocinnamic acid that was tested from 1 to 13 mM due to its lack of solubility in DMSO). A maximum volume of 200 µL $well^{-1}$ was used in 96-well microtiter plates, containing the bacterial test suspension in growth medium and the different concentrations of the chemicals (10 % v v^{-1} for Chapter IV or 5 % v v^{-} Chapter V, VI, VII, VIII (*Appendix A.2.*)). Bacterial growth was measured at 600 nm using a microplate reader (Spectramax M2e, Molecular Devices, Inc.). MIC was determined as the lowest concentration that inhibited microbial growth after 24 h of growth at 30 °C and under 150 rpm agitation (Ferreira *et al.*, 2011). To determine the MBC, 180 µL were removed from each well and an equal amount of neutralizer was added to the well and neutralization was allowed to occur for 10 min (this step was not performed for Chapter IV; Table III.3; A). A volume of 10 µL of each well was plated in plate count agar (PCA, Merck, Germany) and incubated overnight at 30 ± 3°C. The MBC was considered the lowest concentration of chemical were no growth was detected on solid medium (Ferreira *et al.*, 2011). Three independent experiments were performed for each chemical and bacterium.

Table III.3. Methodologies followed along the experimental work of this thesis (Chapters IV, V, VI, VII and VIII)

	Condition	Chapter IV	Chapter V	Chapter VI	Chapter VII	Chapter VIII
A	Neutralization	No	Yes	Yes	NA	Yes
B	Temperature	30 ± 3°C	25 ± 3°C	25 ± 3°C	37 ± 3°C	25 ± 3°C
C	Washing	NaCl (8.5 g L^{-1})	PB pH 7	PB pH 7	PB pH 7	PB pH 7
D	Biocides concentration	10 mM (10% v v^{-1} in NaCl 8.5 g L^{-1})	5 mM with exception of CHX at 0.1 mM (5% v v^{-1} in PB pH 7)	Bacteria were exposed to the phytochemicals and derivatives, LA and CTAB individually and in combination according to the concentrations on Table VI.1 (2.5% v v^{-1} of each in PB pH 7)		CTAB tested from 0.005 to 1 mM (2.5% v v^{-1} in PB pH 7)
E	Phytochemicals or derivatives concentration	10 mM (10% v v^{-1} in NaCl 8.5 g L^{-1})	5 mM (5% v v^{-1} in PB pH 7)		Bacteria were exposed to the phytochemicals and derivatives, accordingly to the concentrations on Table VI.1 (2.5% v v^{-1} of each in PB pH7)	Cinnamaldehyde 0.5, 1 and 2 mM; α-methylhydrocinnamic acid at 5 mM (2.5% v v^{-1} in PB pH7)
F	Contact time	1 hour	30 minutes	30 minutes	10 or 60 minutes	3 or 5 minutes
G	Solution for resuspension	NaCl (8.5 g L^{-1})	PB pH7	PB pH 7	NA	Neutralizer

NA – Not applicable in this condition

III.2.2 Efficacy against early sessile cells

Bacterial suspensions (~10^8 CFU mL^{-1}) were dispersed into 96-well polystyrene plates (200 µL $well^{-1}$) and their adhesion to the surface was measured following the protocol described by Simões *et al.* (2007) in which an adhesion period occurred for 2 h at a constant temperature (Table III.3; B) under agitation at 150 rpm in the absence of phytochemicals, derivatives or biocides. After the adhesion period non-adhered bacteria were discarded by washing (Table III.3; C) the plates prior to exposure to biocides or phytochemicals. Biocides and phytochemicals were tested according to the concentration (D,E), temperature (B) and contact time (F) presented in Table III.3 and under agitation (150 rpm). Chemicals were then removed by pipetting and washed one time (to reduce the concentration to sub-inhibitory levels (Johnston *et al.*, 2002)) or 200 µL of neutralizer was added to the wells for 10 min, followed by another washing step (Table III.3; F). Sessile cells were scraped with a pipette tip for 1 minute (*Appendix A.3.*), re-suspended (Table III.3; G) and their viability was assessed after plating on Mueller-Hinton Agar (MHA, Merck, Portugal). CFU were determined after 24 h at 30 °C incubation and presented as $\log_{10}$ CFU cm^{-2}. Three independent experiments were performed for each condition tested.

III.2.3 Physicochemical characterization of bacterial surfaces

The physicochemical properties of bacteria surfaces were assessed by the sessile drop contact angle measurement on bacterial lawns as described by Busscher *et al.* (1984). Contact angles were determined using an OCA 15 Plus (DATAPHYSICS) video-based optical measuring instrument, allowing image acquisition and data analysis. Measurements (≥ 15 per liquid and chemical) were performed according to Simões *et al.* (2007) after bacteria incubation (Table III.3; F) with the biocides or phytochemicals (Table III.3; D,E). The liquid's surface tension components reference values were obtained from the literature (Janczuk *et al.*, 1993). Hydrophobicity was assessed after contact angle measurement following the van Oss approach (van Oss *et al.*, 1987; 1988; 1989). The degree of hydrophobicity of a given surface (s) is expressed as the free energy of interaction between two entities of that surface when immersed in water (w)$-(\Delta G_{sws} - \mathrm{mJ\ cm^{-2}})$. The surface is considered hydrophobic if the interaction between two entities is stronger than the interaction of each with water ($\Delta G_{sws} < 0$). On the other hand, if $\Delta G_{sws} > 0$ the material is considered hydrophilic. ΔG_{sws} can be

calculated using the surface tension components of the interacting entities by the following equation 1,

$$\Delta G_{sws} = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 - 4\left(\sqrt{\gamma_s^+\gamma_s^-} + \sqrt{\gamma_w^+\gamma_w^-} - \sqrt{\gamma_s^+\gamma_w^-} - \sqrt{\gamma_s^-\gamma_w^+}\right) \quad (1)$$

γ^{LW} represents the Lifshitz-van der Waals component of the free energy of the surface and γ^+ and γ^- are the electron acceptor and donor parameters, respectively, of the Lewis acid-based component (γ^{AB}), where $\gamma^{AB} = 2\sqrt{\gamma^+\gamma^-}$. The surface tension components of a solid material have been obtained by measuring the contact angles of three liquids with different polarities and known surface tension components (1): α-bromonaphthalene (apolar), formamide (polar), and water (polar). Upon obtaining the data, the following equations 2 and 3 can be solved,

$$(1+\cos\theta)\gamma_w^{Tot} = 2\left(\sqrt{\gamma_s^{LW}\gamma_w^{LW}} + \sqrt{\gamma_s^+\gamma_w^-} + \sqrt{\gamma_s^-\gamma_w^+}\right); \quad (2)$$

θ is the contact angle and

$$\gamma^{Tot} = \gamma^{LW} + \gamma^{AB} \quad (3)$$

III.2.4 Bacterial growth evaluation

A bacterial suspension from an overnight culture was adjusted to an optical density of 0.04 at 600 nm (3.05×10^7 CFU mL^{-1} for *E. coli* NCTC 10418, 4.48×10^7 CFU mL^{-1} for *S. aureus* NCTC 10788 and 1.60×10^7 CFU mL^{-1} for *E. hirae* NCTC 13383), in fresh Mueller-Hinton Broth prepared in PB pH7. In a 96-well microtiter plate, 190 µl of bacterial suspension were added to 10 µl (5 % v v^{-1} of well volume) of the phytochemical, derivative or biocide at 5 mM concentration in the well with the exception of CHX which was tested at 0.1 mM, because of the limited solubility of CHX in ethanol. The bacterial growth was measured at 600 nm using a microplate reader (Spectramax M2e, Molecular Devices, Inc.) for 24 h at 30 ± 3 ºC, without continuous agitation. During the first 4 h the optical density was read every 15 min with prior orbital shaking of 700 rpm for 5 seconds, and for the next 20 hours readings were performed every 30 min with prior shaking. The software GrowthRates 2.1 (Bellingham Research Institute) was used to calculate the lag phase, growth rate and doubling time (Hall *et al.*, 2014; Jung *et al.*, 2015). Three independent experiments were performed for each chemical and bacterium.

III.2.5 Determination of quorum sensing inhibition using *Chromobacterium violaceum*

Quorum sensing inhibition (QSI) was determined by modifying the methodology used by Borges *et al.* (2014a). Briefly, *C. violaceum* ATCC 12472 was grown overnight in Luria-Bertani broth (LB, Liofilchem, Italy) prepared in PB pH7 at 30 ± 3°C, under 150 rpm of agitation. In order to determine the QSI concentration for each chemical the bacterium was exposed to each chemical at different concentrations (5% v v^{-1}) in a 96-well plate and it was incubated at 30 °C and under 150 rpm for 24 h. The QSI concentration was considered the lowest concentration that was able to diminish or inhibit the purple pigment without inhibition of growth in the 96-well plates (Adonizio *et al.*, 2006; McLean *et al.*, 2004). Three independent experiments were performed for each chemical.

III.2.6. Bacterial susceptibility by the checkerboard methodology

The checkerboard assay was performed accordingly to Abreu *et al.* (2014) and Chan *et al.* (2011), with some modifications. Bacterial suspensions (10^7 CFU mL^{-1}) were prepared in fresh MHB diluted in PB pH 7. The assay was performed in a 96-well plate that was filled using electronic pipettes and a pipette robot (VIAFLO ASSIST together with INTEGRA's VIAFLO electronic pipettes) to increase reproducibility. Phytochemical solutions (2.5% v v^{-1}) were added to a 96-well plate followed by the bacterial suspension and biocide solutions (2.5% v v^{-1}) in a total of 200 µL. Phytochemicals were tested in a range of 0 to 25 mM along the *y* axis (rows) while CTAB was tested in the range of 0 to 0.1 mM and LA from 0 to 300 mM along the *x* axis (columns). Plates were incubated for 24 h at 30 °C under 150 rpm of orbital agitation. The optical density at 600 nm was read before and after incubation. The MIC of each biocide, phytochemical/derivative was considered when growth inhibition was observed (Ferreira *et al.*, 2011). The fractional inhibitory concentration index (FICI) was calculated according to equation 4:

$$\mathrm{FICI} = \frac{[\mathrm{P}]}{[\mathrm{P}]_{\mathrm{MIC}}} + \frac{[\mathrm{B}]}{[\mathrm{B}]_{\mathrm{MIC}}} \quad (4)$$

where [P]/[B] are the concentration of the phytochemical or derivative (P) or biocide (B) on the combination and $[\mathrm{P}]_{\mathrm{MIC}}/[\mathrm{B}]_{\mathrm{MIC}}$ are the MIC of each phytochemical or derivative or biocide alone.

FICI and MIC were determined for each individual 96-well plate and the final FICI was determined considering three independent results. The phytochemicals and derivatives with a FICI < 1 were considered to potentiate the biocide since it highlights the reduction of concentration of the phytochemical or biocide needed to inhibit bacterial growth when in combination, in comparison with their MIC alone. The concentration of the phytochemical or derivative in combination with the biocide was selected taking into consideration the best results obtained for at least two of the tested bacteria and the solubility. Three independent experiments were performed for each combination and bacteria.

III.2.7. Ethidium Bromide efflux inhibition assay

Bacterial suspensions of initial OD_{600} of 0.1-0.3 (10^8 CFU mL^{-1}) were prepared from overnight cultures with fresh medium and allowed to grow until mid-exponential phase (OD_{600} of 0.6-0.8) at 37 ± 3 ºC. Cells were harvested (16000 g for 5 min) and washed with PB pH 7 and the suspension was adjusted to a final OD_{600} of 0.4. To each well, 100 µL of cell suspension were added (except for the fluorescence control where only pH 7 PB was added) and 50 µL of EB (0.02 g L^{-1}). Fluorescence (Exc: 530; Em: 590) was read at 37 ± 3 ºC on a TECAN Infinite 200 PRO with Te-Inject module every 2 min 30 sec for 10 min (2 sec of linear shaking with 1 mm of amplitude before each reading). Afterwards, each well was injected with 50 µL of the inhibitor or solvent (a duplicate of each well was set up in each plate). The inhibitor or solvent was four times more concentrated to ensure the final concentration in the well (Table VII.1) and volume (200 µL) of the well. The fluorescence reading continued after the injection for additional 50 min (total of 1 h). Fluorescence control was subtracted from each time point of the control (solvent) and the inhibitor. Data was presented as the mean and standard deviation of at least three independent experiments.

III.2.8. Ethidium bromide minimum inhibitory concentration determination in the presence of efflux inhibitors

The MIC for EB was determined using the two-fold microdilution method with the addition of the inhibitor (5 % v v^{-1}) (CLSI, 2007). Each inhibitor was at a sub-inhibitory concentration to avoid any impact on cell growth (Table VII.1). After a 24 h incubation period at 37 ± 3 ºC, bacterial growth was assessed visually and the MIC was

measured as the concentration where no visible growth was detected (Costa *et al.*, 2011; 2015).

III.2.9. Detection of resistance genes (*qacA/B, qacG, qacJ, smr* and *tetK*)

III.2.9.1. Preparation of chromosomal DNA

Genomic DNA was extracted using the modified QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), where 30 min digestion with lysostaphin (150 mg L^{-1}) was performed prior extraction (Costa *et al.*, 2011).

III.2.9.2. Preparation of plasmid DNA

Plasmid DNA was extracted using the Promega Wizard™ Plus SV Minipreps DNA Purification System (centrifugation protocol) with and addition incubation with lysostaphin (35 mg L^{-1}) at 37 ± 3 ºC for 90 minutes prior extraction (Costa *et al.*, 2011).

III.2.9.3. Polymerase Chain Reaction amplification of resistance genes

DNA fragments that correspond to biocide resistance genes (*qac*A/B, *qac*G, *qac*J, *smr*, *tetK*) were amplified by Polymerase Chain Reaction (PCR) using the primers described on Table III.4. Reaction mixtures included $MgCl_2$ (1.75 mM), dNTPs (0.2 mM, 25 pmol of each primer, NZY Taq II (2.5 U) in buffer NZY Tech. For each target gene a positive control (strain with the gene), a negative control (strain without the gene), a water control (no DNA) and a marker (#N0551 or #SM0311, from BioLabs or Thermofisher respectively) were performed in addition to the target gene amplification. DNA amplification conditions were different depending on the target gene (Table III.5). The amplification products were separated in a 1% agarose gel electrophoresis.

Table III.4.Primers used in this study to amplify resistance genes

Primer	Sequence (5'-3')	Amplicon size (bp)	Reference
*qacA/B*_Fw	GCTGCATTTATGACAATGTTTG	628	Anthonisen *et al.* (2002)
*qacA/B*_Rv	AATCCCACCTACTAAAGCAG		
*qacG*_Fw	CAACAGAAATAATCGGAACT	275	Bjorland *et al.* (2005)
*qacG*_Rv	TACATTTAAGAGCACTACA		
*qacJ*_Fw	CTTATATTTAGTAATAGCG	306	Bjorland *et al.* (2005)
*qacJ*_Rv	GATCCAAAAACGTTAAGA		
*smr*_Fw	ATAAGTACTGAAGTTATTGGAAGT	285	Bjorland *et al.* (2001)
*smr*_Rv	TTCCGAAAATGTTTAACGAAACTA		
*tetK*_Fw	GTA GCG ACA ATA GGT AAT AGT	261	Strommenger *et al.* (2003)
*tetK*_Rv	GTA GTG ACA ATA AAC CTC CTA		

Table III.5. PCR Amplification conditions for each target gene used

Gene	Thermocycling Steps	Initial denaturation	Amplification			Final Extension
			Denaturation	Annealing	Extension	
qacAB	Temperature (°C)	95	95	40	72	72
	Time (s)	240	60	45	60	300
	Cycles	1		35		1
qacG	Temperature (°C)	95	95	48	72	72
	Time (s)	180	30	30	30	300
	Cycles	1		35		1
qacJ	Temperature (°C)	95	95	46	72	72
	Time (s)	180	30	30	30	300
	Cycles	1		35		1
smr	Temperature (°C)	94	94	48	72	72
	Time (s)	240	30	30	30	300
	Cycles	1		35		
tetK	Temperature (°C)	94	94	55	72	72
	Time (s)	240	30	30	30	300
	Cycles	1		35		1

III.2.10. Bactericidal suspension test (EN 1276:2009)

The suspension test used was adapted from EN 1276:2009 (CEN, 2009). Briefly, an overnight culture was grown in MHB/PB at 37 ± 3 ºC under 150 rpm. The culture was washed once with PB pH 7 and the bacterial suspension was adjusted to an $OD_{600\,nm}$ of 0.33 with PB pH 7 ($1.5 – 5 \times 10^8$ CFU mL^{-1}). A volume of 900 µL of cell suspension was added to an Eppendorf containing the phytochemical/derivative (5 % v v^{-1} of solvent; several concentrations were tested depending on the phytochemical/derivative - 0.5, 1 and 2 mM for cinnamaldehyde; 5 mM for α-methylhydrocinnamic acid) and CTAB (from 0.005 to 1 mM) for a total of 1 mL of test solution and vortexed for 5 sec. Before adding the bacterial cells, the test solution was incubated statically for 2 min at 25 ± 3 ºC. Incubation at 25 ± 3 ºC was allowed to occur for 5 min. 100 µL of the test solution was placed in 900 µL of neutralizer for 10 min and CFU determination was performed. CFU were determined after 24 h at 30 °C incubation and presented as $\log_{10}$ CFU cm^{-2}. The results are presented as $\log_{10}$ CFU mL^{-1}.

III.2.10.1. Bactericidal suspension test (EN 1276:2009) in clean or dirty conditions

The suspension test in the presence of BSA, representative of organic matter, was performed as described in III.2.10. however the volumes were the following 500 µL of cell suspension ($OD_{600\,nm}$ at 0.6) were added to an Eppendorf containing phytochemical/derivative (5 % v v^{-1} of solvent, 1 or 2 mM of cinnamaldehyde final

concentration), CTAB (several concentrations were tested from 0.02 to 1 mM final concentration), EDTA (final concentration of 10 or 25 mM), BSA (final concentration 0.3 $g.l^{-1}$ of BSA or 3 g L^{-1} depending on which condition was desired - clean or dirty conditions, respectively) and 250 µL of PB pH 7, for a total of 1 mL of test solution. The results are presented as $\log_{10}$ CFU mL^{-1}.

III.2.11. Surface wiping assay

III.2.11.1. Holder and carrier development

The surface wiping assay protocol was developed in the present thesis. The holder and carrier were designed based on EN 16615:2015 - Efficacy evaluation of surface disinfection Wipes, where a block of 2.3 – 2.5 Kg (18.6 × 12.1 × 8.6 cm) is used to mimic the pressure that is used when a surface is cleaned using a wipe (CEN, 2015b). This design was intended to be a smaller scale, where a larger number of samples could be assessed in a shorter period of time with the benefit of a low cost. Therefore, a disc system was developed taking as an example the Wiperator (E2967 − 15) sampling system (ASTM, 2016).

The size and weight of the carrier was determined by using the formula of the area (5), the force (6), pressure (7), density (8) and volume (9):

$$\text{Area}_{\text{rectangle}} = \text{height} \times \text{width};\ \text{Area}_{\text{circle}} = \pi \times \text{radius}^2 \quad (5)$$

$$\text{Force} = \text{mass} \times \text{aceleration} \quad (6)$$

$$\text{Pressure} = \text{Force} \div \text{area} \quad (7)$$

$$\text{d} = \text{mass} \div \text{Volume} \quad (8)$$

$$\text{Volume} = \pi \times \text{radius}^2 \times \text{height} \quad (9)$$

Therefore, taking into consideration the values for the granite block, the pressure that the block exerts to the surface needs to be obtained in order to have a carrier that exerts exactly the same pressure to the disc ($P_{block} = P_{carrier}$). In addition, the radius of the carrier should be half the disc (0.5 cm):

$A_{block} = L \times w = 0.121 \times 0.086 = 0.0104\ m^2$

$F_{block} = m \times a = 2.3 \times 9.8 = 22.5\ N\ (2.3Kg)$

$= 2.5 \times 9.8 = 24.5\ N\ (2.5\ Kg)$

$$\mathbf{P_{block} = F \div A = 2166\ Pa\ (2.3\ Kg)}$$
$$\mathbf{= 2354\ Pa\ (2.5\ Kg)}$$
$$A_{Carrier} = \pi \times r^2 = \pi \times (0.005)^2 = 0.0000785\ m^2$$
$$F_{Carrier} = P \times A = 0.1701\ N\ (2.3\ Kg)$$
$$= 0.1849\ N\ (2.5\ Kg)$$
$$\mathbf{m_{Carrier} = F \div a = 0.0173\ Kg\ (2.3\ Kg)}$$
$$\mathbf{= 0.0189\ Kg\ (2.5\ Kg)}$$

$$d = m \div V \Leftrightarrow 8\frac{g}{cm^2}\ (\text{stainless steel}) = \frac{m}{V} \Leftrightarrow V_{carrier} = 2.17 cm^3\ (2.3\ Kg)$$
$$= 2.36\ cm^3 (2.5\ Kg)$$
$$V_{Carrier} = \pi \times r^2 \times h \ \Leftrightarrow\ \mathbf{h_{carrier} = 2.76\ cm\ (2.3\ Kg)}$$
$$\mathbf{= 3.00\ cm\ (2.5\ Kg)}$$

In Figure III.1 the design of the holder and carrier is shown.

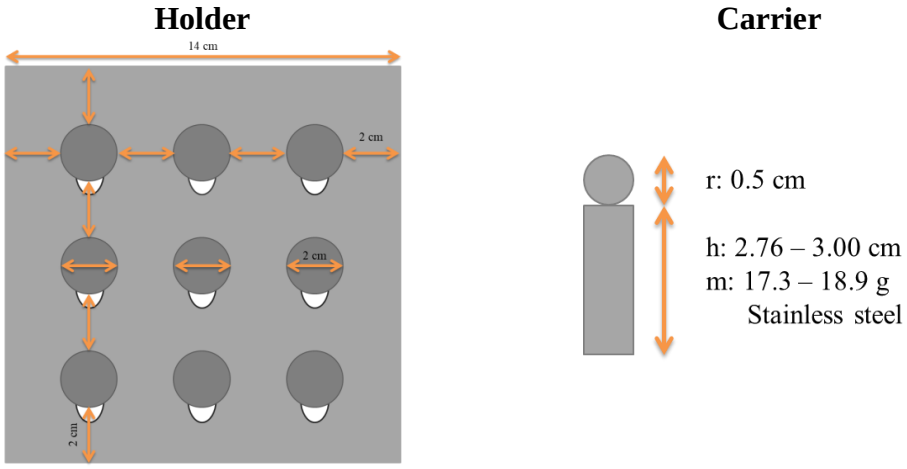


Figure III.1. Holder (left) and carrier (right) design in 2D.

All the components of this assay are presented in Figure III.2. The wipes were purchased from Bastos Viegas SA (Penafiel, Portugal). The holder for the discs, the discs and the carrier were done in stainless steel AISI 316.

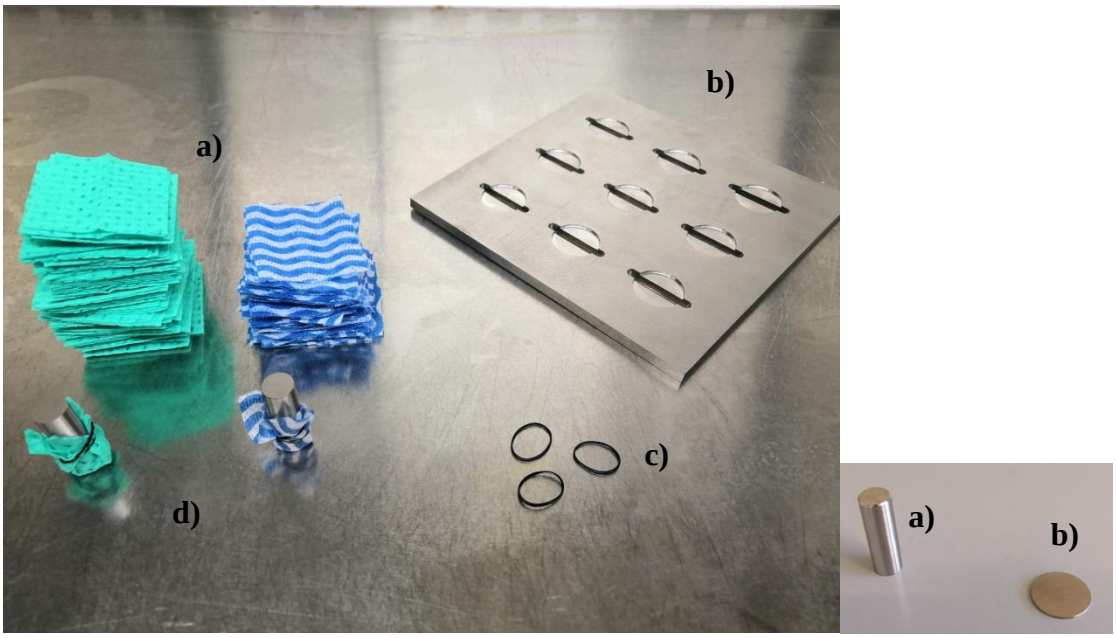


Figure III.2. Components of the surface wiping efficiency test. On the left: a) wipes, b) holder for the discs, c) rubber rings and d) wipe carrier. On the right: a) carrier, b) stainless steel disc.

III.2.11.2. Bacterial Suspension – $OD_{600\ nm}$ optimization

According to the standards used as reference, a bacterial suspension of 1.5×10^9 CFU mL^{-1} to 5.0×10^9 CFU mL^{-1} is used to inoculate the discs and at least $10^{5.5}$ CFU $disc^{-1}$ should be recovered after drying. Taking into consideration the results obtained (Table III.6) a suspension OD_{600nm} of 4 and 2 were selected for *E. coli* and *S. aureus* and a drying time of 30 min at 37 ± 3 ºC.

Table III.6. Bacterial suspension concentration at different optical density (OD_{600nm}) of *E. coli* and *S. aureus*. The drying time was also optimized when an initial OD_{600nm} of 4 and 2 were used for *E. coli* and *S. aureus*

	E. coli	***S. aureus***
OD_{600nm}		
1	1.03×10^9 ± 2.18×10^8CFU mL^{-1}	8.25×10^8 ± 1.25×10^8 CFU mL^{-1}
1.6	3.42×10^9 ± 3.82×10^8 CFU mL^{-1}	1.98×10^9 ± 4.62×10^8 CFU mL^{-1}
2	-	**1.43×10^9 ± 3.51×10^8 CFU mL^{-1}**
3	3.92×10^9 ± 1.38×10^9 CFU mL^{-1}	-
4	**6.13×10^9 ± 2.92×10^9 CFU mL^{-1}**	-
Drying time		
30 min	**1.74×10^6 ± 8.55×10^5 CFU**	**1.27×10^7 ± 1.76×10^6 CFU**
45 min	1.17×10^6 ± 1.42×10^5 CFU	7.67×10^6 ± 1.91×10^6 CFU
1 h	6.50×10^5 ± 2.50×10^4 CFU	5.17×10^6 1.66×10^6 CFU

III.2.11.3. Preparation of the contaminated surface D1

An overnight culture (37 ± 3 ºC) was washed twice with PB pH 7 and the cell suspension was adjusted to 1.5 – 5 x 10^9 CFU mL^{-1}. Right before using the cell suspension, 0.5 mL of ten times concentrated BSA (final concentration 0.3 g L^{-1}) was added to 4.5 mL of cell suspension and homogenization by vortex was performed for 30 sec. A volume of 10 µL of this suspension was transferred to the centre of a clean and sterile stainless steel disc and allowed to completely dry at 37 ± 3 ºC for 30 min (D1; Figure III.3).

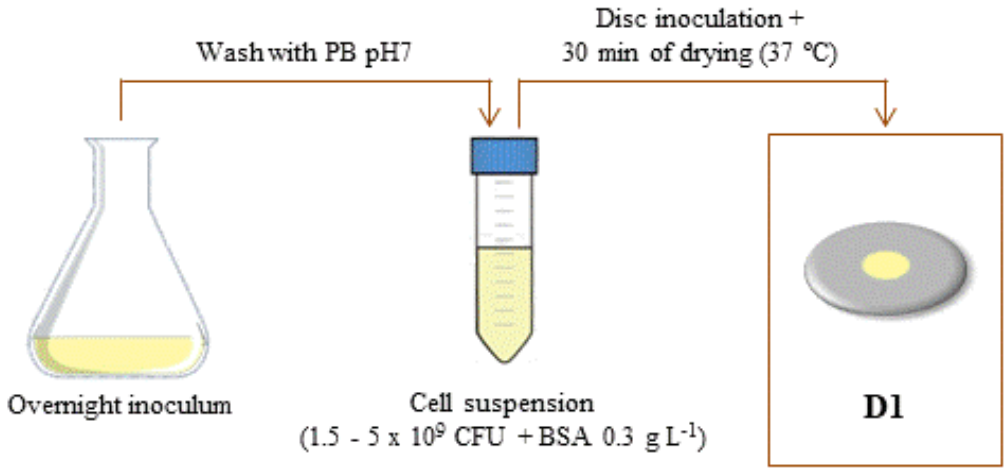


Figure III.3. Schematic representation of the preparation of D1.

III.2.11.4. Preparation of the wipe carrier

The control solution (0.1 % polysorbate 80 in water) and the formulation (1 mM cinnamaldehyde dissolved in isopropanol (5 % v v^{-1} of final volume), 0.5 mM CTAB and 25 mM EDTA in 20 mM PB pH 7) were prepared for each experiment. The wipe (4 × 4 cm) was pre-soaked on 20 mL of solution, ensuring the wipe was completely covered in the solution, for 2 min at room temperature (20 ± 3 ºC). Handled with a clean pair of gloves the wipe was wrung to drain the excess of liquid and weighted after removal of excess liquid; wipe A - 0.233 ± 0.030 g and 0.214 ± 0.031 g; wipe B - 0.370 ± 0.019 g and 0.355 ± 0.017 g for the control and formulation, respectively. The wipe was then wrapped onto the carrier and fixed with a rubber as shown in Figure III.4.

Figure III.4. Wipe carrier – assembly of the carrier with the soaked wipe.

III.2.11.5. Wiping test

The wiping test was performed as shown in Figure III.5. Briefly, D1 was placed on the holder with two clean and sterile stainless steel discs next to it. The wipe carrier was placed on top of D1 and with the help of forceps the wipe carrier is slid (without placing any pressure on the wipe carrier towards the disc) for 1 min vertically and horizontally as described in the diagram showed on Figure III.5. The wipe carrier was then moved to D1.1 and D1.2, repeating the surface wiping movement for 1 min for each disc (Figure III.5). After the wiping, each disc was placed in 5 mL of universal neutralizer with 2 g of glass beads (the efficacy of the neutralizer was tested before – in section III.2.10) and vortexed for 5 sec. Neutralization of the formulation was allowed to occur for 10 min. Discs were then vortexed for 30 sec and CFU determination was performed as described in section III.2.2. The results are presented as $\log_{10}$ CFU reduction or $\log_{10}$ CFU.

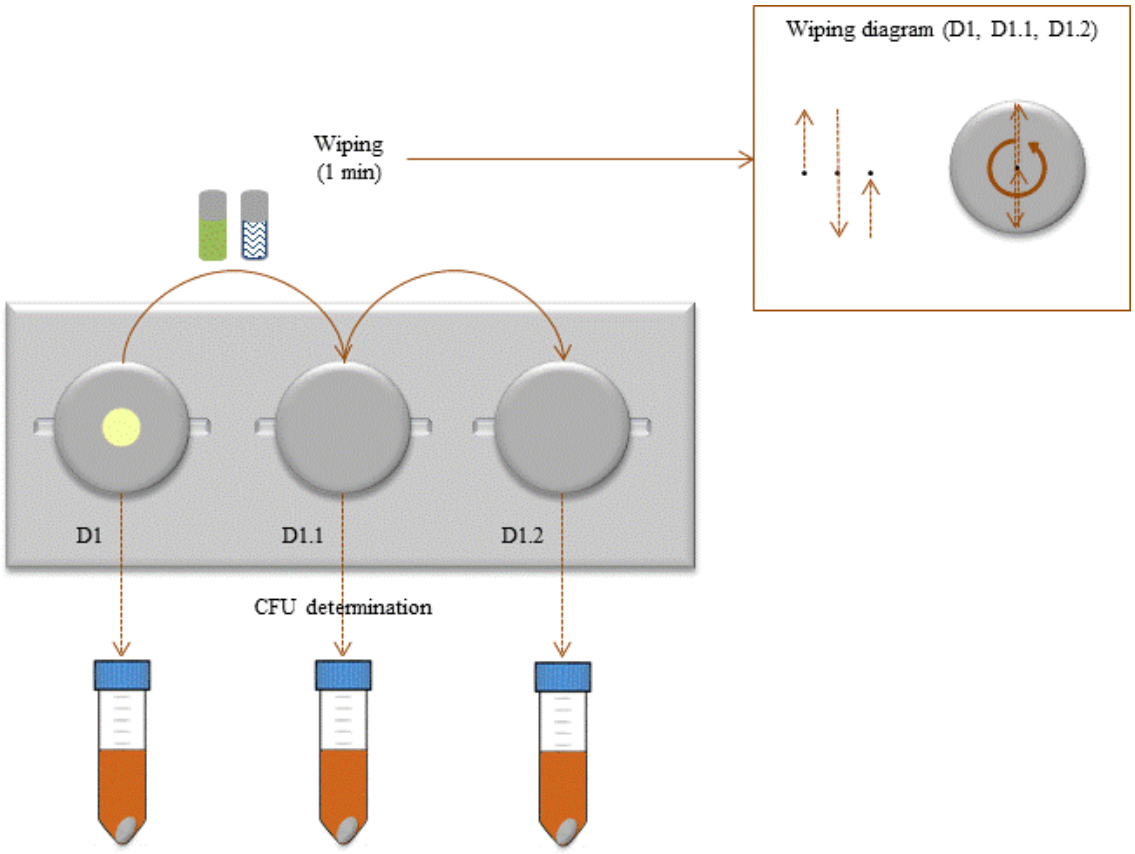


Figure III.5. Schematic representation of the steps of the wiping assay.

III.2.11.6. Wipe characterization

The wipes that were purchased from Bastos Viegas SA (Penafiel, Portugal) had different characteristics, such as composition and structure Figure III.6. The wipes used in this work were 90% fibres (60% viscose/40% polyester) and 10% synthetic ligand named wipe A (white/blue) and wipe B (green) had 94% de fibres (70% viscose/30% polyester) and 6% synthetic ligand. Using optical coherence tomography (Thorlabs Ganymede Spectral Domain OCT system with central wavelength of 930 nm, Thorlabs GmbH, Dachau, Germany, using a LSM03 objective lens (5 × magnification)) it was possible to obtain some data on the thickness and pore diameter of both wipes that is presented on Table III.7.

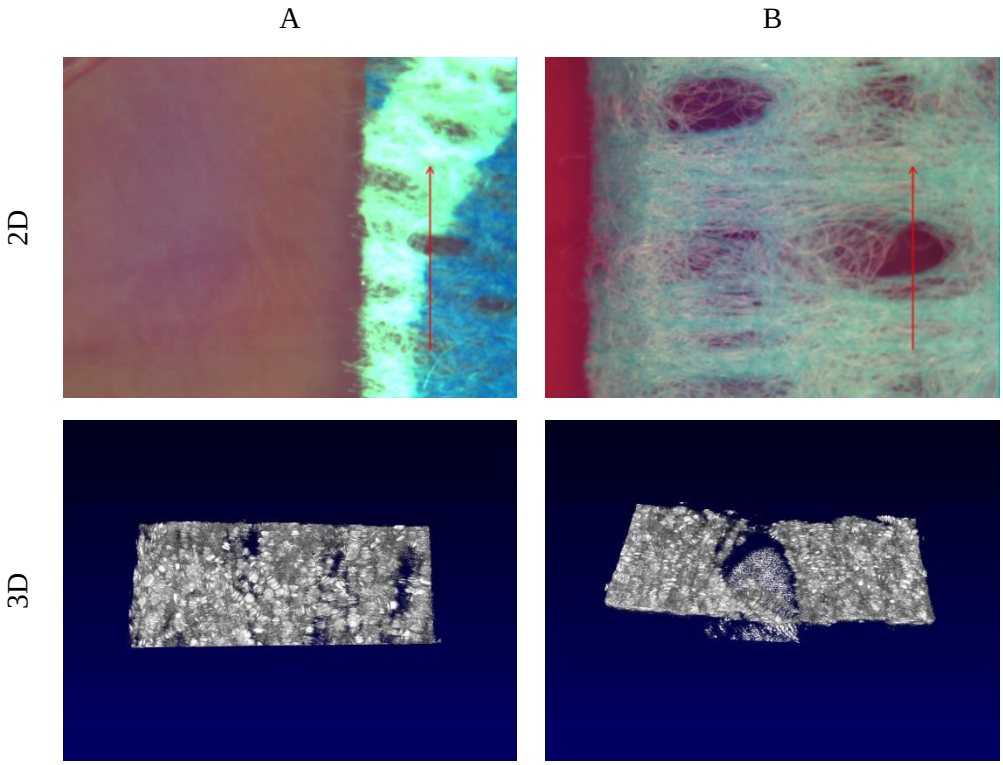


Figure III.6. Optical coherence tomography (OCT) 2D and 3D and images of wipe A (right) and B (left) 5 times amplification.

Table III.7. OCT measurements of wipe thickness and pore diameter based on the section illustrated in Figure III.6

	A	**B**
Thickness (mm)	0.22 ± 0.04	0.28 ± 0.06
Pore diameter (mm)	0.42 ± 0.18	0.98 ± 0.06

III.2.12. Evaluation of formulation stability and phytochemical/biocide chemical interactions

III.2.12.1. Preparation of cinnamaldehyde standard solutions

A stock solution of cinnamaldehyde of 100 mM was prepared using a mixture of acetonitrile:water (1:1). The stock solution was further diluted using the same solvent mixture to obtain working solutions at the desired concentrations (0.01, 0.05, 0.1, 0.5 and 1 mM).

III.2.12.2. Preparation of formulation solution samples

Formulation solution was 10-fold diluted in acetonitrile:water (1:1) to obtain a cinnamaldehyde concentration of 0.1 mM. This procedure was performed with fresh prepared and after 1 month of shelf storage formulation solutions.

III.2.12.3. Stability assessment by High Pressure Liquid Chromatography

A Shimadzu HPLC instrument with an LC 20AP solvent delivery system, a universal loop injector (Rheodyne 7725 i) of injection capacity of 20 µL, and a SPD M20A diode array detector (λ_{max} = 190 nm) was employed in this work. Chromatographic analysis was performed using a Phenomenex Luna C-18 column (150mm × 4.6 mm; 10 μm), a acetonitrile/water mobile phase (gradient mode 30:70 to 100:0). Data acquisition was performed using LabSolutions software (version 5.93).

III.2.12.4. Phytochemical/biocide chemical interaction study by Nuclear Magnetic Resonance spectroscopy

^{1}H NMR (Nuclear Magnetic Resonance) data were acquired on a Bruker Avance III 400 Nuclear Magnetic Resonance (NMR) spectrometer operating at 400.15 MHz. The relaxation delay was 90° pulse, spectral width of 8012 Hz and 65 K data points. ^{1}H NMR spectra of the samples were recorded at room temperature (25 ± 3 °C) in 5-mm outer-diameter tubes. The samples were prepared in deuterated water. TMS (3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt) was used as internal reference.

III.2.13. Statistical analysis

The statistical program GraphPad Prism version 6 was used to analyse the data. One-way analysis of variance (one-way ANOVA) followed by the post hoc Dunnett's multiple comparison test. A confidence level of ≥ 95% ($p < 0.05$), ≥ 99% ($p < 0.01$) and ≥ 99.9% ($p < 0.001$) was used to consider statistical significance. The results are presented as the average and standard deviation (SD) of three independent experiments for each sample.

Chapter IV. Phytochemical profiling as a solution to palliate disinfectant limitations

The indiscriminate use of biocides for general disinfection has contributed to the increased incidence of antimicrobial tolerant microorganisms. The work of this chapter aims to assess the potential of seven phytochemicals (tyrosol, caffeic acid, ferulic acid, cinnamaldehyde, coumaric acid, cinnamic acid and eugenol) in the control of planktonic and sessile cells of E. coli and S. aureus. Cinnamaldehyde and eugenol showed antimicrobial properties, MIC of 3-5 and 5-12 mM and MBC of 10-12 and 10-14 mM against S. aureus and E. coli, respectively. Cinnamic acid was able to completely control adhered bacteria with effects comparable to the biocides peracetic acid and sodium hypochlorite and it was more effective than hydrogen peroxide (all at 10 mM). This phytochemical caused significant changes on bacterial membrane hydrophilicity. The observed effectiveness of phytochemicals makes them interesting alternatives and/or adjuvants for the frequently used biocidal products. Cinnamic acid is of particular interest for the control of sessile cells.

The work included in this chapter resulted in the publication:

Malheiro J., Gomes I., Borges A., Bastos M.M., Maillard J.Y., Borges F., Simões M., 2016. Phytochemical profiling as a solution to palliate disinfectant limitations. Biofouling, 32(9):1007–1016. Doi: 10.1080/08927014.2016.1220550.

IV.1. Experimental details

Bacteria	*E. coli* CECT 434 and *S. aureus* CECT 976
Phytochemicals or derivatives	Caffeic acid, cinnamaldehyde, cinnamic acid, coumaric acid, eugenol, ferulic acid, tyrosol
Biocides	Hydrogen peroxide (HP), peracetic acid (PA), sodium hypochlorite (SH)
Temperature	30 ± 3 ºC
Methodology used	III.2.1 Antibacterial susceptibility testing III.2.2 Efficacy against early sessile cells III.2.3 Physicochemical characterization of bacterial surfaces

IV.2. Results

This study was performed with seven biosynthetically related phytochemicals (Figure IV.1) with the aim to ascertain their biocidal potential. Three commonly used disinfectants (HP, PA and SH) were used for comparison. *E. coli* CECT 434 and *S. aureus* CECT 976 were the microorganisms selected and the MIC and MBC of biocides and phytochemicals were assessed (Table IV.1).

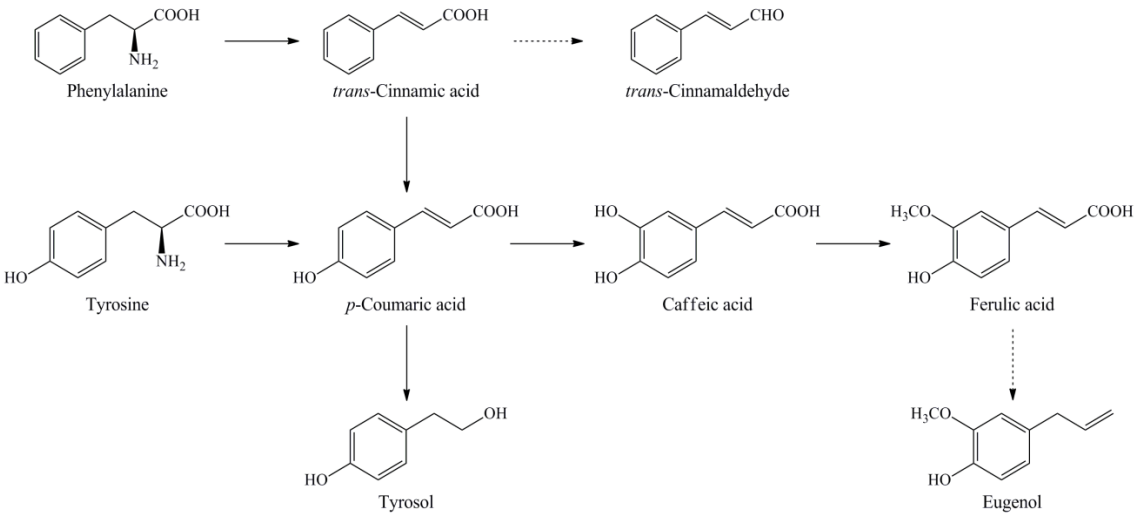
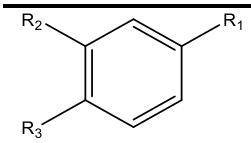
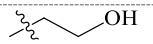
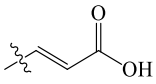
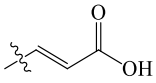
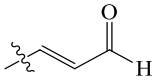
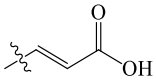
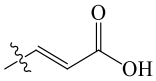
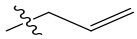


Figure IV.1. Biosynthetic relationship of the phytochemicals used.

Table IV.1. Properties of the selected phytochemicals and MIC and MBC of the selected phytochemicals and biocides against *E. coli* CECT 434 and *S. aureus* CECT 976

				***E. coli* CECT 434**		***S. aureus* CECT 976**	
	R_1	**R_2**	**R_3**	**MIC (mM)**	**MBC (mM)**	**MIC (mM)**	**MBC (mM)**
HP				16	20	400	450
PA				5	7	9	10
SH				3	3	4	5
Tyrosol		-	OH	> 25	> 25	> 25	> 25
Caffeic acid		OH	OH	25	> 25	23	> 25
Ferulic acid		OCH_3	OH	> 25	> 25	25	> 25
Cinnamaldehyde		-	-	3	10	5	12
Coumaric acid		-	OH	15	> 25	25	25
Cinnamic acid		-	-	15	> 25	25	25
Eugenol		OCH_3	OH	5	10	12	14

IV.2.1. MIC determination of selected phytochemicals and biocides

HP had MIC and MBC values more than 20 times lower for *E. coli* (16 to 20 mM for MIC and MBC) than for *S. aureus* (400 and 450 mM). PA and SH were the disinfectants with the lowest MIC and MBC regardless of the bacteria tested. The most efficient phytochemicals were cinnamaldehyde and eugenol, showing the lowest MIC and MBC against both bacteria. Moreover, cinnamaldehyde and eugenol exhibited MIC similar to SH (except MIC of eugenol for *S. aureus*) and MIC and MBC comparable to PA ($p > 0.05$). Cinnamaldehyde and eugenol MIC and MBC were lower than for HP ($p < 0.05$). Caffeic, ferulic, coumaric and cinnamic acids showed similar MIC when tested against *S. aureus* ($p > 0.05$). Coumaric and cinnamic acids had also similar MIC against *E. coli*. Some phytochemicals shown MIC or MBC values above 25 mM. Tyrosol had the lowest antimicrobial activity (MIC and MBC > 25 mM against both bacteria).

IV.2.2. Phytochemicals and biocides are able to remove adhered bacteria from polystyrene surfaces

Additional tests were performed with sessile bacteria on polystyrene surfaces to evaluate the efficacy of the disinfectants and phytochemicals in the removal of monolayer adhered bacteria. After a 2 h adhesion period, 4.89 $\log_{10}$ CFU cm^{-2} of *E. coli* and 5.21 $\log_{10}$ CFU cm^{-2} of *S. aureus* adhered on the polystyrene surface. The polystyrene-adhered bacteria were exposed to the selected disinfectants and phytochemicals for 1 h and the CFU of adhered bacteria are presented in Figure IV.2. Exposure to HP only caused CFU reduction of adhered *E. coli*. PA and SH were the most efficient disinfectants causing total CFU reduction of both bacteria ($p > 0.05$). Considering the selected phytochemicals, it was observed that cinnamic acid promoted a drastic CFU reduction of *E. coli* and *S. aureus* from polystyrene at a concentration 2.5 times lower than the MBC (concentration used: 10 mM). This phytochemical displayed an activity comparable to PA and SH ($p > 0.05$) and it was more efficient than HP against *S. aureus* sessile bacteria ($p < 0.05$). The phytochemicals with poor activity ($\leq$1 $\log_{10}$CFU cm^{-2} reduction from surfaces) against *S. aureus* were cinnamaldehyde, coumaric, caffeic and ferulic acids, tyrosol and eugenol. Tyrosol and eugenol were the less efficient against *E .coli* with reduction from surfaces lower than 1 $\log_{10}$ CFU cm^{-2}, followed by ferulic acid (1< $\log_{10}$ CFU cm^{-2} reduction from surfaces $\leq$ 2), caffeic acid (2 < $\log_{10}$ CFU cm^{-2} reduction from surfaces $\leq$ 3), cinnamaldehyde, coumaric acid and cinnamic acid (3 < $\log_{10}$ CFU cm^{-2} reduction from surfaces $\leq$ 4).

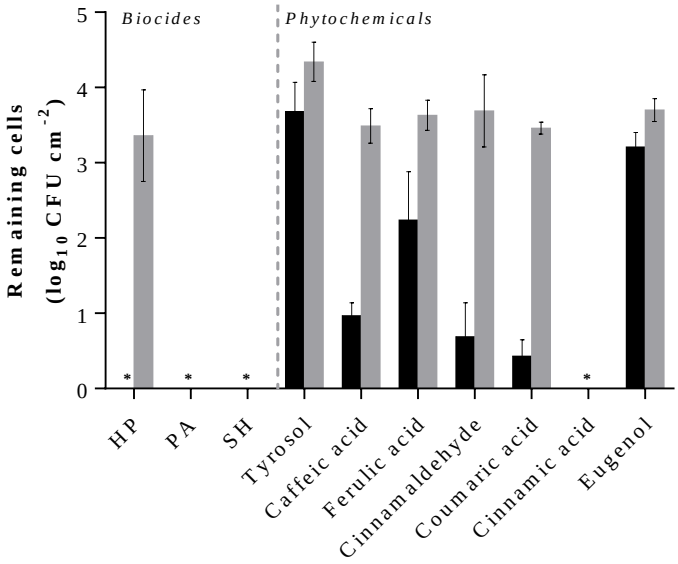


Figure IV.2. Effects of the selected biocides and phytochemicals on the control of sessile *E. coli* CECT 434 (black) and *S. aureus* CECT 976 (grey). The Figure presents the remaining CFU of sessile bacteria after 1 h exposure to the selected biocides and phytochemicals. Values are mean ± SD of three experiments. *- No CFU were detected.

IV.2.3. Bacteria hydrophobicity is altered in the presence of certain phytochemicals

The possibility of changes on membrane hydrophobicity of *E. coli* and *S. aureus* following exposure to the selected disinfectants and phytochemicals was also assessed, Table IV.2. SH was able to enhance the hydrophilicity (ΔG_{sws}) of both bacteria ($p < 0.05$). PA had no significant effects on the membrane hydrophilicity of both bacteria ($p > 0.05$). HP was able to increase the ΔG_{sws} of *E. coli*. Considering the phytochemicals, cinnamic acid was found to increase hydrophilicity of *E. coli* and reduce the hydrophilicity of *S. aureus* ($p < 0.05$). The remaining phytochemicals increased the hydrophilicity of *S. aureus*, with the exception of tyrosol ($p < 0.05$). In fact, tyrosol did not influence the membrane properties of *E. coli* or *S. aureus* ($p > 0.05$). Caffeic, *p*-coumaric and ferulic acids, and cinnamaldehyde increased the hydrophilicity of *E. coli* ($p < 0.05$). Eugenol increased membrane hydrophilicity, however, the effect on *E. coli* was not as evident as it was against *S. aureus* ($p < 0.05$).

Table IV.2. Effects of the selected disinfectants and phytochemicals on the hydrophobicity of *E. coli* CECT 434 and *S. aureus* CECT 976

	Hydrophobicity (mJ m^{-2}) - ΔG_{sws}^{TOT}	
	***E. coli* CECT 434**	***S. aureus* CECT 976**
Control (Water)	25.22 ± 5.22	20.78 ± 5.45
HP	42.38 ± 3.80	21.50 ± 4.69
PA	21.05 ± 2.51	27.93 ± 4.94
SH	33.81 ± 3.96	42.45 ± 4.79
Control (DMSO)	28.14 ± 4.30	23.28 ± 5.77
Tyrosol	29.39 ± 0.48	23.81 ± 1.99
Caffeic acid	37.67 ± 8.78	28.77 ± 2.08
Ferulic acid	32.26 ± 3.35	26.81 ± 5.02
Cinnamaldehyde	34.03 ± 4.98	27.98 ± 2.43
Coumaric acid	32.58 ± 3.65	27.73 ± 4.26
Cinnamic acid	31.68 ± 6.76	10.09 ± 5.75
Eugenol	27.94 ± 0.97	30.17 ± 5.14

IV.3. Discussion

Over the years, natural products have assumed an important role as alternative sources of novel bioactive molecules. In the work presented in this chapter, seven phytochemicals were selected based on their related chemical structures. Their effects were assessed against planktonic and sessile cells of two bacteria, *E. coli* and *S. aureus*, previously used in diverse antimicrobial screening studies (Borges *et al.*, 2013; Simões *et al.*, 2008). For comparison, three commonly used disinfectants (HP, PA and SH) were also tested. The selected disinfectants are recognized for their broad antimicrobial spectrum (McDonnell *et al.*, 1999; Pericone *et al.*, 2000; Rasmussen *et al.*, 2013; Rutala *et al.*, 1997).

IV.3.1. Biocides and phytochemicals antibacterial action

An initial screening was performed with the selected disinfectants and phytochemicals to ascertain their MIC and MBC against *E. coli* and *S. aureus*. HP was the least effective benchmark disinfectant. The lower susceptibility of *S. aureus* to HP in the concentration used in this study, compared to *E. coli* could be explained with the expression of catalase by *S. aureus* (Park *et al.*, 2008), although this was not ascertained

in this study. PA and SH are powerful oxidizing agents that are effective against both Gram-positive and Gram-negative bacteria (Penna *et al.*, 2001). The data attained in the present study (Table IV.1) confirmed their reported biocidal efficacy (Penna *et al.*, 2001; Spoering *et al.*, 2001). Despite a high efficacy against bacteria, they present distinct advantages and disadvantages that influence their use (Estrela *et al.*, 2002; Ferraris *et al.*, 2005; Kitis, 2004; McDonnell *et al.*, 1999).

Although some of the selected phytochemicals presented high (~ 25 mM) MIC and MBC values, cinnamaldehyde and eugenol presented MIC and MBC comparable to benchmark disinfectants. Differences on the MIC and MBC of the phytochemicals against *E. coli* and *S. aureus* were observed. In general, *E. coli* was more susceptible than *S. aureus*, contrarily to what is commonly observed. Gram-negative bacteria are more tolerant than Gram-positive bacteria to biocides due to the presence of an outer membrane (Livermore, 2012). The higher resistance of Gram-positive bacteria can be related with phytochemicals selectivity. Cinnamic acid derivatives are organic acids (pKa ~ 4.2) and their efficacy as antimicrobials is thought to be dependent on the concentration of undissociated acid (Campos *et al.*, 2009; Johnston *et al.*, 2003). In fact, this small molecules can cross the cell membrane by passive diffusion as undissociated chemicals, disturb or even disrupt the cell membrane structure, acidify the cytoplasm and cause denaturation of proteins as well as increase bacterial permeability (Campos *et al.*, 2009; Johnston *et al.*, 2003). Therefore, the presence of a thinner peptidoglycan layer in Gram-negative bacteria may facilitate the antimicrobial action of phytochemicals. Considering the promising antibacterial activities observed, their activity as quorum sensing inhibitors was also assessed since several phytochemicals anti-quorum sensing properties have been described which can confer them an importance role in biofilm control (Borges *et al.*, 2014a). However, in this study and in these experimental conditions only eugenol demonstrated a slight anti-quorum sensing activity against *C. violaceum* (*Appendix A.4.)*. This property cannot be discarded for the other phytochemicals tested since the inhibition of quorum sensing by eugenol, cinnamaldehyde, curcumin and *p*-coumaric acid was already described (Bodini *et al.*, 2009; Brackman *et al.*, 2011b; Zhou *et al.*, 2013). In this study only the quorum sensing system of *C. violaceum*, homologs of LuxI/LuxR system, was studied (Borges *et al.*, 2014a). So, the possibility of inhibition of other quorum sensing systems cannot therefore be discarded.

IV.3.2. Phytochemicals ability to remove polystyrene adhered bacteria and their action on bacterial surface

Despite the absence of anti-quorum sensing activity observed in these experimental conditions, the phytochemicals were assessed for their ability to control adhered cells and their effects were compared with the disinfectants. Monolayer adhered bacteria were used in this study rather than three-dimensional biofilm structures. According to previous studies, contaminated hospital surfaces are mostly colonized by monolayer adhered cells with densities of 10^4 - 10^6 CFU cm^{-2} (values in the range of those found in this study for *E. coli* and *S. aureus*) (Dancer, 2004; Otter *et al.*, 2015; Wren *et al.*, 2008). Moreover, it was found that the effects of selected disinfectants were similar on CFU reduction of monolayer adhered cells (2 h adhesion) and biofilms (24 h-old) (Meireles *et al.*, 2015).

HP was the least efficient disinfectant. Its biocidal activity is based on a bimodal killing pattern where the first mode occurs when *E. coli* is exposed to low concentrations of HP that damages DNA. The second mode occurs when *E. coli* is exposed to higher concentrations and cell membrane damage can be observed (Imlay *et al.*, 1986; Linley *et al.*, 2012). The influence of HP on *E. coli* surface properties was observed in this study as an increase in the surface hydrophilicity. The high effectiveness of PA and SH can be explained by their mode of action. PA action can occur by disruption of cell wall permeability, proteins denaturation, and oxidation of sulfhydryl and sulfur bonds in proteins (Al-Adham *et al.*, 2013; Kitis, 2004). Furthermore, it was hypothesized that it can disrupt the chemiosmotic function of the lipoprotein from cytoplasmic membrane and transport function through dislocation or even rupture of cell walls (Kitis, 2004). This hypothesis is also supported by the increase of the hydrophilic character of *S. aureus* and the decrease of the hydrophilic character of *E. coli*. The biocidal activity of SH can be largely attributed to the undissociated hypochlorous acid (HOCl) and to its dissociate form hypochlorite ion (OCl^-), whose formation is pH dependent. Hypochlorous acid can penetrate the bacteria, cross the cell wall and membranes, inhibit the activity of essential enzymes that modulates growth, damage the membrane and DNA and cause damage in the membrane transport system (Estrela *et al.*, 2002; Fukuzaki, 2006). The hydrophobicity data attained in this work also reinforces this hypothesis. The exposure of *E. coli* and *S. aureus* to SH led to a significant increase on their surface hydrophilicity. The data is in accordance with the findings of Gottardi *et al.* (2005) where the action of active chlorine (hypochlorous acid) in bacteria can be divided in two effects: non-lethal

and lethal. In the first stage reversible chlorination of the bacterial surface occurs. In the second stage penetration into the bacteria combined with irreversible cell changes occurs. In another study it was found that bacterial membrane damage was also related to changes in membrane hydrophilicity (Borges *et al.*, 2013).

In general, phytochemicals were highly efficient in causing sessile bacteria reduction from surfaces, with the exception of tyrosol and eugenol. Although tyrosol has been described as an antimicrobial agent it can be also converted to other phenolic intermediates by bacteria reducing its antimicrobial activity (Brooks *et al.*, 2006; Liebgott *et al.*, 2008; Liebgott *et al.*, 2007). On the other hand, eugenol demonstrated antimicrobial effectiveness at low concentrations (10 mM). This effect was also observed by Ali *et al.* (2005) with eugenol and cinnamaldehyde against *Helicobacter pylori*. However, in this study eugenol was not effective in the control of sessile bacteria, even if other studies were able to observe antibiofilm potential of this phytochemical against *Pseudomonas* spp., *Candida albicans* and oral bacteria (de Paula *et al.*, 2014; Magesh *et al.*, 2013; Niu *et al.*, 2004). These observations suggest that the efficacy of eugenol to control sessile bacteria appears to be species dependent.

Cinnamaldehyde, *p*-coumaric, caffeic and ferulic acids exhibited similar activities against the sessile cells, which supports the fact that these phytochemicals are known to have similarities in their mode of action, regarding bacterial surface interaction (Campos *et al.*, 2009; Johnston *et al.*, 2003; Lou *et al.*, 2012). Ghosh *et al.* (2013) demonstrated that cinnamaldehyde is able to promote bacterial surface disruption especially in association with silver nanoparticles. Cinnamaldehyde was also described as being capable to control *Pseudomonas* spp. biofilms (Niu *et al.*, 2004). The observed increase in hydrophilicity of bacteria surface after the exposure to eugenol, caffeic, *p*-coumaric and ferulic acids as well as cinnamaldehyde for both bacteria is correlated with the mechanism of action proposed for the generality of phytochemicals that includes membrane disturbance with increase in permeability (Campos *et al.*, 2009; Gill *et al.*, 2004; Lou *et al.*, 2012).

Interestingly, the action of cinnamic acid on the control of sessile bacteria was comparable to that of benchmark disinfectants and its efficiency was similar against both bacteria. Actually, it was the only phytochemical that demonstrated a high efficiency in the control of sessile bacteria. The results on the assessment of the bacterial physicochemical surface properties have shown that cinnamic acid acts on bacterial surface hydrophilicity, an effect that was more noticeable against *S. aureus.* This result is

in accordance with previous studies performed with cinnamic acid against *Listeria monocytogenes*, *E. coli* and *Pseudomonas aeruginosa* (Chambel *et al.*, 1999; Ramos-Nino *et al.*, 1996) and the yeast *Saccharomyces cerevisiae*, proposing that cinnamic acid can change the membrane properties of bacteria. Since the phytochemicals were chosen based on rational structure differences it is possible to hypothesize that the effects of cinnamic acid on the bacterial surface properties can be related to the absence of substituents in the benzene ring and the presence of the carboxylic function in its structure (Campos *et al.*, 2009; Johnston *et al.*, 2003). Although this phytochemical is recognized by several authors for its bioactive properties such as anticancer, antidiabetic, antimicrobial, antifungal and antiviral, the antibacterial mode of action of cinnamic acid is not yet completely understood (Korošec *et al.*, 2014; Sharma, 2011; Zhang *et al.*, 2014). This study provides additional data and demonstrates the potential of cinnamic acid to control sessile *E. coli* and *S. aureus*.

IV.4. Conclusion

New biocides are required for general disinfection practices, both in hospital settings and industry. This challenge has led to the search for new and alternative molecules to be used as biocides or as adjuvants/potentiators to commonly used disinfectants. In this context, phytochemicals emerged as a sustainable source of new and environmentally friendly molecules. In this work it was observed that cinnamaldehyde and eugenol can be considered antimicrobials as their MIC and MBC are comparable to the selected and frequently used biocides. Moreover, it was also found that phytochemicals, despite the absence of evident antimicrobial properties, could be used as dispersing agents of sessile cells, particularly cinnamic acid which caused total reduction of sessile *E. coli* and *S. aureus* after exposure to sub-MIC/MBC. The efficacy of cinnamic acid was similar to PA and SH and higher than that of HP, especially in the control of *S. aureus*. This phytochemical was able to modify the bacteria surface properties by decreasing their hydrophilic character. The results achieved in this study, and the accepted status of environmentally friendly and low cytotoxic of phytochemicals (Abreu *et al.*, 2012; Fresco *et al.*, 2006), reinforce their potential as new biocides and/or new adjuvants for biocidal formulations to be used in daily disinfection.

IV.5. Relevancy for thesis development

In this work, cinnamic acid stood out as well as cinnamaldehyde (Table IV.3), therefore, caffeic acid, coumaric acid, eugenol, ferulic acid and tyrosol were excluded for further studies. Based on the data, cinnamic acid was used as framework for the selection of a new set of derivatives.

In order to explore the possibility of phytochemicals and derivatives to be used for disinfection the methodology in the following work of the thesis project was based on EN 1276:2009 "Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas" (CEN, 2009). Briefly, 25 ± 3 ° was used as test temperature instead of 30 ± 3 ° and PB pH7 replaced 8.5 g L^{-1} NaCl in order to stablish a working pH of 7.

Table IV.3. Results compilation of the data presented on chapter IV, highlighting the selected phytochemicals to be used in the subsequent work

Chapter IV				
	Growth Inhibition	Kill	Removal of adhered bacteria	Alteration of bacterial hydrophobicity
Cinnamic acid	√	+	√	√
Cinnamaldehyde	√	√	+	+
Caffeic acid	√		+	+
Coumaric acid	√	+	+	+
Eugenol	√	√		+
Ferulic acid	+		+	+
Tyrosol				

√ - Accomplished for all the bacteria tested; + - Accomplished for at least one of the bacteria tested

Chapter V. Evaluation of cinnamaldehyde and cinnamic acid derivatives in microbial growth control

The conspicuous absence of novel and effective strategies to control microbial growth, both in the food and healthcare industries and the evidence of increasing microbial resistance to conventional biocides has led to a search for novel antimicrobials and growth prevention strategies. In this work, 15 phytochemicals and derivatives, structurally related to cinnamic acid and cinnamaldehyde, were assessed for their effects on the growth of E. coli, S. aureus and E. hirae. Their effects were compared to seven commonly used biocides (hydrogen peroxide, sodium hypochlorite, chlorhexidine, cetyltrimethylammonium bromide, triclosan, o-phthalaldehyde and lactic acid). All phytochemicals and derivatives increased lag phase and culture doubling time of the bacteria tested. Cinnamic acid and other related derivatives inhibited bacterial growth. The MIC of cinnamaldehyde was comparable to o-phthalaldehyde and sodium hypochlorite and lower than hydrogen peroxide and lactic acid. The effect of methyl trans-cinnamate on bacterial growth was more evident against Gram-negative bacteria. It also inhibited quorum sensing in C. violaceum. The effect of phytochemicals against sessile bacteria was low: log_{10} CFU $cm^{-2}<1$. Inhibition of quorum sensing and of bacterial growth supports the fact that phytochemicals are an interesting source of antimicrobials with potential use as biocides or more likely as additives to improve the efficacy of current biocidal formulations and preservative systems.

The work included in this chapter resulted in the publication:

Malheiro J.F., Maillard J.-Y., Borges F., Simões M., 2018. Evaluation of cinnamaldehyde and cinnamic acid derivatives in microbial growth control. International Biodeterioration & Biodegradation, in press. Doi: 10.1016/j.ibiod.2018.06.003.

V.1. Experimental details

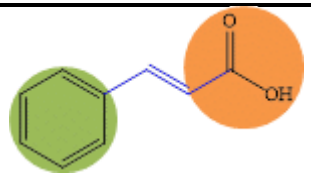
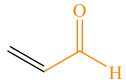
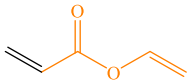
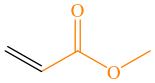
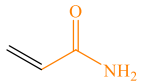
Bacteria	*E. coli* NCTC 10418, *S. aureus* NCTC 10788, *E. hirae* NCTC 13383, *C. violaceum* ATCC 12472
Phytochemicals or derivatives	3,4-(Methylenedioxy)cinnamic acid, 4-(dimethylamino)cinnamic acid, 4-chlorocinnamic acid, 4-methoxycinnamic acid, 4-nitrocinnamic acid, allyl cinnamate, cinnamaldehyde, cinnamamide, cinnamic acid, cinnamyl alcohol, hydrocinnamic acid, methyl trans-cinnamate, phenylacetone, trans-4-(trifluoromethyl)cinnamic acid, α-fluorocinnamic acid α-methylcinnamic acid, α-methylhydrocinnamic acid
Biocides	Cetyltrimethylammonium bromide (CTAB), chlorhexidine (CHX), hydrogen peroxide (HP), lactic acid (LA), o-phthalaldehyde (OPA), peracetic acid (PA), sodium hypochlorite (SH), triclosan (TRI)
Temperature	25 ± 3 ºC
Methodology used	III.2.1 Antibacterial susceptibility testing III.2.2 Efficacy against early sessile cells III.2.4 Bacterial growth evaluation III.2.5 Determination of quorum sensing inhibition using *Chromobacterium violaceum*

V.2. Results

V.2.1. Phytochemicals and derivatives antibacterial properties

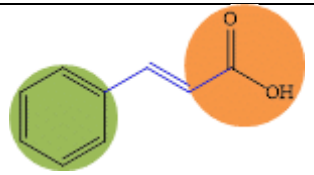
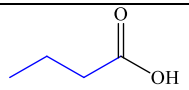
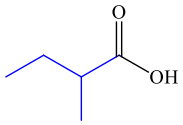
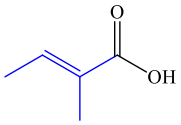
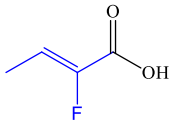
The structure and properties of the selected phytochemicals and derivatives are presented in Table V.1. The three series of compounds used in this study are presented as well as their structures, molecular weight (MW), partition coefficient (logP), topological polar surface area (TPSA), number of hydrogen bonds and chemical volume.

Table V.1. Structure and properties of the selected phytochemicals and derivatives. The three series of compounds are presented as well as their structures, molecular weight (MW), partition coefficient (logP), topological polar surface area (TPSA), number of hydrogen bonds and chemical volume. The structural and molecular properties were determined with Molinspiration Calculation Software and Chemdraw (Andrade *et al.*, 2015)

			MW (g/mol)	logP	TPSA	n- ROTB	n-OH acceptors	n- OHNH donors	Volume (A^3)
	Cinnamic acid		148.16	1.91	37.30	2	2	1	138.46
Series 1	Cinnamaldehyde		132.16	2.48	17.07	2	1	0	130.44
	Cinnamyl alcohol		134.18	2.03	20.23	2	1	1	136.28
	Allyl cinnamate		188.23	3.17	26.30	5	2	0	183.96
	Methyl trans-cinnamate		162.19	2.53	26.30	3	2	0	155.99
	Cinnamamide		147.18	1.40	43.09	2	2	2	141.73
	Phenylacetone		134.18	1.66	17.07	2	1	0	136.39

continues next page

Table V.1. Continue

			MW (g/mol)	logP	TPSA	n- ROTB	n-OH acceptors	n- OHNH donors	Volume (A^3)
Series 2	Hydrocinnamic acid		150.18	1.88	37.30	3	2	1	144.65
	α-Methylhydrocinnamic acid		164.20	2.11	37.30	3	2	1	161.24
	α-Methylcinnamic acid		162.19	2.46	37.30	2	2	1	155.02
	α-Fluorocinnamic acid		166.15	1.94	37.30	2	2	1	143.39
Series 3	4-(Dimethylamino)cinnamic acid	4-$N(CH_3)_2$	191.23	2.01	40.54	3	3	1	184.37
	4-Chlorocinnamic acid	4-Cl	182.61	2.59	37.30	2	2	1	152.00
	4-(Trifluoromethyl)cinnamic acid	4-CF_3	216.16	2.81	37.30	3	2	1	169.76
	4-Nitrocinnamic acid	4-NO_2	193.16	1.87	83.12	3	5	1	161.80
	4-Methoxycinnamic acid	4-OCH_3	178.19	1.97	46.53	3	3	1	164.01
	3,4-(Methylenedioxy)cinnamic acid	3,4-OCH_2O-	192.17	1.80	55.77	2	4	1	162.39

MIC and MBC of the phytochemicals, derivatives and biocides used along the study are provided in Table V.2. HP was ineffective against *S. aureus* as it was not possible to determine an MIC or MBC even at the highest concentration tested (450 mM). Despite being able to inhibit growth (MIC), CTAB (0.3 mM) and OPA (10 mM) were not bactericidal for *E. coli* and *E. hirae*, respectively. In the case of phytochemicals and derivatives, the maximum concentration tested was 25 mM with the exception of 4-nitrocinnamic acid (13 mM) due to solubility drawbacks in DMSO. Cinnamic acid, cinnamaldehyde, cinnamyl alcohol, cinnamamide, hydrocinnamic acid, α-methylhydrocinnamic acid, α-methylcinnamic acid and α-fluorocinnamic acid inhibited bacterial growth. Cinnamaldehyde was able to inhibit growth of all bacteria tested at low concentrations (3-8 mM) and to kill *S. aureus* at concentrations of 8-10 mM. Cinnamaldehyde MICs were comparable to SH and OPA and lower than HP and LA. Cinnamic, hydrocinnamic, α-methylhydrocinnamic, α-methylcinnamic and α-fluorocinnamic acids inhibited bacterial growth at concentrations ranging from 15-25 mM. Cinnamyl alcohol inhibited *E. coli* (8-15 mM) and *S. aureus* (20 mM) whereas cinnamamide (20-25 mM) only inhibited *E. coli* growth. Neither MIC or MBC were obtained for allyl cinnamate, methyl trans-cinnamate, phenylacetone and 4-(dimethylamino)cinnamic , 4-chlorocinnamic, trans-4-(trifluoromethyl)cinnamic, 4-nitrocinnamic, 4-methoxycinnamic, 3,4-(methylenedioxy)cinnamic acids. Compounds that have a modification on the unsaturated side-chain (series 2) presented MIC for all the bacteria tested whereas the chemicals with substituents on the benzene ring (series 3) did not result in the determination of MIC or MBC in our experimental conditions.

Considering the gathered data (lower MICs and MBCs) only three biocides were selected for further experiments, CHX, CTAB and TRI.

Table V.2. MIC and MBC of biocides, phytochemicals and derivatives used in this study. The chemicals were tested on *E. coli* NCTC 10418, *S. aureus* NCTC 10788 and *E. hirae* NCTC 13383. The concentration necessary for QSI was also determined for the phytochemicals and derivatives.* Partial inhibition, only a decrease in the purple pigment was observed

Chemical		*E. coli* NCTC 10418		*S. aureus* NCTC 10788		*E. hirae* NCTC 13383		QSI
		MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)	(mM)
HP		25—30	30	>450	> 450	3—10	5	
SH		5	5—20	8	8—20	5	15—20	
CHX		0.005	0.02—0.1	0.005	0.04—0.05	0.005	0.04—0.05	
CTAB		0.03	> 0.3	0.01—0.03	0.01—0.05	0.02	0.02—0.04	
TRI		0.003	0.01—0.03	0.003	0.02—0.03	0.02	0.02—0.04	
OPA		3—4	4—10	4	6—8	6—8	>10	
LA		50	100—200	50	100—300	50	250	
Cinnamic acid		15—20	> 25	18—20	> 25	20—25	> 25	5
Series 1	Cinnamaldehyde	3	> 25	3—5	8—10	5—8	> 25	0.5*
	Cinnamyl alcohol	8—15	> 25	20	> 25	> 25	> 25	1
	Allyl cinnamate	> 25	> 25	> 25	> 25	> 25	> 25	1
	Methyl trans-cinnamate	> 25	> 25	> 25	> 25	> 25	> 25	1
	Cinnamamide	20—25	> 25	> 25	> 25	> 25	> 25	5
	Phenylacetone	> 25	> 25	> 25	> 25	> 25	> 25	5*
Series 2	Hydrocinnamic acid	20—25	> 25	20	> 25	25	> 25	-
	α-Methylhydrocinnamic acid	15—20	> 25	15—20	> 25	20—25	> 25	-
	α-Methylcinnamic acid	15—20	> 25	15—25	> 25	15—25	> 25	5
	α-Fluorocinnamic acid	15—20	> 25	15—25	> 25	18—25	> 25	5*
Series 3	4-(Dimethylamino)cinnamic acid	> 25	> 25	> 25	> 25	> 25	> 25	5*
	4-Chlorocinnamic acid	> 25	> 25	> 25	> 25	> 25	> 25	5
	trans-4-(Trifluoromethyl)cinnamic acid	> 25	> 25	> 25	> 25	> 25	> 25	5*
	4-Nitrocinnamic acid	> 13	> 13	> 13	> 13	> 13	> 13	5
	4-Methoxycinnamic acid	> 25	> 25	> 25	> 25	> 25	> 25	5*
	3,4-(Methylenedioxy)cinnamic acid	> 25	> 25	> 25	> 25	> 25	> 25	5

V.2.2. Phytochemicals and derivatives are able to modify bacterial growth parameters

The effects of the selected phytochemicals and derivatives on bacterial growth dynamics, including the length of lag phase and doubling time, were measured (Table V.3). The results showed that the selected biocides completely inhibited bacterial growth (CHX at 0.1 mM, and CTAB and TRI at 5 mM). All the phytochemicals and derivatives decreased bacterial growth rate of at least one of the bacteria tested and were also able to affect bacterial lag phase. Cinnamaldehyde inhibited growth of *E. coli* ($p < 0.05$) and *S. aureus* ($p < 0.05$) and increased significantly ($p < 0.05$) *E. hirae* doubling time from 1.58 h to 11.82 h. The duration of the lag phase was increased by allyl cinnamate with *E. coli* (2 fold; $p < 0.05$), *S. aureus* (2.2 fold; $p < 0.05$) and *E. hirae* (2.3 fold; $p < 0.05$), by methyl trans-cinnamate with *E. coli* (10 fold; $p < 0.05$), and by 4-(dimethylamino)cinnamic acid with *S. aureus* (3.5 fold; $p < 0.05$). 4-Chlorocinnamic acid only affected the growth of *S. aureus* with a significant increase in doubling time (2.6 fold; $p < 0.05$) and a significant decrease in lag phase (2.4 fold; $p > 0.05$). Cinnamamide increased *E. coli* doubling time by 2.8 fold ($p < 0.05$) whereas 4-(trifluoromethyl)cinnamic acid increased significantly *S. aureus*' doubling time (4.9 fold; $p < 0.05$). 4-Methoxycinnamic acid and 3,4-(methylenedioxy)cinnamic acid increased *S. aureus* doubling time by 2.3 ($p < 0.05$) and 2.1 fold ($p < 0.05$), respectively.

Table V.3. Growth parameters determined for *E. coli* NCTC 10418, *S. aureus* NCTC 10788 and *E. hirae* NCTC 13383 when exposed to the phytochemicals or derivatives. Doubling time (Dt) and the length of lag phase (min) was determined with GrowthRates software (Hall *et al.*, 2014; Jung *et al.*, 2015). Values are mean ± SD of three experiments. ND – not determined.

		***E. coli* NCTC 10418**		***S. aureus* NCTC 10788**		***E. hirae* NCTC 13383**	
		Dt (h)	**Lag phase (min)**	**Dt (h)**	**Lag phase (min)**	**Dt (h)**	**Lag phase (min)**
	Control (water)	1.08 ± 0.05	70.55 ± 17.42	2.48 ± 0.18	79.55 ± 17.88	1.25 ± 0.10	72.58 ± 4.07
	Control (DMSO)	1.56 ± 0.04	77.45 ± 19.47	2.70 ± 0.25	92.33 ± 23.98	1.58 ± 0.09	91.09 ± 5.93
	Cinnamic acid	2.54 ± 0.10	71.04 ± 23.55	4.64 ± 0.49	84.38 ± 29.62	2.39 ± 0.34	89.97 ± 10.88
Series 1	Cinnamaldehyde	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	11.82 ± 2.75	ND
Series 1	Cinnamyl alcohol	2.61 ± 0.24	73.28 ± 12.76	4.07 ± 0.38	101.85 ± 36.45	2.05 ± 0.26	101.58 ± 15.36
Series 1	Allyl cinnamate	2.66 ± 0.30	157.07 ± 18.89	4.26 ± 0.32	199.38 ± 44.15	2.96 ± 0.40	213.06 ± 55.49
Series 1	Methyl trans-cinnamate	2.92 ± 0.57	778.11 ± 48.97	4.06 ± 0.13	146.99 ± 59.51	3.24 ± 1.04	133.88 ± 29.75
Series 1	Cinnamamide	2.22 ± 0.13	107.45 ± 29.62	3.52 ± 0.11	95.80 ± 24.42	1.85 ± 0.14	96.17 ± 12.16
Series 1	Phenylacetone	1.82 ± 0.19	86.53 ± 13.76	3.32 ± 0.27	88.49 ± 32.64	1.68 ± 0.26	105.69 ± 13.05
Series 2	Hydrocinnamic acid	2.80 ± 0.39	63.00 ± 12.55	4.04 ± 0.26	105.48 ± 36.91	1.99 ± 0.40	97.50 ± 14.16
Series 2	α-Methylhydrocinnamic acid	2.66 ± 0.36	71.31 ± 16.01	4.40 ± 0.51	136.84 ± 25.89	2.17 ± 0.25	97.81 ± 21.51
Series 2	α-Methylcinnamic acid	2.41 ± 0.26	95.25 ± 13.73	5.13 ± 0.85	110.98 ± 41.38	2.37 ± 0.40	105.62 ± 20.66
Series 2	α-Fluorocinnamic acid	2.04 ± 0.20	91.76 ± 22.84	5.14 ± 0.87	103.13 ± 33.46	3.38 ± 0.17	53.31 ± 15.82
Series 3	4-(Dimethylamino)cinnamic acid	1.70 ± 0.04	90.71 ± 17.55	5.18 ± 0.64	323.59 ± 56.07	2.24 ± 0.48	127.71 ± 11.13
Series 3	4-Chlorocinnamic acid	2.83 ± 0.06	99.75 ± 33.16	7.08 ± 2.61	38.56 ± 4.00	3.41 ± 0.18	78.04 ± 26.89
Series 3	Trans-4-(Trifluoromethyl)cinnamic acid	2.48 ± 0.17	98.76 ± 19.82	9.85 ± 1.56	ND	4.53 ± 0.70	87.84 ± 13.79
Series 3	4-Nitrocinnamic acid	2.24 ± 0.34	70.93 ± 19.55	6.80 ± 1.78	104.52 ± 40.66	1.89 ± 0.21	98.79 ± 14.55
Series 3	4-Methoxycinnamic acid	2.08 ± 0.10	85.69 ± 23.15	6.10 ± 1.53	109.35 ± 45.28	2.31 ± 0.50	96.50 ± 19.50
Series 3	3,4-(Methylenedioxy)cinnamic acid	2.40 ± 0.03	117.36 ± 21.58	5.74 ± 1.51	115.93 ± 43.28	2.72 ± 0.26	99.56 ± 20.50

ND – not determined

V.2.3. Sessile bacteria removal efficacy

The effect of the phytochemicals, derivatives and biocides was evaluated against monolayer sessile bacteria after 30 min exposure to (Figure V.1). The three biocides tested caused total CFU reduction of sessile cells regardless of the bacteria (data not shown). Considering the phytochemicals and derivatives, a generally low CFU reduction ($\log_{10}$ CFU cm^{-2} < 1) was observed. Cinnamaldehyde caused 0.8 $\log_{10}$ CFU cm^{-2} reduction ($p > 0.05$) of *E. coli*. Against sessile *S. aureus*, only 0.4 $\log_{10}$ CFU cm^{-2} reduction was achieved with α-fluorocinnamic ($p < 0.05$) and α-methylcinnamic ($p < 0.05$) acids. Cinnamic, hydrocinnamic, α-methylhydrocinnamic, α-methylcinnamic and α-fluorocinnamic acids were able to cause 0.8 to 0.9 $\log_{10}$ CFU cm^{-2} reduction of sessile *E. hirae* ($p < 0.05$). Cinnamic acid and the derivatives with a modification on the unsaturated side-chain (series 2) demonstrated the best CFU reduction against all the bacteria tested. The remaining chemicals were ineffective in CFU sessile reduction from the polystyrene surfaces. The derivatives with substituents on the benzene ring (series 3) did not show any activity.

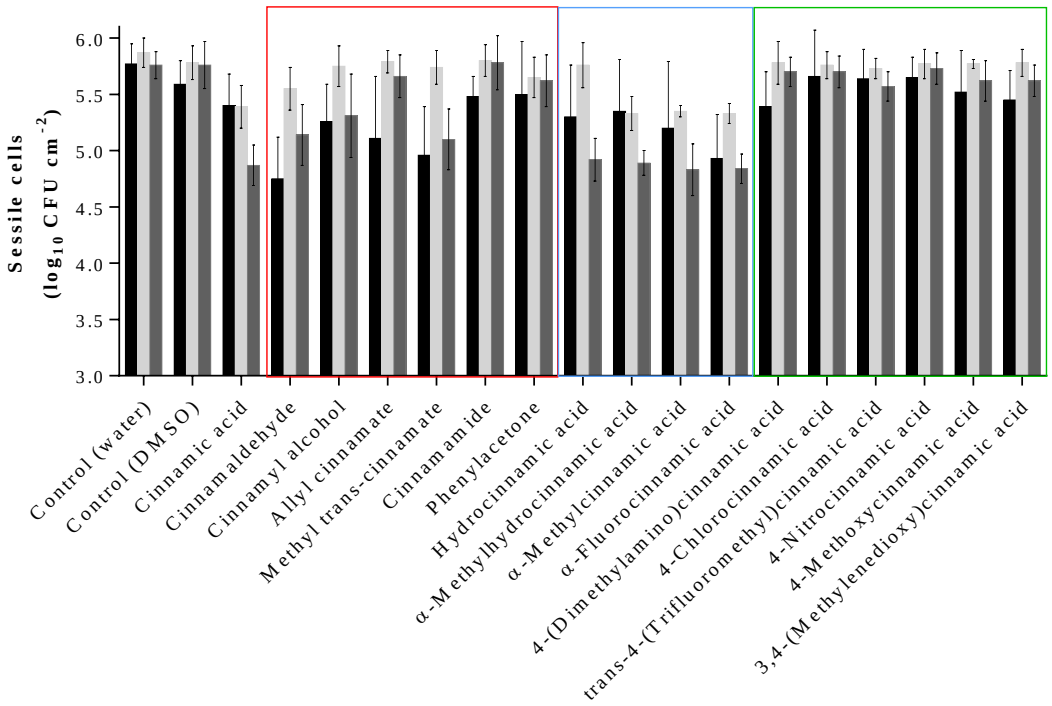


Figure V.1. Effects of the selected phytochemicals and derivatives on the control of sessile *E. coli* NCTC 10477 (black), *S. aureus* NCTC 10788 (light grey) and *E. hirae* NCTC 13383(dark grey). The figure presents the remaining CFU of sessile bacteria after 30 min exposure to the selected chemicals from series 1 (orange), 2 (blue) and 3 (green). Values are mean ± SD of three experiments.

V.2.4. Quorum sensing inhibition by phytochemicals and derivatives

Results of quorum sensing inhibition are shown in Table V.2. All the phytochemicals and derivatives, with the exception of hydrocinnamic and α-methylhydrocinnamic acids, were able to inhibit quorum sensing of *C. violaceum*. Moreover, cinnamaldehyde, cinnamyl alcohol, allyl cinnamate and methyl trans-cinnamate were able to inhibit *C. violaceum* quorum sensing system with low concentrations; 0.5 mM in the case of cinnamaldehyde and 1 mM for the other three cinnamic derivatives.

V.3. Discussion

Phytochemicals have the potential to be used as antimicrobial agents directly or as adjuvants for improving the efficacy of existing formulations against both planktonic and sessile bacteria (Aldulaimi, 2017; Barbieri *et al.*, 2017; Khatri *et al.*, 2016; Monte *et al.*, 2014). From the data included in chapter IV, it was concluded that cinnamaldehyde and cinnamic acid are the best phytochemicals as they demonstrated to have significant antibacterial and dispersal activities. Cinnamaldehyde had a MIC (3 and 5 mM) and MBC (10 and 12 mM) against *E. coli* and *S. aureus* while cinnamic acid was able to completely remove the adhered bacteria after their exposure to the phytochemical for 1 hour. This knowledge was taken into consideration for the selection of the 15 phytochemicals and derivatives used in the work presented in this chapter. The selected phytochemicals and derivatives present structure similarities with cinnamaldehyde and cinnamic acid having chemicals modifications on carboxyl group (series 1), unsaturated side-chain (series 2) or in the benzene ring (series 3).

Phytochemicals and derivatives were characterized in terms of MW, logP, TPSA, number of hydrogen bonds and chemical volume (Table V.1). This information helps to understand putative membrane permeation and absorption ability by the chemicals (Lipinski, 2004; Lipinski *et al.*, 1997; Silva *et al.*, 2016). Particularly LogP, which is a measurement of hydrophobicity (ratio of concentrations of a chemical in a mixture of octanol/water, and TPSA can give an insight on the membrane permeability properties (Lipinski *et al.*, 1997). These parameters were chosen based on their correlation with the drug-likeness of a molecule for cell membranes (Andrade *et al.*, 2015; Kerns *et al.*, 2008b;

Lipinski, 2004). The term drug-like is always dependent on the mode of administration but is intrinsically associated with aqueous solubility, lipophilicity and membrane permeability and bioavailability (Kerns *et al.*, 2008a). In the work presented in this chapter, this strategy was used to understand the role of the structure of the selected phytochemicals and derivatives on their interaction with the bacteria.

V.3.1. Antimicrobial action

Initially the determination of MIC and MBC of the biocides and all the phytochemicals and derivatives as well as of the biocides was performed to confirm their antibacterial activity. The ineffectiveness of HP against *S. aureus* may have resulted from the expression of catalase (Park *et al.*, 2008), although this was not confirmed in this study. The majority of the biocides killed all the bacteria tested (excepting HP against *S. aureus*, CTAB against *E. coli* and OPA against *E. hirae*), confirming their broad spectrum of action (Abreu *et al.*, 2013; Otter *et al.*, 2015; Russell, 2003; Wand, 2017). In addition, in this work 5% of ethanol was used in the experiments that involved CHX, therefore, a synergistic effect, and a high activity of CHX was observed (Mulberrry *et al.*; Sogawa *et al.*, 2010). The ineffectiveness of CTAB against *E. coli* and OPA against *E. hirae* was possibly associated with the concentration used, which can be low for these bacteria. For the MIC and MBC assay, the higher concentration of each biocide tested was limited by their solubility. Another possibility is that the bacteria tested could be resistant to CTAB and OPA. Although this mechanism was not ascertained in this study, we must highlight that bacterial resistance to both biocides has been extensively reported in the literature (Chapman, 2003; Coulon *et al.*, 2010; Herruzo *et al.*, 2017; Nakata *et al.*, 2011; Rajagopal *et al.*, 2014). The majority of the phytochemicals and derivatives tested were not able to inhibit or kill the bacteria used in this study (MIC and MBC > 25 mM). The most promising was cinnamaldehyde with MIC (*E. coli*, *S. aureus* and *E. hirae*) and MBC (*S. aureus*) comparable or lower than those of HP, SH, OPA and LA. Cinnamaldehyde antibacterial activity has already been documented for other bacterial strains (Kollanoor Johny *et al.*, 2010; Siddiqua *et al.*, 2015). In fact, the MIC determined in the work presented in Chapter IV for *S. aureus* (5 mM) and *E. coli* (3 mM) are in accordance with the values presented in this Chapter. However, Siddiqua *et al.* (2015) was able to determine MIC of cinnamaldehyde for *E. coli* (38 mM), *S. aureus* (14 mM), *Bacillus cereus* (15 mM) and *Yersinia enterocolitica* (38 mM) and Kollanoor Johny *et al.* (2010) has observed that the exposure of *Salmonella enteritis* to 10 mM of cinnamaldehyde

resulted in 6 $\log_{10}$ CFU mL^{-1} reduction after 8 h exposure or in complete killing after 24 h, in the case of *Campylobacter jejuni*.

V.3.2. Influence on bacterial growth

To evaluate the effect of phytochemicals and derivatives on bacterial growth, doubling time and lag phase length were calculated. The efficacy (MIC/MBC) obtained for the biocides and cinnamaldehyde was confirmed by the complete inhibition of growth. Interestingly, despite the antimicrobial inefficiency (MIC > 25 mM) of the majority of the phytochemicals and derivatives, they were able to affect bacterial growth at 5 mM, either increasing doubling time or increasing the duration of the lag phase. Methyl trans-cinnamate was particularly efficient in increasing *E. coli* lag phase (by 10 fold: from 77.45 ± 19.47 to 778.11 ± 48.97 min). Huang *et al.* (2009) also reported that methyl trans-cinnamate increased lag phase length and decreased exponential phase at 3.1 mM for *E. coli* and completely inhibited growth at 3.1 mM for *S. aureus* and 6.2 mM for *E. coli*.

V.3.3. Exposure of sessile cells to phytochemicals and derivatives

Bacteria adhere and contaminate hospital surfaces at levels of 10^4-10^6 CFU cm^{-2}, and, therefore, this was the number used in this study for all the bacteria tested (Dancer, 2004; Otter *et al.*, 2015). The biocides tested in this study completely controlled the adhered bacteria reinforcing their high efficiency (Abreu *et al.*, 2013; Otter *et al.*, 2015; Wand, 2017). Despite the low efficacy of all the phytochemicals and derivatives ($\log_{10}$ CFU cm^{-2} removal< 1) it was possible to observe that sessile *S. aureus* was the most resistant (maximum of 0.4 $\log_{10}$ CFU cm^{-2} reduction), whereas *E. hirae* was the most susceptible bacterium (0.9 $\log_{10}$ CFU cm^{-2} reduction). α-Methylcinnamic and α-fluorocinnamic were the only chemicals capable of removing *E. coli* (0.4 and 0.7 $\log_{10}$ CFU cm^{-2}, respectively), *S. aureus* (0.4 $\log_{10}$ CFU cm^{-2}) and *E. hirae* (0.9 $\log_{10}$ CFU cm^{-2}).

Previous studies and the data presented in Chapter IV, have reported, higher levels of efficacy for some of the phytochemicals used along this project, namely cinnamic acid and cinnamaldehyde (Ali *et al.*, 2005). However, it is important to take into account experimental differences, particularly the bacterial culture methods. In the case of the assays described in Chapter IV, cinnamic acid caused total CFU reduction (4.89 $\log_{10}$ CFU cm^{-2} of *E. coli* and 5.21 $\log_{10}$ CFU cm^{-2} of *S. aureus*) of sessile bacteria at 30 ± 3 ºC after 1 h contact time in NaCl (8.5 g L^{-1}) solution. In the present work the pH

was constantly at 7 (controlled by the use of PB pH7), the contact time was 30 min and the bacteria used were *S. aureus* NCTC 10788, *E. coli* NCTC 10418 and *E. hirae* NCTC 13383 (instead of *S. aureus* CECT 976 and *E. coli* CECT 434). pH is an important parameter since in this case, the chemicals are organic acids with a pKa of ≈ 4.2 and it is thought that their effectiveness is dependent on the concentration of undissociated acid (Ali *et al.*, 2005; Campos *et al.*, 2009; Johnston *et al.*, 2003). When a lower pH is used, the antimicrobial activity of phenolic acids tend to increase since the concentration of undissociated phenolic acids (more lipophilic) is higher, making them more permeable in cytoplasmic membranes (Sánchez-Maldonado *et al.*, 2011).

Since the selection of the phytochemicals and derivatives was made considering structural similarities, it is interesting to note that cinnamic, hydrocinnamic, α-methylhydrocinnamic, α-methylcinnamic and α-fluorocinnamic acids share a structural similarity that includes the presence of the carboxylic group and the absence of substitutes in the benzene ring. These similarities also result in similar logP (1.91, 1.88, 2.11, 1.94 and 2.46) and equal TPSA (37.30 Å) and hydrogen acceptor (2) and donor (1) bonds. Therefore, it was shown in this work the significance of the carboxylic group, and the absence of substituents in the benzene ring due to their efficacy in sessile bacteria. The same tendency was observed in previous studies (Campos *et al.*, 2009). Despite the low CFU reduction, these chemicals were able to affect all the bacteria tested.

V.3.4. Quorum sensing inhibition

Quorum sensing is an important mechanism of bacterial communication that can influence antimicrobial susceptibility, bacterial growth and ability to form biofilms as it controls enzyme secretion, bioluminescence, virulence factors production and biofilm formation (Gilbert *et al.*, 2003b; Niu *et al.*, 2006; Otter *et al.*, 2015; Si *et al.*, 2017). In fact, Borges *et al.* (2017) demonstrated that the antibiotic furvina, by interaction with LasI/LasR system, inhibits the 3-oxo-C12-HSL-dependent QS system of *P. aeruginosa* and was also able to reduce biofilm formation and QS controlled virulence factors. Myszka *et al.* (2016) has also demonstrated that *Thymus vulgare* essential oil, carvacrol and thymol were effective against the production of quorum sensing autoinducers and therefore bacterial motility (reduction of flagella gene) was significantly suppressed and consequently, biofilm formation was inhibited. Several studies already reported the ability of phytochemicals to inhibit quorum sensing (Amaya *et al.*, 2012; Borges *et al.*, 2017; Si *et al.*, 2017; Zhou *et al.*, 2013). In fact, the quorum sensing inhibition properties

of cinnamaldehyde has already been described against *E. coli* (Niu *et al.*, 2006). In this work, the selected phytochemicals and derivatives were also assessed on their ability to inhibit quorum sensing system of *C. violaceum*, homologs of the LuxI/LuxR system (Borges *et al.*, 2014a; Borges *et al.*, 2017; Niu *et al.*, 2006). This system is well described, and proposed to be the main quorum sensing system for Gram-negative bacteria (Borges *et al.*, 2014a). In this work it was found that cinnamic acid, cinnamamide, α-methylcinnamic acid, 4-chlorocinnamic acid, 4-nitrocinnamic acid and 3,4-(methylenedioxy)cinnamic acid were able to completely inhibit quorum sensing at 5 mM and cinnamyl alcohol, allyl cinnamate and methyl trans-cinnamate at 1 mM. Cinnamic acid, cinnamamide and 4-chlorocinnamic acid capacity to interfere with quorum sensing mechanism had already been described for bacteria that produce *N*-acyl homoserine lactone quorum sensing signals (Brackman *et al.*, 2011a; Joshi *et al.*, 2016). The lack of quorum sensing inhibition properties of hydrocinnamic acids had also been observed by Brackman *et al.* (2011a) in *Vibrio* spp. quorum sensing system. In the same study, cinnamyl alcohol and methyl trans-cinnamate were ineffective as quorum sensing inhibitors. On the other hand, cinnamaldehyde quorum sensing inhibition of *Vibrio* spp. was more noticeable than on *C. violaceum* system (Brackman *et al.*, 2011a). However, in this study it was a observed complete inhibition of *C. violaceum* quorum sensing at a concentration of 1 mM.

V.4. Conclusion

Cinnamic acid, cinnamaldehyde and their derivatives have shown to cause a significant bacterial growth control. Almost all derivatives affected bacterial growth parameters (doubling time and lag phase length) although cinnamaldehyde was able to inhibit planktonic growth at low concentrations. It was also possible to observe that the structure of phytochemicals and derivatives influenced their antimicrobial activity against both planktonic and sessile bacteria. In general, the presence of carboxylic group and the absence of substituents on the benzene ring lead to an enhancement of the antimicrobial action. However, the effect of the compounds against sessile cells were almost negligible compared to the action of the benchmarked biocides. Moreover, the selected phytochemicals and derivatives inhibited quorum sensing. Altogether, the

phytochemicals and derivatives positively affect the antimicrobial activity, bacterial growth and quorum sensing. Furthermore, their modest action against sessile cells reinforces their potential to be used as additives and/or potentiators of currently used biocidal formulations.

V.5. Relevancy for thesis development

In the work included in this chapter cinnamic acid, cinnamaldehyde, cinnamyl alcohol, allyl cinnamate, methyl tans-cinnamate, cinnamamide and the derivatives belonging to series 2 stood out for their relevant action (Table V.4). So, phenylacetone, and all the derivatives from series 3 were excluded from further studies.

Among the tested biocides CTAB and LA were selected taking into consideration the results obtained in this chapter, their chemical stability and toxicity properties. These widely used biocides, will be employed in a subsequent combination study with the selected phytochemicals and derivatives.

Table V.4. Results compilation of the data presented on chapter V, highlighting the selected phytochemicals to be used in the subsequent work

		Chapter V					
		Growth inhibition	Kill	QSI	Growth curve modification		Removal of adhered bacteria
					Lag phase	dt	
	Cinnamic acid	√		√		√	+
Series 1	Cinnamaldehyde	√	+	√	√	√	+
	Cinnamyl alcohol	+		√		√	
	Allyl cinnamate			√	√	√	+
	Methyl trans-cinnamate			√	√	√	+
	Cinnamamide	+		√	+	√	
	Phenylacetone			√		+	
Series 2	Hydrocinnamic acid	√			+	√	+
	α-Methylhydrocinnamic acid	√			+	√	+
	α-Methylcinnamic acid	√		√	+	√	+
	α-Fluorocinnamic acid	√		√	+	√	+
Series 3	4-(Dimethylamino)cinnamic acid			√	+	√	
	4-Chlorocinnamic acid			√	√	√	
	trans-4-(Trifluoromethyl)cinnamic acid			√	+	√	
	4-Nitrocinnamic acid			√		√	
	4-Methoxycinnamic acid			√	+	√	
	3,4-(Methylenedioxy)cinnamic acid			√	+	√	

√ - Accomplished for all the bacteria tested; + - Accomplished for at least one of the bacteria tested

Chapter VI. Biocide potentiation using cinnamic phytochemicals and derivatives

Surface disinfection is of utmost importance in the prevention of bacterial infections. This work aims to assess the ability of ten phytochemicals and related derivatives as potentiators of two commonly used biocides — CTAB and LA. LA in combination with cinnamic, hydrocinnamic, α-methylcinnamic, and α-fluorocinnamic acids had a FICI ≤ 1 for E. coli and S. aureus. Several phytochemicals or derivatives in combination with biocides improved the biocidal efficacy against early sessile bacteria. The most effective combination was LA with allyl cinnamate (2.98 ± 0.76 log_{10} CFU cm^{-2} reduction) against E. coli. The combination with CTAB of phytochemicals or derivatives was successful with a maximum bactericidal efficacy against sessile E. coli when combined with allyl cinnamate (2.20 ± 0.07 log_{10} CFU cm^{-2} reduction) and for S. aureus when combined with α-methylcinnamic acid (1.68 ± 0.30 log_{10} CFU cm^{-2} reduction). This work highlights the potential of phytochemicals and their derivatives to be used in biocide formulations

The work included in this chapter resulted in the publication:

Malheiro J.F., Maillard J.-Y., Borges F., Simões M., 2019. Biocide potentiation using cinnamic phytochemicals and derivatives. Molecules, 24(21):1-15. Doi: 10.3390/molecules24213918.

VI.1. Experimental details

Bacteria	*E. coli* NCTC 10418, *S. aureus* NCTC 10788, *E. hirae* NCTC 13383
Phytochemicals or derivatives	Allyl cinnamate, cinnamaldehyde, cinnamamide, cinnamic acid, cinnamyl alcohol, hydrocinnamic acid, methyl trans-cinnamate, α-fluorocinnamic acid, α-methylcinnamic acid, α-methylhydrocinnamic acid
Biocides	Cetyltrimethylammonium bromide (CTAB), lactic acid (LA)
Temperature	25 ± 3 ºC
Methodology used	III.2.2. Efficacy against early sessile cells III.2.3 Physicochemical characterization of bacterial surfaces III.2.6. Bacterial susceptibility by the checkerboard methodology

VI.2. Results

VI.2.1. Phytochemicals and derivatives potentiate biocides in growth control

The checkerboard method was used to test a wide range of concentrations of phytochemicals/derivatives and biocides (LA and CTAB). Among the concentrations tested, a combination for each phytochemical/derivative with each biocide was selected (Table VI.1). Cinnamyl alcohol and cinnamamide were the only phytochemicals/derivatives that did not potentiate any biocide (FICI > 1). Cinnamic acid, allyl cinnamate and all the phytochemicals and derivatives tested that have a modified side chain (hydrocinnamic, α-methylhydrocinnamic, α-methylcinnamic and α-fluorocinnamic acids) were able to potentiate LA (FICI ≤ 1). The combination of LA with cinnamic acid, hydrocinnamic acid or α-methylcinnamic acid had FICI ≤ 1 for *E. coli* and *S. aureus*, while α-fluorocinnamic acid was able to potentiate LA effect towards *E. coli* (FICI = 0.8), *S. aureus* (FICI = 0.7) and *E. hirae* (FICI = 1). Cinnamaldehyde and methyl trans-cinnamate were able to decrease the CTAB concentration needed to inhibit bacterial growth.

Table VI.1. Phytochemicals and derivatives concentration used in combination with LA or CTAB. The concentrations were determined by the checkerboard methodology where FICI was calculated for all the bacteria under study and the best value for at least two bacteria was chosen. If no potentiation was detected, the combination was chosen taking into consideration the phytochemical or derivative concentration used with both biocides and where solubility was not compromised. Potentiation with a given biocide is highlighted in bold and the FICI value is presented in parentheses as well as the correspondent bacterium (*E. coli* NCTC 10418, *S. aureus* NCTC 10788, *E. hirae* NCTC 13383)

Phytochemical/derivative (mM)		**Combination with**			
		LA (mM)		**CTAB (mM)**	
		Concentration (mM)	Bacterium (FICI)	Concentration (mM)	Bacterium (FICI)
Cinnamic acid	5	**20**	*E. coli* (0.8) *S. aureus* (0.9)	0.01	-
Cinnamaldehyde	0.5	300	-	**0.015**	*S. aureus* (0.9)
Cinnamyl alcohol	5	40	-	0.015	-
Allyl cinnamate	5	**200**	*E. coli* (1)	0.015	-
Methyl trans-cinnamate	5	40	-	**0.015**	*E. coli* (0.8)
Cinnamamide	5	40	-	0.015	-
Hydrocinnamic acid	8	**15**	*E. coli* (0.9) *S. aureus* (0.9)	0.015	-
α-Methylhydrocinnamic acid	5	**30**	*S. aureus* (1)	0.015	-
α-Methylcinnamic acid	3	**20**	*E. coli* (0.8) *S. aureus* (0.7)	0.015	-
α-Fluorocinnamic acid	5	**15**	*E. coli* (0.8) *S. aureus* (0.7) *E. hirae* (1)	0.015	-

VI.2.2. Biocide-phytochemical or derivative combinations reduced early sessile bacteria

As some of the combinations tested have shown to potentiate bactericidal efficacy against the selected bacteria further studies were performed to understand their effects against bacteria in an early sessile state. Two hours adhered bacteria were exposed to the phytochemical or derivatives and the biocide, alone or in combination, for 30 min. When the phytochemicals or derivatives and LA were used alone it was observed that *E. coli* CFU reduction was higher than for *S. aureus* (Figure VI.1). LA was particularly efficient in reducing the viability of sessile *E. coli.* The highest reduction was 2.26 $\log_{10}$ CFU cm^{-2} after exposure to 40 mM LA. LA was only able to reduce *S. aureus* viability by 0.39

$\log_{10}$ CFU cm^{-2} at a concentration higher than 40 mM, with a maximum reduction of 0.87 $\log_{10}$ CFU cm^{-2} observed after exposure to 200 mM of LA. CTAB caused similar CFU reduction of *E. coli* and *S. aureus*. The efficiency of the majority of the combinations of phytochemicals or derivatives with LA on sessile *E. coli* (Figure VI.1, top left) and *S. aureus* (Figure VI.1, top right) was not significant ($p > 0.05$) in comparison with the exposure to LA. However, the CFU reduction of adhered *E. coli* when LA was used in combination with cinnamic acid was significant in comparison to the biocide ($p < 0.05$) and the phytochemical alone ($p < 0.001$). The same effect was observed when LA was combined with allyl cinnamate ($p < 0.05$) or hydrocinnamic acid ($p < 0.01$). On the other hand, CFU reduction efficiency of CTAB combined with phytochemicals or derivatives highlight some promising results (Figure VI.1 - down left for *E. coli* and right for *S. aureus*). In fact, the combination of α-methylhydrocinnamic acid ($p < 0.001$) and α-fluorocinnamic acid ($p < 0.001$) with CTAB had a higher efficiency in reducing CFU of sessile *E. coli* and *S. aureus* than the phytochemical or derivative or the biocide alone. The combination of cinnamaldehyde ($p < 0.01$) and allyl cinnamate ($p < 0.01$) with CTAB when used against sessile *E. coli* was more efficient than exposing the bacterium to these compounds or the biocide alone. For *S. aureus*, the combinations with increased efficiency were CTAB with cinnamic acid ($p < 0.01$), cinnamyl alcohol ($p < 0.01$), hydrocinnamic acid ($p < 0.001$) and methylcinnamic acid ($p < 0.001$). Considering the significant CFU reduction caused by the combination of a phytochemical or derivatives with CTAB, the most significant effects were achieved using allyl cinnamate and α-methylhydrocinnamic acid, a sessile *E. coli* reduction of 2.20 ± 0.07 $\log_{10}$ CFU cm^{-2} and 2.12 ± 0.03 $\log_{10}$ CFU cm^{-2}, respectively. Exposing *S. aureus* to the combinations caused reductions of 1.68 ± 0.30 $\log_{10}$ CFU cm^{-2}, 1.59 ± 0.04 $\log_{10}$ CFU cm^{-2} and 1.43 ± 0.37 $\log_{10}$ CFU cm^{-2} when exposed to α-methylcinnamic acid ($p < 0.001$), hydrocinnamic acid ($p < 0.001$) and α-methylhydrocinnamic acid ($p < 0.001$), respectively.

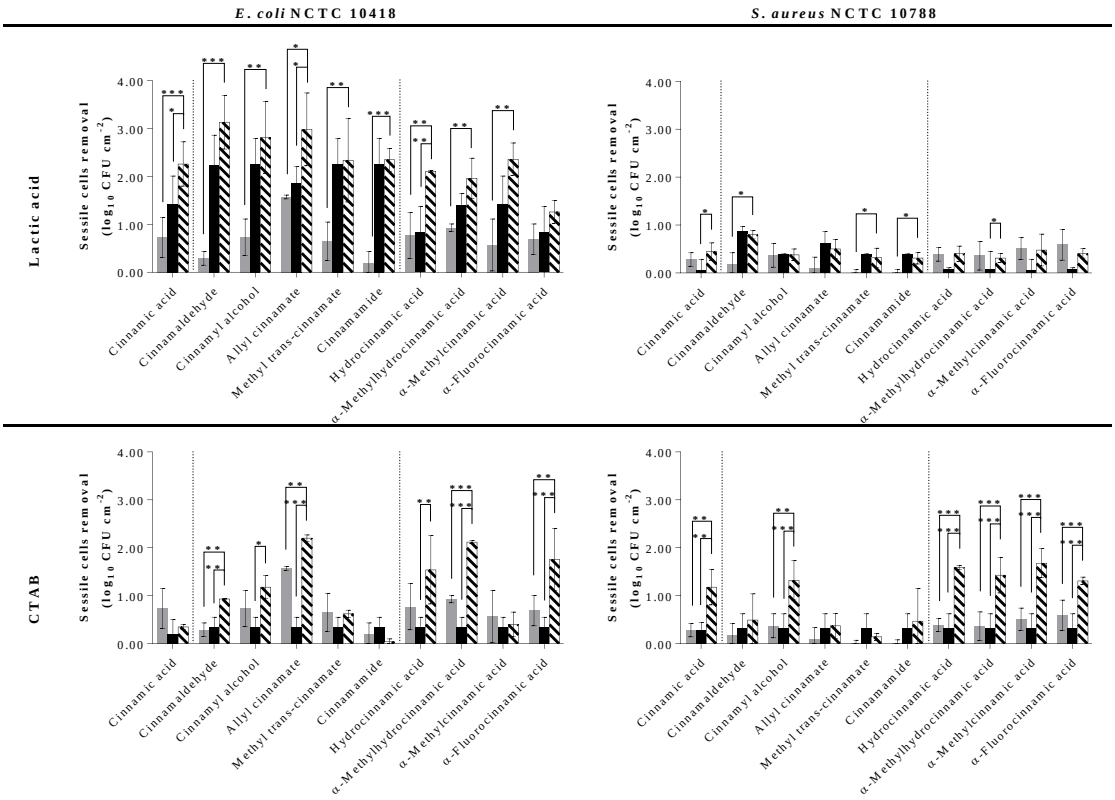


Figure VI.1. Effect of the combination of lactic acid (top) or CTAB (bottom) with the phytochemicals or derivatives on *E. coli* NCTC 10418 (left) and *S. aureus* NCTC 10788 (right). Each bacterium was exposed for 30 min to the established concentration of the phytochemical or derivative (grey columns) and biocide (lactic acid or CTAB; black columns) alone and in combination (dashed columns). Bacteria were exposed to the phytochemicals or derivatives concentrations presented in Table VI.1. Values are mean ± SD. The statistical significance is represented (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

VI.2.3. Phytochemicals and derivatives effects on bacterial surface hydrophobicity

The phytochemicals and derivatives causing the highest biocidal potentiation were selected to study their effects on the cell surface physico-chemical parameters, particularly in the hydrophobicity. Allyl cinnamate was excluded as its antimicrobial activity was limited to *E. coli* and α-fluorocinnamic acid was not considered due to its high cost price (Table VI.2). Exposing *S. aureus* and *E. hirae* to the phytochemicals or derivatives for 30 min did not exert any significant alteration to the bacteria hydrophobicity or the surface tension parameters ($p > 0.05$) (Table VI.3). Howeverα-methylcinnamic acid was able to decrease the surface hydrophobicity ($p < 0.01$) on *E. coli* after 30 min exposure. In addition, it decreased the apolar properties ($p < 0.001$) and increased the polar ones ($p < 0.001$) as well as the capacity to accept electrons ($p < 0.001$).

α-Methylhydrocinnamic acid was also able to increase the polar properties ($p < 0.001$) and the ability to accept electrons ($p < 0.05$) by *E. coli* surface. Interestingly, cinnamic acid increased *E. coli* polar properties and the capacity to accept electrons ($p > 0.05$).

Table VI.2. Technical information of the phytochemicals and derivatives and biocides used in this study. [a] The price per 1 g of product corresponds to the price of the chemicals when purchased by the research group in 2015. Some information was adapted from Chapters III and V

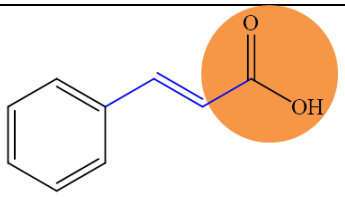
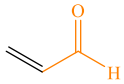
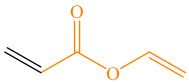
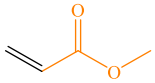
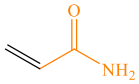
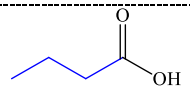
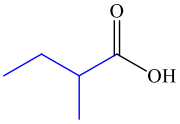
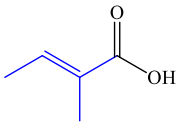
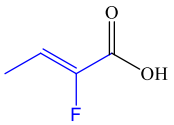
Phytochemical/derivative	Brand	CAS number	Price per 1 g (€)[a]
 Cinnamic acid	Merck	140-10-3	4.86
Cinnamaldehyde 	Sigma Aldrich	14371-10-9	0.05
Cinnamyl alcohol 	Acros Organics	104-54-1	0.15
Allyl cinnamate 	Sigma Aldrich	1866-31-5	0.49
Methyl trans-cinnamate 	Merck	1754-62-7	0.12
Cinnamamide 	Alfa Aesar	621-79-4	7.22
Hydrocinnamic acid 	Acros Organics	501-52-0	0.31
α-Methylhydrocinnamic acid 	Acros Organics	1009-67-2	12.02
α-Methylcinnamic acid 	Acros Organics	1199-77-5	2.64
α-Fluorocinnamic acid 	Sigma Aldrich	350-90-3	91.90
Biocide	**Brand**	**CAS number**	**Price per 1 g (€)[a]**
CTAB	Acros Organics	57-09-0	0.26
LA	Fluka	50-21-5	0.14

Table VI.3. Effect of the selected phytochemicals and derivatives on the surface tension parameters and hydrophobicity of *E. coli* NCTC 10418, *S. aureus* NCTC 10788 and *E. hirae* NCTC 13383. Statistically significant values (* $p<0.05$; ** $p<0.01$;*** $p<0.001$) when compared to the control of DMSO are highlighted in bold. Bacteria were exposed to the phytochemicals and derivatives concentrations presented in Table VI.1. Values are mean ± SD

		Surface tension parameters (mJ m^{-2})				**Hydrophobicity (mJ m^{-2})**
		γ_s^{LW}	γ_s^{AB}	γ_s^{+}	γ_s^{-}	ΔG_{sws}^{TOT}
E. coli NCTC 10418	Control (water)	33.43 ± 1.98	13.74 ± 3.65	1.03 ± 0.52	48.78 ± 3.08	28.98 ± 4.49
	Control (DMSO)	31.99 ± 1.36	15.78 ± 1.99	1.26 ± 0.32	50.11 ± 4.16	29.91 ± 4.98
	Cinnamic acid	29.67 ± 3.33	21.63 ± 1.76	2.45 ± 0.34	47.81 ± 1.49	24.65 ± 1.64
	Cinnamaldehyde	32.96 ± 0.61	12.78 ± 2.02	0.92 ± 0.27	45.02 ± 3.13	24.84 ± 3.73
	Hydrocinnamic acid	30.48 ± 1.04	19.14 ± 2.34	1.95 ± 0.49	47.63 ± 1.14	25.69 ± 2.90
	α-Methylhydrocinnamic acid	28.55 ± 1.19	**24.40 ± 1.86****	**3.00 ± 0.45***	49.87 ± 0.68	25.75 ± 0.80
	α-Methylcinnamic acid	**21.91 ± 4.42*****	**31.60 ± 4.80*****	**5.51 ± 1.76*****	46.42 ± 1.86	**19.08 ± 4.13****
S. aureus NCTC 10788	Control (water)	35.26 ± 1.18	18.01 ± 2.09	1.71 ± 0.52	48.68 ± 4.23	25.80 ± 5.62
	Control (DMSO)	36.24 ± 1.19	17.56 ± 0.68	1.58 ± 0.09	48.92 ± 4.14	25.80 ± 4.73
	Cinnamic acid	34.79 ± 1.73	19.35 ± 0.75	1.83 ± 0.14	51.07 ± 0.66	27.94 ± 1.31
	Cinnamaldehyde	35.70 ± 0.52	17.25 ± 3.06	1.51 ± 0.51	50.19 ± 2.08	27.76 ± 3.06
	Hydrocinnamic acid	34.36 ± 1.55	19.60 ± 1.25	1.89 ± 0.14	50.79 ± 3.03	27.62 ± 3.05
	α-Methylhydrocinnamic acid	34.49 ± 2.20	16.93 ± 0.90	1.34 ± 0.09	53.59 ± 2.47	32.40 ± 3.09
	α-Methylcinnamic acid	35.82 ± 1.04	16.68 ± 1.09	1.32 ± 0.18	52.98 ± 1.21	31.34 ± 2.18
E. hirae NCTC 13383	Control (water)	35.65 ± 1.77	13.20 ± 2.62	0.86 ± 0.33	52.45 ± 2.79	32.88 ± 3.80
	Control (DMSO)	33.93 ± 0.59	17.72 ± 2.40	1.52 ± 0.52	53.02 ± 3.10	31.58 ± 4.83
	Cinnamic acid	32.26 ± 2.25	20.96 ± 1.62	2.15 ± 0.34	51.38 ± 1.27	28.31 ± 1.34
	Cinnamaldehyde	33.02 ± 1.72	20.03 ± 3.58	2.00 ± 0.75	51.64 ± 2.64	28.94 ± 4.11
	Hydrocinnamic acid	30.61 ± 1.70	22.87 ± 2.29	2.61 ± 0.46	50.25 ± 1.73	26.48 ± 1.76
	α-Methylhydrocinnamic acid	33.15 ± 0.50	18.76 ± 2.37	1.69 ± 0.50	53.41 ± 3.69	31.70 ± 5.47
	α-Methylcinnamic acid	34.33 ± 2.72	17.22 ± 2.81	1.41 ± 0.40	53.17 ± 2.43	31.75 ± 3.02

VI.3. Discussion

The use of biocides is essential to control the spread of pathogens in public and industrial settings. Biocide efficacy is affected by several factors such as concentration, contact time and environmental conditions under which it is applied (Bremer *et al.*, 2002; Campana *et al.*, 2017; Humayoun *et al.*, 2018; Maillard *et al.*, 2012). Bacterial state, whether in suspension, adhered on a surface or in a biofilm will impact the biocide efficacy (Humayoun *et al.*, 2018; Mah *et al.*, 2001). The use of a biocide is a balance between a concentration that is low enough not to be hazardous for humane or for the environment, and high enough to kill or inhibit bacteria and consequently prevent the development of antimicrobial resistance (Fraise, 1999).

Taking into account the characteristics of the selected phytochemicals and derivatives (Table VI.2), this work was developed with the purpose of understanding their action in combination with biocides. In this study, ten phytochemicals/derivatives, previously evaluated for their antimicrobial and anti-quorum sensing were selected for this study (data presented in Chapter IV and V). Initially, LA and CTAB were tested in combination with each phytochemical/derivative and the concentration that induced growth inhibition was determined by the checkerboard method. Taking into consideration the concentration determined in this study for the biocides and phytochemicals and derivatives in combination, their effect was assessed to prospect their use on CFU reduction of sessile bacteria.

α-Methylhydrocinnamic acid and α-methylcinnamic acid modified *E. coli* surface properties. The effect of α-methylcinnamic acid was more evident when compared to α-methylhydrocinnamic acid with decrease in bacteria hydrophilicity, an effect that may be related with their structural properties in particular with molar refractivity. This property has been described as an indicator to improve biological activity, and is related to the real volume of the chemical and the London dispersion forces that influence chemical-biological interactions (Hansch *et al.*, 2003; Padrón *et al.*, 2002). In this case, α-methylhydrocinnamic acid and α-methylcinnamic acid were the chemicals with the highest molar refractivity values of 46.54 cm^3 mol^{-1} and 47.42 cm^3 mol^{-1}, respectively – hypothesising the involvement of specific interactions with the membrane (Dambolena *et al.*, 2011; Habicht *et al.*, 1983; Rastija *et al.*, 2009). A higher susceptibility of *E. coli* compared to *S. aureus* when in contact with phytochemicals or derivatives was also

observed in the results shown in Chapter IV. This result can be related to the presence of a thinner peptidoglycan layer in Gram-negative bacteria. In fact, phytochemicals and derivatives can be able to disturb and even disrupt the cell membrane structure. Moreover, as they are small molecules, they can also be able to cross the cell membrane by passive diffusion. In particular, those that are organic acids can increase bacterial membrane permeability, acidify the cytoplasm and cause protein denaturation (Campos *et al.*, 2009; Johnston *et al.*, 2003).

VI.3.1. Phytochemicals and derivatives combination with the biocide lactic acid

In general, the present data demonstrated that Gram-negative bacteria were generally more affected by the use of LA compared to the Gram-positive ones, corroborating previous studies (Corry *et al.*, 1995; Virto *et al.*, 2006). The efficacy of the combination of LA with the phytochemicals or derivatives measured by the checkerboard method pointed out the most promising that are those that have a carboxylic group (cinnamic, hydrocinnamic, α-methylhydrocinnamic and α-methylcinnamic acids). Although in this study a phosphate buffer of pH 7 was used, the addition of LA may alter the pH of the solution and lowered the pH of the combination to levels enough to modify the ratio of dissociated/undissociated forms of the organic acids. In fact, the pH is an important parameter when using organic acids like cinnamic, hydrocinnamic, α-methylhydrocinnamic and α-methylcinnamic acids as it can contribute to change of the dissociated/undissociated ratio and destabilize the cytoplasmic membrane (Ali *et al.*, 2005; Campos *et al.*, 2009; Johnston *et al.*, 2003; Sánchez-Maldonado *et al.*, 2011; Yilmaz *et al.*, 2018). It is important to note that lactic acid has a pKa of 3.79 and the mentioned organic acids a pKa around 4.34 (Ali *et al.*, 2005; Campos *et al.*, 2009; Johnston *et al.*, 2003). The same type of effect was observed when medium-chain fatty acids (caprylic, capric and lauric acid) were combined with organic acids (acetic, lactic, malic, and citric acids) against *E. coli* O157:H7 (Kim *et al.*, 2013). LA may have caused physiological and morphological modifications in bacterial membranes which may have facilitated the entrance of both LA and phytochemicals or derivatives into the cell (Gyawali *et al.*, 2012). The findings of Wang *et al.* (2015) and Boomsma *et al.* (2015) also support this hypothesis as they observed 0.5% LA efficacy on the inhibition of planktonic growth of *Salmonella*, *E. coli* and *L. monocytogenes.* Additionally, they also observed the release of intracellular proteins from these microorganisms following exposure to LA. In fact, LA antimicrobial action is known to be strongly dependent on

the concentration of the acid and on the pH under which the experiment is carried out (Keeton *et al.*, 2008). Some authors (Al-Adham *et al.*, 2012; Chotigarpa *et al.*, 2018) demonstrated that under low pH the biocide permeabilises the outer membrane of Gram-negative bacteria and others suggested that LA can act as a protonator of anionic components like carboxyl and phosphate groups and consequently the molecular interactions between components within the membrane are weakened (Al-Adham *et al.*, 2012; Alakomi *et al.*, 2000).

LA combination with cinnamic acid (pKa 4.09) or hydrocinnamic acid (pKa 4.54) was able to reduce the CFU of sessile *E. coli*. In this case, the activity of these combinations may be related to the lower molecular weight of these two compounds among the phytochemicals/derivatives that have a carboxylic group (cinnamic acid 148.16 g mol^{-1}; hydrocinnamic acid 150.18 g mol^{-1}; α-methylhydrocinnamic acid 164.20 g mol^{-1}, α-methylcinnamic acid 162.19 g mol^{-1}; α-fluorocinnamic acid 166.15 g mol^{-1}), Table V.1. Allyl cinnamate effectiveness when combined with LA may be a result of its lipophilicity (logP of 3.17) and capacity to disturb membranes (Sikkema *et al.*, 1995; Wang *et al.*, 2015) as it has an additional Michael acceptor moiety in its structure, which can act as a covalent modifier affecting bacterial biosynthetic pathways and cellular redox state (Gverzdys *et al.*, 2016; Jackson *et al.*, 2017).

VI.3.2. Phytochemicals and derivatives combination with the biocide cetyltrimethylammonium bromide

Despite the lack of potentiation observed when using the checkerboard methodology, it was possible to detect growth inhibition when CTAB was used in combination with cinnamaldehyde and methyl trans-cinnamate, two phytochemicals/derivatives that have no carboxylic acid function in their chemical structure. Membrane disruption and consequent leakage can be promoted by the CTAB mode of action, which in turn may facilitate the access of the phytochemicals/derivatives and of the biocide to the cell cytoplasm, and consequently react with proteins and other cell components (Azeredo *et al.*, 2003; Nakata *et al.*, 2011; Rodrigues *et al.*, 2013; Yakabe *et al.*, 2011). It has been also described that CTAB can bind to the negative cell surfaces of bacteria, as a consequence of an electrostatic attraction, by chemisorption facilitating membrane permeabilization (Azeredo *et al.*, 2003; Rajagopal *et al.*, 2014; Rodrigues *et al.*, 2013). Azeredo *et al.* (2003) proposed that the use of a concentration of CTAB higher than its MBC, lead to the bacteria becoming more hydrophilic and

positively charged. After interacting with the membrane, CTAB promotes the disorganisation of bacteria cell membrane and disruption (McDonnell *et al.*, 1999; Simões *et al.*, 2005). Additionally, the generation of reactive oxygen species during *E. coli* stress response to CTAB treatment has been reported (Nakata *et al.*, 2011). In the present study, both *E. coli* and *S. aureus* were affected by the combination of phytochemicals or derivatives with CTAB. The mechanism of action of QACs is described as being primarily active against Gram-positive bacteria but higher concentrations are also lethal to Gram-negative bacteria (Al-Adham *et al.*, 2012; Weber *et al.*, 2007). As described for the combination with LA, allyl cinnamate was able to potentiate CTAB action probably due to its lipophilicity (logP of 3.17) and capacity to disturb membranes facilitating CTAB access to *E. coli* cytoplasm (Sikkema *et al.*, 1995; Wang *et al.*, 2015; Yakabe *et al.*, 2011). The combination of cinnamaldehyde and CTAB was also able to potentiate the action of the biocide against *E. coli*. Cinnamaldehyde is known to be able to enter the cell and to interact with the cell membrane constituents, modifying its components such as enzymes and transcriptome, and promote cell death (Friedman, 2017; Garcia-Salinas *et al.*, 2018). In addition, it can also act as a reactive electrophile species and a substrate of the aldehyde dehydrogenase and can disturb the bacteria detoxification pathways. According to Gill *et al.* (2004) the action of cinnamaldehyde against *L. monocytogenes* and *E. coli* is related to a rapid inhibition of the energy metabolism.

S. aureus was affected by the combinations with phytochemicals/derivatives that possess a carboxylic group giving them the ability to be hydrogen bond donors. This feature is relevant for their interaction with the membrane and/or bacteria internal components that is aid by the action of the biocide CTAB. The importance of the carboxylic group for the activity of this type of chemicals was already described in the literature (Campos *et al.*, 2009; Minatel *et al.*, 2017; Singh, 2017). Interestingly, this effect is observed in the combination with CTAB but not when they were combined with LA.

Taking into consideration the results obtained in the combination studies with both LA and CTAB as well as the data presented in the previous chapters for the efficacy of the phytochemicals and their derivatives individually, six phytochemicals/derivatives stand out, cinnamic acid, cinnamaldehyde, hydrocinnamic acid, α-methylhydrocinnamic acid, α-methylcinnamic acid and α-fluorocinnamic acid. As one important aspect on the

development of a disinfection formulation is the cost of each product in order to commercialize a cost-efficient biocide α-fluorocinnamic acid was excluded from future combination and formulation studies due to its high price, in comparison with the phytochemicals/derivatives tested (Table VI.2) (Rutala *et al.*, 2008, updated 2019).

VI.4. Conclusion

Surface disinfection is a frontline strategy to control bacterial contamination and spread. In the work presented in this chapter, a combinatorial approach has been considered to improve disinfection efficacy, where different antimicrobials with different mode of action were combined and their effectiveness in combination excels the individual. Overall, the combination of LA or CTAB with phytochemicals/derivatives was successfully accomplished. The combinations of LA with the phytochemicals or derivatives that possess a carboxylic group were able to inhibit the growth of *E. coli* and *S. aureus*. Phytochemicals/derivatives combination with LA only increased the bbiocide efficacy against *E. coli* sessile cells, and only when combined with cinnamic acid, allyl cinnamate or hydrocinnamic acid. CTAB was particularly successful in reducing CFU of sessile bacteria when combined with allyl cinnamate or with α-methylcinnamic acid.

VI.5. Relevancy for thesis development

In the work presented in this chapter some phytochemicals/derivatives stood out, namely cinnamic acid, cinnamaldehyde, hydrocinnamic acid, α-methylhydrocinnamic acid, α-methylcinnamic acid and α-fluorocinnamic acid, Table VI.4. Therefore, cinnamyl alcohol, allyl cinnamate, methyl trans-cinnamate and cinnamamide were excluded for further studies. In addition, α-fluorocinnamic acid was excluded due to its high price. Since resistance to antimicrobials is an important factor that needs to be overcame these phytochemicals/derivatives were tested against *S. aureus* strains overexpressing resistance efflux pumps in order to understand their ability to modulate efflux.

Table VI.4. Results compilation of the data presented on chapter V and VI, highlighting the selected phytochemicals to be used in the subsequent work

		Chapter V						**Chapter VI**				
		Growth inhibition	Kill	QSI	Growth curve modification		Removal of adhered bacteria	Growth Inhibition (potentiation)		Removal of adhered bacteria (potentiation)		Price per 1 g (€)
					Lag phase	dt		LA	CTAB	LA	CTAB	
	Cinnamic acid	√		√		√	+	+		+	+	4.86
Series 1	Cinnamaldehyde	√	+	√	√	√	+		+		+	0.05
	Cinnamyl alcohol	+		√		√					+	0.15
	Allyl cinnamate			√	√	√	+	+		+	+	0.49
	Methyl trans-cinnamate			√	√	√	+		+			0.12
	Cinnamamide	+		√	+	√						7.22
	Phenylacetone			√		+						
Series 2	Hydrocinnamic acid	√			+	√	+	+		+	+	0.31
	α-Methylhydrocinnamic acid	√			+	√	+	+			√	12.02
	α-Methylcinnamic acid	√		√	+	√	+	+			+	2.64
	α-Fluorocinnamic acid	√		√	+	√	+	√			√	91.90
Series 3	4-(Dimethylamino)cinnamic acid			√	+	√						
	4-Chlorocinnamic acid			√	√	√						
	trans-4-(Trifluoromethyl)cinnamic acid			√	+	√						
	4-Nitrocinnamic acid			√		√						
	4-Methoxycinnamic acid			√	+	√						
	3,4-(Methylenedioxy)cinnamic acid			√	+	√						

√ - Accomplished for all the bacteria tested; + - Accomplished for at least one of the bacteria tested

Chapter VII. Phytochemical derivatives as *Staphylococcus aureus* efflux pump modulators

Bacterial resistance to antimicrobials has become one of the most important concerns worldwide as it results in severe infection rates and has a high economic impact. Some global resistance mechanisms have emerged probably following the misuse of the available antimicrobials (biocides and antibiotics). One predominant mechanism is the overexpression of efflux pumps that when inhibited should result in decreased antimicrobial resistance. This chapter aims to assess the possibility of using cinnamic acid, cinnamaldehyde, hydrocinnamic acid, α-methylhydrocinnamic acid and α-methylcinnamic acid as efflux modulators. S. aureus strains harboring norA, mrsA, tetK, qacA or smr resistance genes were tested for their ability to extrude EB in the presence of the phytochemicals/derivatives. Cinnamaldehyde promoted EB accumulation on all the strains tested with the exception of S. aureus SM39 (qacA) and S. aureus ATCC25923_EB (norA). MIC of EB in the presence of cinnamaldehyde resulted in a 4 fold decrease for the strain harboring tetK gene (S. aureus XU212). This EB accumulation was hypothesized as being caused by membrane destabilization with consequent disruption of the proton motive force necessary for the efflux pump to extrude EB. In addition, the phytochemicals and derivatives that possess a hydroxyl group were also able to promote EB accumulation in S. aureus XU212. This strain possesses a chromosome encoded tetK gene and is negative for the presence of qacA/B, qacG, qacJ and smr. The general results reinforced the concept that phytochemicals and derivatives have great potential to be used in combinations to potentiate the efficiency of antimicrobials that have become less effective against resistant strains.

The work included in this chapter resulted from the collaboration of:

Malheiro J. F., Costa S. S. *, Borges F., Couto I. *, Maillard J. Y., Simões M.

* Grupo de Micobactérias, Unidade de Microbiologia Médica, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (IHMT, UNL). The publication is in preparation.

VII.1. Experimental details

Bacteria	*S. aureus* NCTC 10788, *S. aureus* SA1199b, *S. aureus* RN4220:pUL5054, *S. aureus* XU212, *S. aureus* SM39, *S. aureus* SM52, *S. aureus* ATCC 25923, *S. aureus* ATCC 25923_EB
Phytochemicals or derivatives	Cinnamic acid, cinnamaldehyde, hydrocinnamic acid, α-methylhydrocinnamic acid, α-methylcinnamic acid
Biocides	-
Temperature	37 ºC
Methodology used	III.2.7. Ethidium Bromide efflux inhibition assay III.2.8. Ethidium Bromide minimum inhibitory concentration determination in the presence of efflux inhibitors III.2.9. Detection of resistance gene (*qacA/B*, *qacG*, *qacJ*, *smr* and *tetK*)

VII.2. Results

VII.2.1. Efflux modulation by phytochemicals and derivatives

In the work included in this chapter the ability of phytochemicals and derivatives to modulate efflux of EB was assessed. Initially, suspensions of different strains of *S. aureus* were exposed to a phytochemical, derivative or reserpine, a known efflux inhibitor (Garvey *et al.*, 2008; Gibbons *et al.*, 2000), after 10 minutes of being in contact with EB alone.

S. aureus NCTC 10788 (Figure VII.1) was selected as a susceptible strain, while *S. aureus* SA1199b (Figure VII.2), RN4220:pUL5054 (Figure VII.3) and XU212 (Figure VII.4) were selected for their expression of a NorA MDR efflux protein, MsrA macrolide efflux protein and a TetK efflux pump, respectively. As expected, reserpine ($p < 0.05$) was able to promote EB accumulation inside the cell after being added to the suspension (Figure VII.1), which was detected by an increase in EB fluorescence. The same effect was observed after the addition of cinnamaldehyde ($p < 0.05$). On the other hand, adding cinnamic ($p < 0.05$ after 30 min of addition), hydrocinnamic ($p < 0.05$ after 15 min of

addition), α-methylhydrocinnamic ($p < 0.05$ from 2.5 to 30 min of addition) and α-methylcinnamic ($p > 0.05$) acids lead to a decrease in EB accumulation.

The behaviour of *S. aureus* SA1199b when exposed to the phytochemicals and derivatives was similar to *S. aureus* NCTC 10788 (Figure VII.2). Reserpine ($p < 0.05$) and cinnamaldehyde ($p < 0.05$) promoted accumulation of EB and cinnamic ($p < 0.05$), hydrocinnamic ($p > 0.05$), α-methylhydrocinnamic ($p > 0.05$) and α-methylcinnamic ($p > 0.05$) acids decreased the accumulation of EB.

Cinnamaldehyde has promoted EB accumulation on *S. aureus* RN4220:pUL5054 ($p < 0.05$). With this strain only a slight accumulation of EB was observed by the addition of reserpine ($p > 0.05$). All the other phytochemicals and derivatives led to a decrease of EB inside the cell ($p < 0.05$ for cinnamic and hydrocinnamic acids after 27.5 and 37.5 min of addition, respectively).

The addition of cinnamaldehyde to a suspension of *S. aureus* XU212 (Figure VII.4) led to the accumulation of EB ($p < 0.05$). This accumulation also happened when cinnamic ($p < 0.05$ after 27.5 min of addition), hydrocinnamic ($p < 0.05$ after 15 min of addition), α-methylhydrocinnamic ($p < 0.05$ after 15 min of addition) and α-methylcinnamic ($p > 0.05$ after 17.5 min of addition) acids were injected. Reserpine did not exert any effect on *S. aureus* XU212, contrarily to what was observed with *S. aureus* NCTC 10788, SA1199b and RN4220:pUL5054.

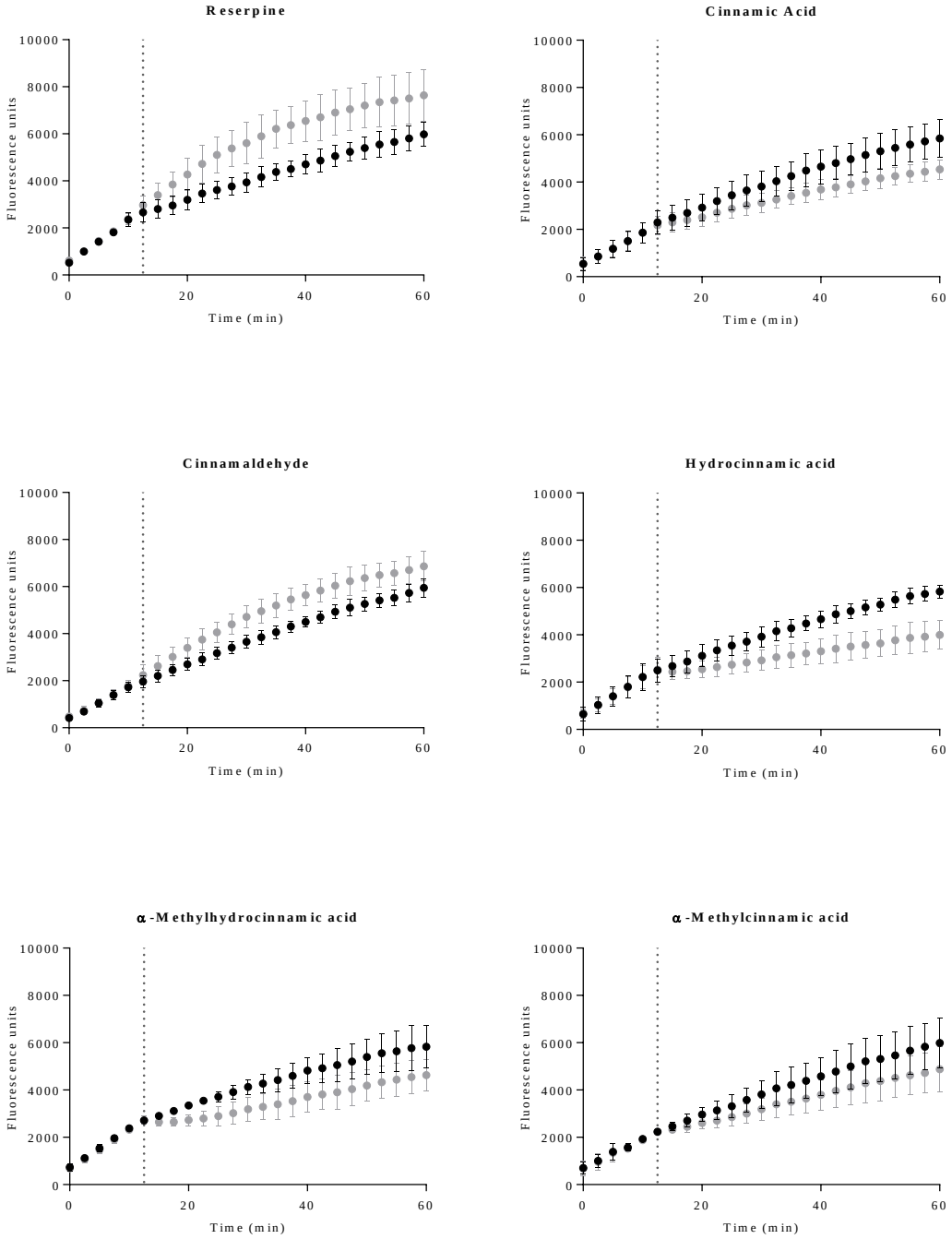


Figure VII.1. EB accumulation by *S. aureus* NCTC 10788 when exposed to the phytochemicals and derivatives at the concentrations presented in Table VII.1. The solvent (black) and a phytochemical/derivative (grey) were added into the well at 12.5 min (vertical dashed line). The data are presented as the mean ± SD of at least three independent experiments.

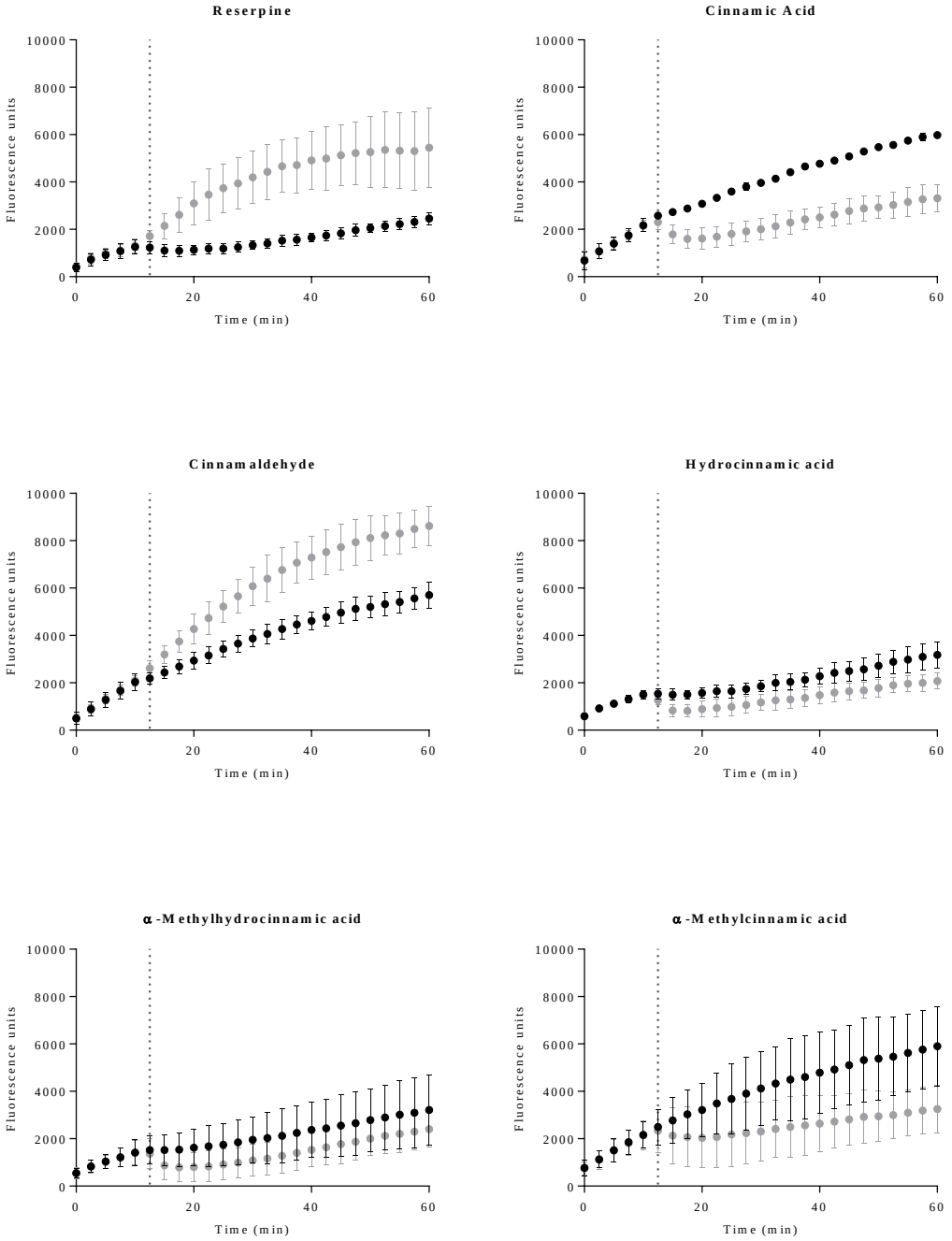


Figure VII.2. EB accumulation by *S. aureus* SA1199b when exposed to the phytochemicals and derivatives at the concentration presented in Table VII.1. The solvent (black)and a phytochemical/derivative (grey) were added into the well at 12.5 min (vertical dashed line). The data are presented as the mean ± SD of at least three independent experiments.

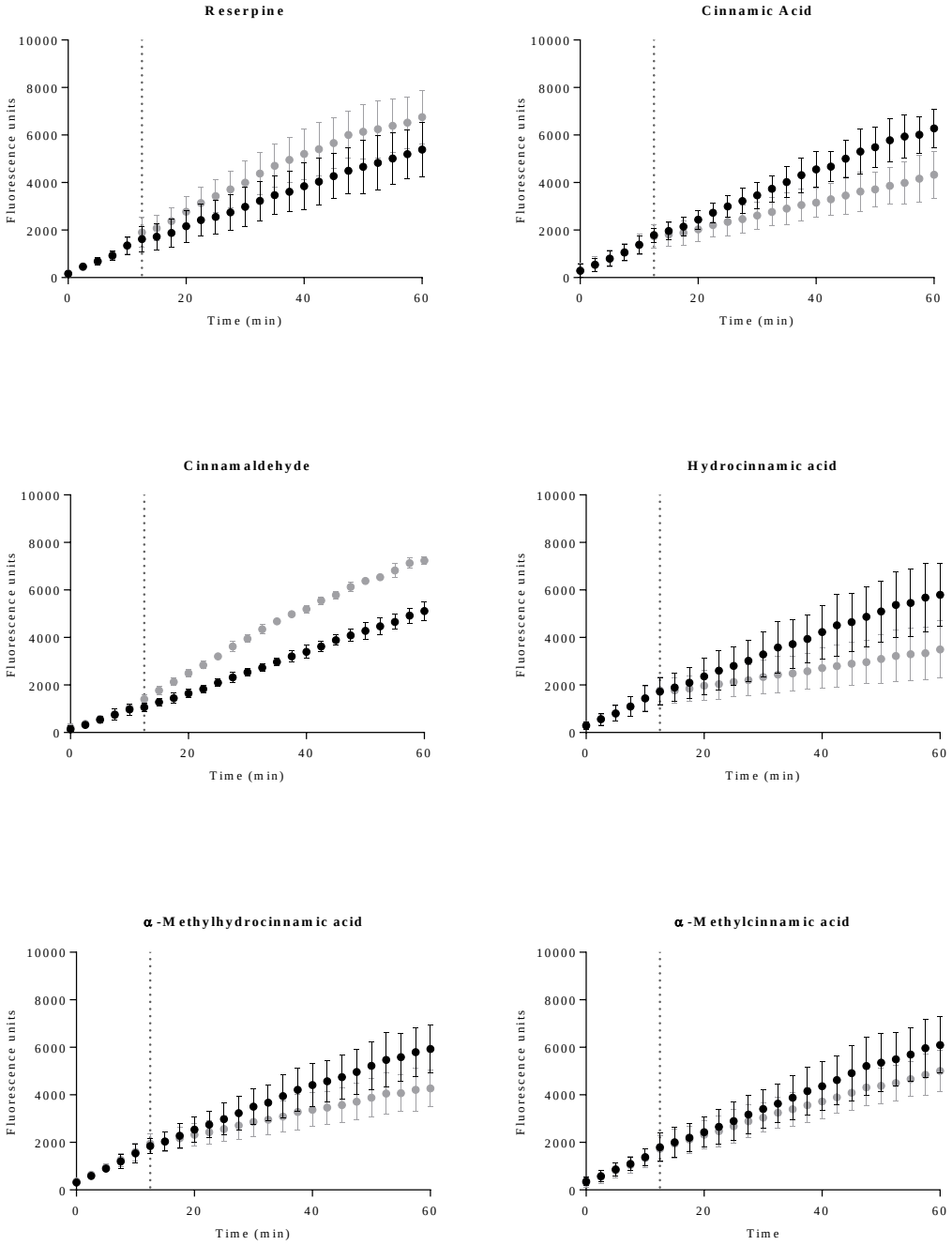


Figure VII.3. EB accumulation by *S. aureus* RN4220:pUL5054 when exposed to the phytochemicals and derivatives at the concentration presented in Table VII.1. The solvent (black) and a phytochemical/derivative (grey) were added into the well at 12.5 min (vertical dashed line). The data are presented as the mean ± SD of at least three independent experiments.

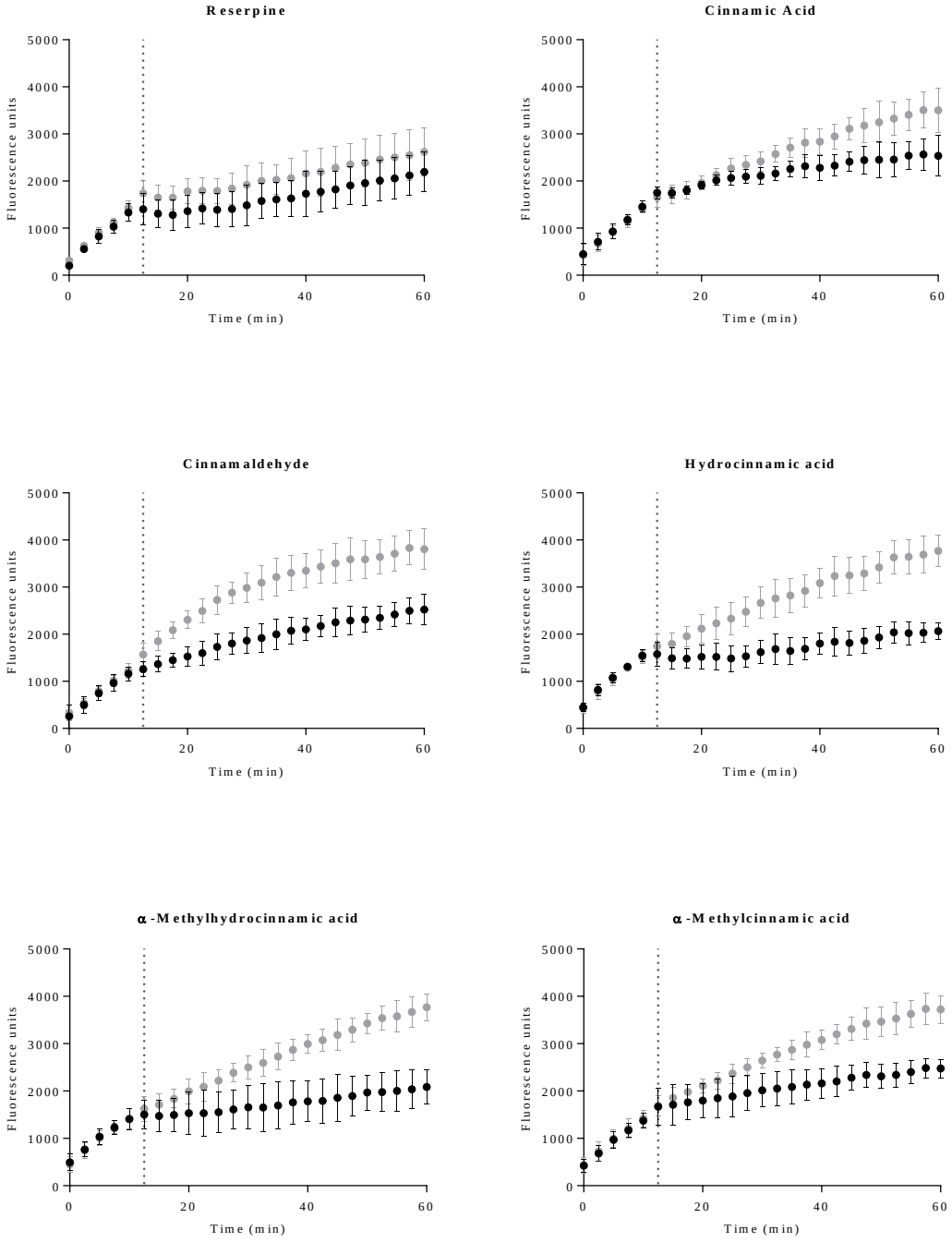


Figure VII.4. EB accumulation by *S. aureus* XU212 when exposed to the phytochemicals and derivatives at the concentration presented in Table VII.1. The solvent (black) and a phytochemical/derivative (grey) were added into the well at 12.5 min (vertical dashed line). The data are presented as the mean ± SD of at least three independent experiments.

VII.2.2. Bacteria tolerance to EB changes in the presence of phytochemicals and derivatives

Considering the results obtained for *S. aureus* NCTC 10788 (Figure VII.1), SA1199b (Figure VII.2), RN4220:pUL5054 (Figure VII.3) and XU212 (Figure VII.4), it was interesting to further explore the mode of action of these phytochemicals and derivatives. Therefore, *S. aureus* ATCC 25923_EB (adapted to EB and overexpressing NorA efflux pump) and the corresponding susceptible strain *S. aureus* ATCC 25923 (negative for *tetK*, *qacAB*, *smr*, *qacJ* and *qacG*) were also included in this study (Costa *et al.*, 2011). In addition, *S. aureus* SM39 (harbouring *qacAB* gene) and SM52 (harbouring *smr* gene) were also included to explore putative resistance to antiseptics (Bjorland *et al.*, 2001; Costa *et al.*, 2011; Costa *et al.*, 2013b). Two additional efflux pumps inhibitors verapamil and thioridazine were used.

For this approach, phytochemicals and derivatives action was explored in combination with EB and their activity was assessed by recording EB MIC for each combination. As it is possible to observe from Table VII.1, the MIC of EB when used alone was 64 mg L^{-1} for *S. aureus* XU212, 256 mg L^{-1} for *S. aureus* SM39, 32 mg L^{-1} for *S. aureus* SM52, 32 mg L^{-1} for *S. aureus* SA1199b, 8 mg L^{-1} for *S. aureus* ATCC 25923 and 256 mg L^{-1} for *S. aureus* ATCC 25923_EB. Cinnamic acid was able to reduce EB MIC in half for *S. aureus* XU212 and SA1199b (to 32 mg L^{-1} and 16 mg L^{-1}, respectively). Cinnamaldehyde promoted a 4 fold MIC reduction for *S. aureus* XU212 and ATCC 25923 (to 16 mg L^{-1} and 2 mg L^{-1}, respectively) and a 2 fold MIC reduction for *S. aureus* SM39, SM52 and ATCC 25923_EB (to 128 mg L^{-1},16 mg L^{-1} and 128 mg L^{-1}, respectively). Hydrocinnamic acid also decreased 4 fold the MIC of EB for *S. aureus* XU212 and to half the MIC for SM39 and SA1199b (to 16 mg L^{-1} for all strains). α-methylhydrocinnamic acid combination with EB has only decreased the MIC to 16 mg L^{-1} (4 fold) of XU212 while α-methylcinnamic acid decreased EB MIC to 16 mg L^{-1} for XU212 (4 fold) and SA1199b (2 fold).

When efflux pump inhibitors were analysed, it was possible to observe that verapamil was the only inhibitor that was effective against all the strains tested (8, 2, 16, 32, 4 and 4 fold reduction for XU212, SM39, SM52, SA1199b, ATCC 25923 and ATCC 25923_EB, respectively). Reserpine was only able to reduce the MIC for *S. aureus* XU212 (2 fold), SM52 (2 fold), SA1199b (8 fold) and ATCC 25923 (4 fold), while thioridazine reduced EB MIC for *S. aureus* XU212 (2 fold), SM52 (16 fold), ATCC 25923 (4 fold) and ATCC 25923_EB (2 fold).

Table VII.1. EB MIC (mg L^{-1}) for several *S. aureus* strains (XU212, SM39, SM52, SA1199b, ATCC 25923, ATCC 25923_EB) alone and in combination with a specific concentration of different phytochemicals/derivatives (cinnamic acid, cinnamaldehyde, hydrocinnamic acid, α-methylhydrocinnamic acid and α-methylcinnamic acid), as well as with known efflux pumps inhibitors (reserpine, verapamil and thioridazine).

		XU212	**SM39**	**SM52**	**SA1199b**	**ATCC 25923**	**ATCC 25923_EB**
EB MIC alone		*64*	*256*	*32*	*32*	*8*	*256*
Phytochemical/derivative							
Cinnamic acid	5 mM	32	256	32	16	16	256
Cinnamaldehyde	0.5 mM	16	128	16	32	2	128
Hydrocinnamic acid	8 mM	16	256	16	16	8	256
α-Methylhydrocinnamic acid	5 mM	16	256	32	32	8	256
α-Methylcinnamic acid	3 mM	16	256	32	16	16	256
Verapamil	0.880 mM	8	128	2	1	2	64
Thioridazine	0.034 mM	32	256	2	32	2	128
Reserpine	0.033 mM	32	256	16	4	2	256

VII.2.3. Cinnamaldehyde promotes EB accumulation on *S. aureus* strains with resistance determinants

The results presented in this chapter have shown that cinnamaldehyde is able to promote EB accumulation on several *S. aureus* strains that overexpress NorA, MrsA and TetK efflux pumps. Therefore, another efflux assay was performed where *S. aureus* suspensions were exposed to EB combined with cinnamaldehyde or one efflux inhibitor (verapamil, thioridazine or reserpine), and EB accumulation was measured for 60 min. For this assay, *S. aureus* XU212 was selected since its efflux was modulated by all the phytochemicals and derivatives tested. *S. aureus* ATCC 25923_EB, the correspondent susceptible strain *S. aureus* ATCC 25923, *S. aureus* SM39 and SM52 were also used to assess putative resistance to EB and antiseptics (Figure VII.5). The data showed that regardless the strain, verapamil was the most efficient inhibitor tested ($p < 0.05$). Thioridazine was able to promote EB accumulation in all the strains ($p < 0.05$), with the exception of *S. aureus* XU212, where EB was pumped out the cell more efficiently in comparison to the control. Reserpine was inefficient against all the strains tested. However, when used against *S. aureus* XU212, EB was pumped out the cell more efficiently. The same effect was observed when thioridazine was in contact with *S. aureus* XU212. Cinnamaldehyde, when used against the strains that possess *tetK* and *smr*

resistance genes, was able to modulate efflux resulting in EB accumulation ($p < 0.05$). The control strain *S. aureus* ATCC 25923 also accumulated EB when exposed to the phytochemical ($p < 0.05$).

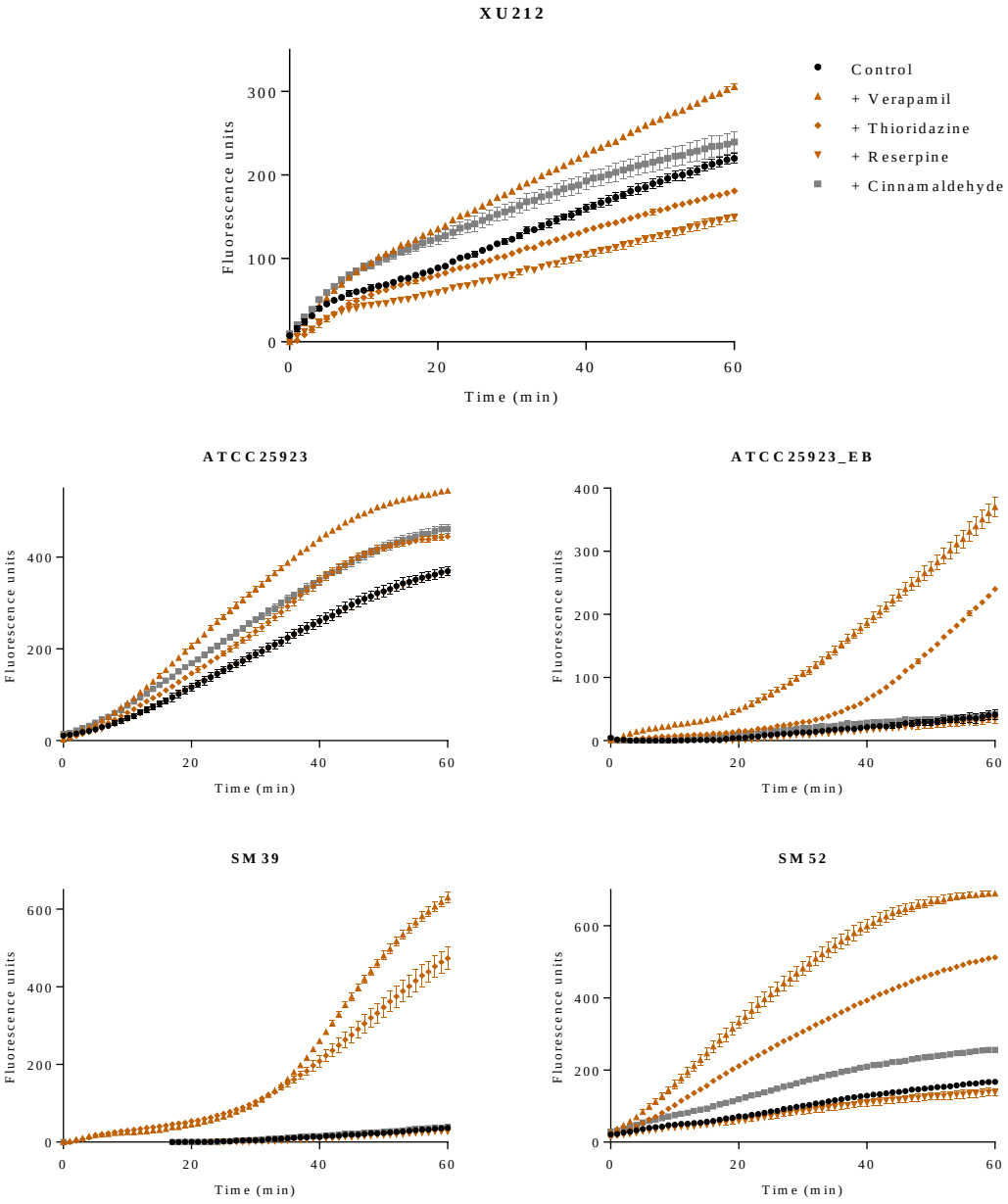


Figure VII.5. EB accumulation by *S. aureus* XU212, ATCC 25923, ATCC 25923_EB, SM39 and SM52 (●) and when exposed to verapamil (▲), thioridazine (◆), reserpine (▼) or cinnamaldehyde (■). The data are presented as the mean ± SD.

VII.2.4. Characterization of *S. aureus* XU212 resistance

S. aureus XU212 was characterised in terms of resistance based on the fact that this strain is reported only as having a *tetK* gene (Gibbons *et al.*, 2000). In fact, in this work it was possible to confirm by PCR the presence of a chromosomic *tetK* resistance gene (Figure VII.6). In addition, the existance of antiseptic resistance genes, *qacAB* and *smr*, was also looked for (Figure VII.7). However, null results were obtained when *S. aureus* XU212 plasmid DNA was tested. Additionally, *S. aureus* XU212 was also tested to evaluate the presence of the resistance genes *qacG* and *qacJ*, but the results were also negative (Figure VII.8).

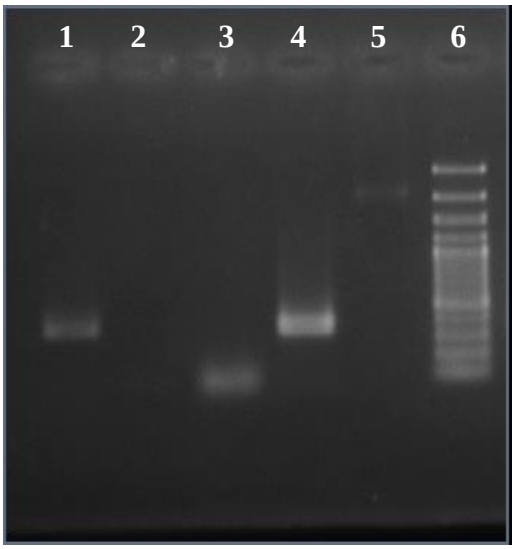


Figure VII.6. Assessment of the presence of the described resistance gene *tetK* on *S. aureus* XU212. Lane 1 - *tetK* positive control (*S. epidermidis* ATCC 12228); lane 2 - *tetK* negative control (*S. aureus* ATCC 25923); lane 3 - *tetK* water control; lane 4 - *tetK S. aureus* XU212 chromosomic DNA; lane 5 - *tetK S. aureus* XU212 plasmid DNA, lane 6 - Marker #N0551.

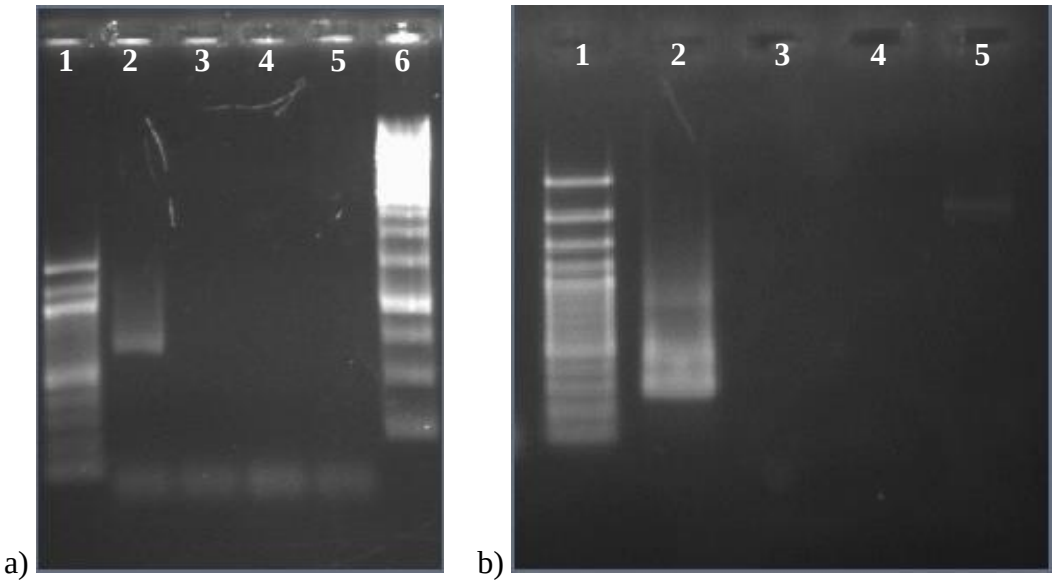


Figure VII.7. Assessment of the presence of *qacAB* (a) or *smr* (b) genes on *S. aureus* XU212. a) Lane 1 - Marker #N0551; lane 2 - *qacAB* positive control (*S. aureus* SM39); lane 3 - *qacAB* negative control (*S. aureus* ATCC 25923); lane 4 - *qacAB* water control; lane 5 – *qacAB S. aureus* XU212; lane 6 - Marker #SM0311. b) Lane 1 - Marker #N0551; lane 2 - *smr* positive control (*S. aureus* SM52); lane 3 - *smr* negative control (*S. aureus* ATCC 25923); lane 4 - *smr* water control; lane 5 - *smr S. aureus* XU212.

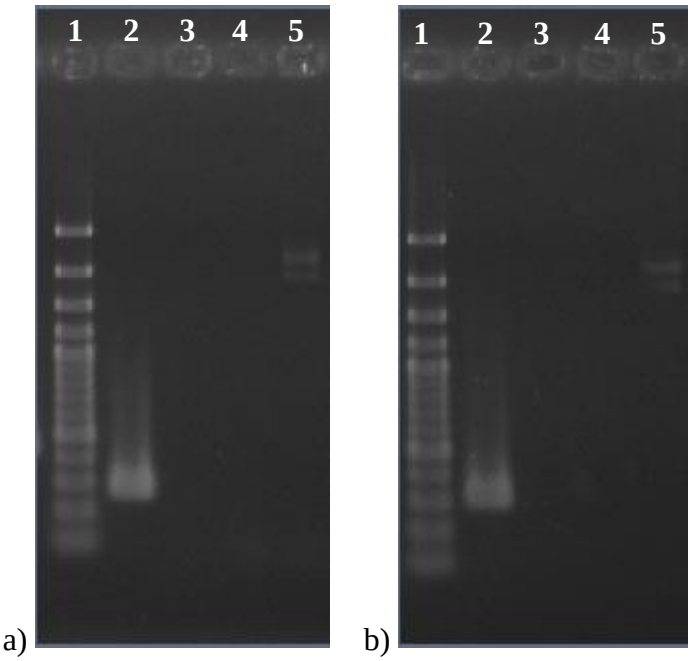


Figure VII.8. Assessment of the presence of *qacG* (a) or *qacJ* (b) genes on *S. aureus* XU212. a) Lane 1 - Marker #N0551; lane 2 - *qacG* positive control (*S. aureus* H4/09); lane 3 - *qacG* negative control (*S. aureus* ATCC 25923); lane 4 - *qacG* water control; lane 5 - *qacG S. aureus* XU212; lane. b) Lane 1 - Marker #N0551; lane 2 - *qacJ* positive control (*S. aureus* 25.1); lane 3 - *qacJ* negative control (*S. aureus* ATCC 25923); lane 4 - *qacJ* water control; lane 5 - *qacJ* XU212.

VII.3. Discussion

The widespread use of biocides in different areas such as industrial and hospital settings has led to an increase in selective pressure in bacteria. Selective pressure may lead to an increase in the resistance towards antimicrobials as well as cross-resistance to antibiotics (Maillard, 2005). The use of chemicals that can positively interfere with bacterial resistance mechanisms is a promising approach to ensure the efficacy of antibiotics. Phytochemicals have been described as effective adjuvants in antimicrobial therapy (Abreu *et al.*, 2017; Abreu *et al.*, 2016a; Abreu *et al.*, 2016b; Barbieri *et al.*, 2017; Monte *et al.*, 2014) acting by inhibiting microbial resistance mechanisms as well as by improving solubility and stability of active substances (Abreu *et al.*, 2016b; Gibbons *et al.*, 2003). Abouelhassan *et al.* (2015) observed auspicious results of several halogenated quinolones potentiated by gallic acid against pathogenic bacteria. When the combination was used against *S. aureus* 11800 fold potentiation was observed, however the mechanism of action was not elucidated. The development of resistance to phytochemicals is considered minimal or inexistent, possibly due to their multiple mechanisms of action that can negate bacterial adaptation (Bello *et al.*, 2016). One example of the combinatorial approach already implemented in clinic settings is the combination of clavulanic acid with amoxicillin, where clavulanic acid inhibits *β*-lactamases despite its weak antibacterial activity (Abreu *et al.*, 2016a).

VII.3.1. Phytochemicals and derivatives promote EB accumulation in *S. aureus* strains overexpressing efflux pumps

In the work presented in this chapter, five of the previously studied phytochemicals and derivatives, were chosen to further understand their mode of action – focusing on *S. aureus* efflux mechanisms. Cinnamic acid, cinnamaldehyde, hydrocinnamic acid, α-methylhydrocinnamic acid and α-methylcinnamic acid were tested against the standard strain used in the previous chapters V and VI (*S. aureus* NCTC 10788) as well as against additional strains that overexpress specific efflux pumps: *S. aureus* SA1199b (NorA), RN4220:pUL5054 (MsrA), XU212 (TetK), ATCC25923_EB (NorA), SM39 (QacAB), SM52 (Smr) and ATCC25923 (negative for *tetK*, *qacAB*, *qacG*, *qacJ* and *smr*).

Reserpine, verapamil and thioridazine were used as controls as they are efflux pump inhibitors. Reserpine was used as positive control since it is an antihypertensive drug that has been reported as a multidrug efflux inhibitor (Garvey *et al.*, 2008; Gibbons *et al.*, 2003; Gibbons *et al.*, 2000; Kaatz *et al.*, 2003). In fact, reserpine is able to modulate resistance in bacteria that possess *norA, bmr* or *pmrA* efflux genes (Abreu *et al.*, 2017; Borges *et al.*, 2016; Gibbons *et al.*, 2000; Putman *et al.*, 2000). The accumulation of EB inside the cells results in fluorescence increase due to the interaction of this molecule with bacterial DNA (Blair *et al.*, 2016). In fact, in this work, the inhibition of NorA overexpressing *S. aureus* SA1199b was observed when reserpine was added to a bacterial suspension in combination with EB. Verapamil is a calcium channel antagonist and thioridazine disturbs the membrane potential and inhibits K^+ and Ca^+ transport channels (Amaral, 2015; Amaral *et al.*, 2012; Chatterjee *et al.*, 1990; Kaatz *et al.*, 2003). Their efficiency was also observed in this study as verapamil and thioridazine were highly efficient in promoting EB accumulation or diminishing its MIC in all the strains tested.

All the phytochemicals and derivatives were able to promote EB accumulation, with the exception of the positive control reserpine, in a suspension of *S. aureus* XU212 and EB. The opposite effect was observed for both the phytochemicals and derivatives and reserpine against *S. aureus* NCTC 10788, SA1199b and RN4220:pUL5054. Reserpine was able to decrease the MIC for EB for *S. aureus* XU212, an effect that was also observed by Smith *et al.* (2007). Reserpine inefficacy to modulate *S. aureus* XU212 efflux and ability to decrease EB's MIC may be explained by the different methodologies used since the exposure time to the phytochemical differs from 60 min in the efflux assay to 24 h for MIC determination. Gibbons *et al.* (2000) observed the ability of reserpine to promote MIC reduction in clinical isolates of MRSA harboring *tetK* efflux gene. In fact, several phytochemicals, such as reserpine, pyrrolidine, quinine, morin and quercetin, had also been reported to potentiate antibiotic activity against methicillin-resistant *S. aureus* strains (Abreu *et al.*, 2016b; 2014). The ability of reserpine to decrease EB MIC in a Smr overexpressing strain (*S. aureus* SM52) was also described by Mourato (2012), where EB's MIC decreased from 16 mg L^{-1} to 4 mg L^{-1} in the presence of the inhibitor. The influence of the time of exposure to the phytochemicals and derivatives is also noticeable for *S. aureus* SA1199b. In this case, the absence of EB accumulation in the efflux assay was contradicted by a reduction on EB's MIC in the presence of cinnamic acid, hydrocinnamic acid and α-methylcinnamic acid. This was also observed for *S. aureus* SM52 in the presence of hydrocinnamic acid. Cinnamaldehyde was highly efficient in

promoting EB accumulation in *S. aureus* XU212, ATCC25923 and SM52 in the conditions used in the efflux assay. In fact, it equates thioridazine efficacy against *S. aureus* ATCC 25923, and it surpasses this inhibitor activity against *S. aureus* XU212. Cinnamaldehyde effect was negligible on the MIC of EB against *S. aureus* SA1199b, which may suggest that the strain was able to overcome the effect of cinnamaldehyde.

The efflux pumps families present in this work belong to the major facilitator superfamily (MFS; NorA, TetK and QacAB), the small multidrug resistance family (SMR; Smr) and the ATP-bonding cassette (ABC; MrsA). MFS and SMR rely on proton motive force or sodium ions, and ABC pumps use ATP hydrolysis (Andersen *et al.*, 2015; Blair *et al.*, 2016; Costa *et al.*, 2013c; Hsieh *et al.*, 1998; Krulwich *et al.*, 2001; Putman *et al.*, 2000). Bacteria proton motive force drives the electrogenic (membrane potential-dependent) efflux pumps and is composed by a balance of a chemical proton gradient (ΔpH) in which inside is alkaline and an electrical potential (ΔΨ) that is negative inside the cell. (Nair *et al.*, 2016; Putman *et al.*, 2000). Ng *et al.* (1994) showed that NorA efflux pump activity was abolished by the antibiotic nigericin, which selectively dissipates ΔpH, demonstrating that the major driving force for this pump is ΔpH. QacA as well as Smr-mediated efflux are driven by both ΔpH and the ΔΨ (Grinius *et al.*, 1994; Mitchell *et al.*, 1999). Considering the balance between ΔpH and the ΔΨ it is possible to hypothesise that cinnamaldehyde action against *S. aureus* efflux is based on disturbing its membrane. In fact, this action affects both the proton and electrical gradient balance regardless of the efflux pump overexpressed and in turn can explain the ability of cinnamaldehyde to promote EB accumulation. According to the literature (Campos *et al.*, 2009; Doyle *et al.*, 2019; Gill *et al.*, 2004; Lou *et al.*, 2012), this small lipophilic molecule can pass the cell membrane by passive diffusion, affect the membrane integrity and permeability of the cell membrane causing the acidification of the cytoplasm. Cinnamic acid, hydrocinnamic acid, α-methylhydrocinnamic acid and α-methylcinnamic acid fall under the class of cinnamic acid derivatives that possess a carboxylic group and can operate by a different mode of action and, therefore, can also sequester H^+, affecting ΔpH (Gill *et al.*, 2004). However, their activity is dependent on the concentration of undissociated acid which at pH 7 (controlled in this work by the use of PB pH7) is not sufficiently high to inhibit overexpressed MFS pumps such as NorA, QacAB and Smr, known to efficiently extrude EB (Campos *et al.*, 2009; Chambel *et al.*, 1999; Johnston *et al.*, 2003; Ramos-Nino *et al.*, 1996; Sánchez-Maldonado *et al.*, 2011). The fact that all the phytochemicals and derivatives tested were able to promote EB accumulation in *S. aureus* XU212 may

suggest that the accumulation of EB may be caused by the inhibition of the TetK efflux pump by affecting the proton motive force and/or due to membrane destabilization (Doyle *et al.*, 2019).

VII.3.2. *S. aureus* XU212 resistance is not due to the presence of the most common efflux determinants

S. aureus XU212 efflux of EB was altered by the presence of the phytochemicals and derivatives but only verapamil was able to inhibit efflux. The *tetK* gene present in this strain encodes for a hydrophobic (50 KDa) membrane bound protein that is capable to actively efflux tetracycline, contributing for bacterial resistance to the antibiotic (Gibbons *et al.*, 2000). *tet* efflux determinants are harbored only by Gram-negative bacteria. However, *tetL* and *tetK* are usually associated with Gram-positive bacteria (Poole, 2007). Also, *tet* genes are typically encoded on mobile genetic elements such as plasmids which contrast with our findings for *S. aureus* XU212 that has a chromosomally encoded *tetK* gene (Poole, 2007).

Several authors reported a relationship between the tolerance to EB and the detection of *qacA* and *qacB* on bacteria (Furi *et al.*, 2013; Marchi *et al.*, 2015; Patel *et al.*, 2010). *qacAB* are resistance genes found in multi-resistance plasmids that contain *bla* and *tet* resistance genes in *S. aureus* and are known to confer increased resistance to benzalkonium chloride (Costa *et al.*, 2010; Sidhu *et al.*, 2002; Wand, 2017). *qacG* and *qacJ* are plasmid-borne efflux pump genes that confer resistance to antiseptics and disinfectants (Costa *et al.*, 2013c). Therefore, the acquisition of these plasmids by bacteria will correspond to an increase in resistance to a biocide (benzalkonium chloride) and antibiotics (penicillin and tetracycline) (Bjorland *et al.*, 2005; Costa *et al.*, 2010; Wand, 2017). Another important biocide resistant gene is *smr*, found in non-transmissible plasmids or in large conjugative plasmids that confers resistance to QACs and phosphonium derivatives (Bjorland *et al.*, 2001). So, *S. aureus* XU212 was screened for the presence of the main efflux pumps genes that are known to confer biocide resistance to *S. aureus* strains. However, it was found that *S. aureus* XU212 is negative for the presence of *qacAB*, *qacG*, *qacJ* and *smr*.

IV.4. Conclusion

Biocide and antibiotic resistance development can be ascribed to the selective pressure exerted by the misuse of antimicrobials. To overcome this issue it is urgent to explore new solutions to achieve new and better outcomes. One of the main mechanisms of bacterial resistance is the overexpression of efflux pumps and, therefore, efflux pump inhibitors are of extreme importance. Phytochemicals are, by now, an importance source of efflux pump inhibitors and, some of them have been used in combinatorial antimicrobial studies. In the work included in this chapter several phytochemicals and derivatives based on cinnamic acid and cinnamaldehyde were used to study EB efflux by several *S. aureus* strains with different resistance determinants. The data showed that cinnamaldehyde is able to promote EB accumulation in *S. aureus* strains with different resistance determinants possibly by interacting with the cell membrane, affecting the proton motive force and consequently inhibiting MFS pumps. Also, the phytochemicals and derivatives based on cinnamic acid were able to reduce the MIC of EB of some *S. aureus* strains harbouring *norA*, *tetK* and *smr* possibly by sequestering of H^+ and therefore affecting ΔpH. The results also demonstrated that *tetK* gene for *S. aureus* XU212 is encoded in the chromosome.

Phytochemicals and derivatives can, therefore, be interesting adjuvants for combinatorial approaches aimed to restore or enhance the activity of antimicrobials. The combination of generally low toxic phytochemicals with antimicrobials that are already used and have known mechanisms of action may facilitate the implementation of these formulations.

VII.5. Relevancy for thesis development

In the work presented in this chapter, cinnamaldehyde was found to be the most promising phytochemical, Table VII.2. However, considering the results included in chapters V and VI, the selected phytochemicals/derivatives chosen to carry on this project aimed to the development of a formulation to be used for surface wiping tests, were cinnamaldehyde and α-methylhydrocinnamic acid.

Table VII.2. Results compilation of the data presented on chapter V, VI and VII, highlighting the selected phytochemicals to be used in the subsequent work

		Chapter V						Chapter VI					Chapter VII	
		Growth inhibition	Kill	QSI	Growth curve modification		Removal of adhered bacteria	Growth Inhibition (potentiation)		Removal of adhered bacteria (potentiation)		Price per 1 g (€)	Efflux inhibition	Growth inhibition in the presence of EB
					Lag phase	dt		LA	CTAB	LA	CTAB			
	Cinnamic acid	√		√		√	+	+		+	+	4.86	+	+
Series 1	Cinnamaldehyde	√	+	√	√	√	+		+		+	0.05	√	+
	Cinnamyl alcohol	+		√		√					+	0.15		
	Allyl cinnamate			√	√	√	+	+		+	+	0.49		
	Methyl trans-cinnamate			√	√	√	+		+			0.12		
	Cinnamamide	+		√	+	√						7.22		
	Phenylacetone			√		+								
Series 2	Hydrocinnamic acid	√			+	√	+	+		+	+	0.31	+	+
	α-Methylhydrocinnamic acid	√			+	√	+	+			√	12.02	+	+
	α-Methylcinnamic acid	√		√	+	√	+	+			+	2.64	+	+
	α-Fluorocinnamic acid	√		√	+	√	+	√			√	91.90		
Series 3	4-(Dimethylamino)cinnamic acid			√	+	√								
	4-Chlorocinnamic acid			√	√	√								
	trans-4-(Trifluoromethyl)cinnamic acid			√	+	√								
	4-Nitrocinnamic acid			√		√								
	4-Methoxycinnamic acid			√	+	√								
	3,4-(Methylenedioxy)cinnamic acid			√	+	√								

√ - Accomplished for all the bacteria tested; + - Accomplished for at least one of the bacteria tested

Chapter VIII. Surface wiping test to study biocide -cinnamaldehyde combination to improve surface disinfection efficiency

Surface disinfection is crucial to improve the prevention and control of microbial contaminations. Nonetheless, the misuse of the disinfectants used for routine disinfection has led to an increased concern on the selective pressure that the microorganisms are exposed to and consequently on their impact on bacteria resistance and cross-resistance. The aim of this work was to develop a formulation to be used for surface disinfection that is based on the combination of a natural product (cinnamaldehyde) and a widely used biocide - a quaternary ammonium compound (CTAB). The wiping method was based on the Wiperator test (E2967 − 15) and the efficacy evaluation of surface disinfection wipes test (EN 16615:2015). After some steps of concentration optimization of the formulation, the wiping of a contaminated surface, with 6.20 ± 0.21 log_{10} CFU of E. coli and 7.10 ± 0.06 log_{10} CFU of S. aureus), a total reduction of 4.35 ± 0.22 log_{10} CFU and 4.27 ± 0.22 log_{10} CFU was achieved when the wipe was impregnated with the formulation in comparison with 2.45 ± 0.41 log_{10} CFU and 1.50 ± 0.35 log_{10} CFU of removal performed just by mechanical action (for E. coli and S. aureus, respectively). Furthermore, the formulation has prevented the transfer of bacteria to clean surfaces. Preliminary data showed that the obtained formulation had a 4-fold reduction of cinnamaldehyde concentration after 1 month of shelf storage. The work presented in this chapter highlights the potential of a combinatorial approach of classic biocides with phytochemicals for the development of disinfectant formulations. This approach can also reduce the concentration of synthetic biocides and therefore reduce the potential environmental and public health burdens from their use.

The work included in this chapter resulted in the publication:

Malheiro J. F., Oliveira C., Cagide F., Borges F., Simões M., Maillard J-Y., 2020. Surface wiping test to study biocide -cinnamaldehyde combination to improve efficiency in surface disinfection. International Journal of Molecular Science, 21(21):1-14. Doi: 10.3390/ijms21217852.

VIII.1. Experimental details

Bacteria	*E. coli* NCTC 10418 and *S. aureus* NCTC 10788
Phytochemicals or derivatives	Cinnamaldehyde, α-methylhydrocinnamic acid
Biocides	CTAB
Temperature	25 ± 3 ºC
Methodology used	III.2.10. Bactericidal suspension test (EN 1276:2009) III.2.11. Surface wiping assay III.2.12. Evaluation of formulation stability and phytochemical/biocide chemical interactions

VIII.2. Results

VIII.2.1. Formulation optimization – without soil load

Considering the requirements of EN 1276:2009, the development of a formulation based on the combination of a QAC and a phytochemical was performed (CEN, 2009). With this purpose, it was selected the best CTAB concentration that allows a bacterial reduction of around 5 $\log_{10}$ CFU mL^{-1} but, at the same time, allows to observe the effect of the phytochemical when in combination. A CTAB concentration of 0.04 mM was chosen (*Appendix A.5.*) and the study was initiated using *S. aureus* as reference strain.

CTAB was combined with different concentrations (0.5, 1 and 2 mM) of cinnamaldehyde and 5 mM of α-methylhydrocinnamic acid as shown in Figure VIII.1. The CFU reduction caused by the different concentrations of cinnamaldehyde and by 5 mM of α-methylhydrocinnamic alone was almost negligible (cinnamaldehyde 0.5 mM: 0.08 ± 0.04 $\log_{10}$ CFU mL^{-1}, 1 mM: 0.07 ± 0.08 $\log_{10}$ CFU mL^{-1}, 2 mM: 0.05 ± 0.00 $\log_{10}$ CFU mL^{-1}; α-methylhydrocinnamic 5 mM: 0.06 ± 0.12 $\log_{10}$ CFU mL^{-1}). However, after 5 min of contact with the bacteria, the combination of CTAB/cinnamaldehyde showed a higher efficacy when compared to CTAB alone. The best result was 0.04 mM CTAB combined with 1 mM of cinnamaldehyde ($p > 0.05$) with a cell reduction of 5.97 ± 0.33 $\log_{10}$ CFU mL^{-1}. Due to

the lack of potentiation effect of α-methylhydrocinnamic to CTAB this cinnamic acid derivative was excluded from further testing

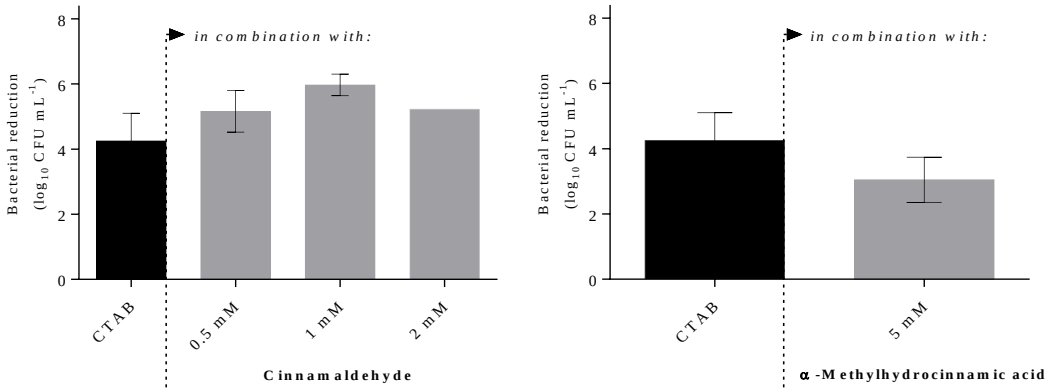


Figure VIII.1. *S. aureus* NCTC 10788 CFU reduction after exposing a bacterial suspension to 0.04 mM CTAB (black) alone or in combination (grey) with different concentrations of cinnamaldehyde or α-methylhydrocinnamic acid, for 5 min. Values are mean ± SD. No statistically significance was observed ($p > 0.05$).

A reduction of 4.25 ± 0.85 $\log_{10}$ CFU mL^{-1} was achieved when CTAB was used alone, the concentration of CTAB was reduced in half to better detect the influence of cinnamaldehyde when in combination. Considering that DMSO, the solvent often used for compounds' solubilization, is cytotoxic, it was strategic to look for a user-friendly solvent. In this case, the solubilisation of cinnamaldehyde was done in isopropanol and the volume of this solvent in the formulation was 5% v v^{-1}. As it is possible to observe in Figure VIII.2, the combination of CTAB/cinnamaldehyde had a higher efficiency when compared to CTAB alone. The combination achieved a reduction of 1.99 ± 0.48 and 4.00 ± 0.76 $\log_{10}$ CFU mL^{-1} whether cinnamaldehyde was dissolved in DMSO or isopropanol ($p < 0.001$), respectively. CTAB alone promoted a reduction of 1.31 ± 0.18 and 1.73 ± 0.33 $\log_{10}$ CFU mL^{-1} when in the presence of 5% v v^{-1} of DMSO or isopropanol, respectively. Exposure of a *S. aureus* bacterial suspension for 5 min to 1 mM of cinnamaldehyde alone has reduced 0.05 ± 0.02 $\log_{10}$ CFU mL^{-1} or 0.04 ± 0.05 $\log_{10}$ CFU mL^{-1}, if dissolved in DMSO or isopropanol, respectively. The CFU reduction obtained by the combination just by changing the solvent from DMSO to isopropanol was significantly higher ($p < 0.01$). Therefore, further investigation was carried out using isopropanol as cinnamaldehyde solvent.

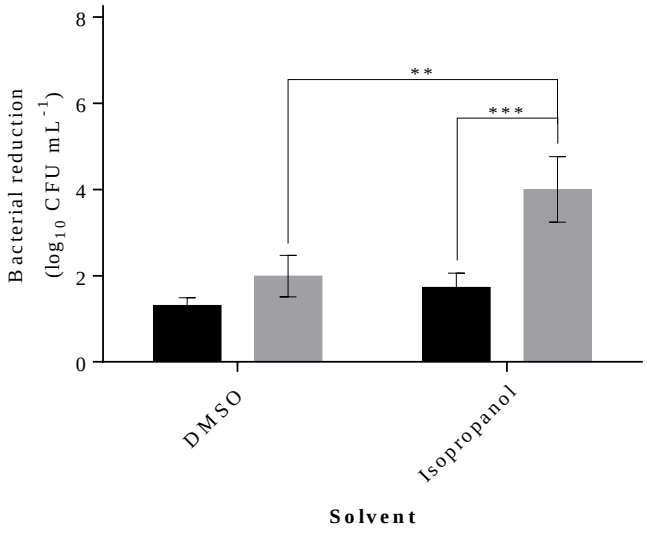


Figure VIII.2. *S. aureus* NCTC 10788 CFU reduction after exposing a bacterial suspension to 0.02 mM CTAB (black) alone or in combination 1 mM of cinnamaldehyde (grey), when two different solvents were used on the formulation (DMSO and isopropanol), for 5 min. Values are mean ± SD. The statistical significance is represented (** $p < 0.01$; *** $p < 0.001$).

Another important aspect to consider is the pH under which the formulation efficacy is higher. In order to evaluate the impact of pH on the biocidal efficacy, two concentrations of CTAB (0.02 and 0.03 mM) and two bacteria (*E. coli* and *S. aureus*) were used. Therefore, the combination CTAB/cinnamaldehyde was tested at pH 7 and 8 against *E. coli* and *S. aureus* (Figure VIII.3). Exposing bacterial suspensions of *E. coli* and *S. aureus* to 1 mM of cinnamaldehyde alone has led to a CFU reduction of 0.05 ± 0.06 $\log_{10}$ CFU mL^{-1} and 0.04 ± 0.05 $\log_{10}$ CFU mL^{-1} at pH 7, respectively. At pH 8, it was observed a reduction of 0.06 ± 0.07 $\log_{10}$ CFU mL^{-1} and 0.02 ± 0.10 $\log_{10}$ CFU mL^{-1}. Considering the study of CTAB alone, at both concentrations, on *E. coli* (Figure VIII.3), it is possible to observe that its effect was higher at pH 8 ($p > 0.05$). In addition, only at pH 7 it was possible to observe a positive effect of 1 mM of cinnamaldehyde on *E. coli* reduction ($p > 0.05$). A maximum *E. coli* reduction was obtained with the combination of 0.02 mM of CTAB with 1 mM of cinnamaldehyde at pH 8 (reduction of 3.15 ± 0.99 $\log_{10}$ CFU mL^{-1}). When the effect of the biocide and the phytochemical were assessed against *S. aureus* (Figure VIII.3), it was possible to observe that the pH was able to influence the efficiency of CTAB alone. In this case, a reduction of 1.73 ± 0.33 $\log_{10}$ CFU mL^{-1} (0.02 mM) and 4.05 ± 0.04 $\log_{10}$ CFU mL^{-1} (0.03 mM) were achieved at pH 7, while a reduction of 3.89 ± 0.60 $\log_{10}$ CFU mL^{-1} (0.02 mM; $p < 0.001$) and 4.95 ± 0.00 $\log_{10}$ CFU mL^{-1} (0.03 mM) were

obtained for pH 8. The highest *S. aureus* reduction was obtained with 0.03 mM of CTAB and with 1 mM of cinnamaldehyde (4.19 ± 0.30 $\log_{10}$ CFU mL^{-1}) at pH 7. Considering that only at pH 7 a positive effect of the presence of cinnamaldehyde in the combination was observed together with the auspicious results obtained for *S. aureus* the subsequent studies were performed at pH 7.

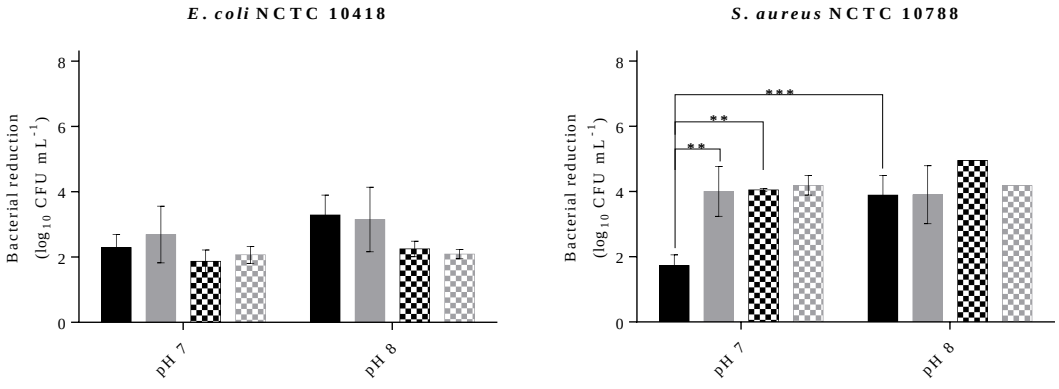


Figure VIII.3. *E. coli* NCTC 10418 and *S. aureus* NCTC 10788 CFU reduction after exposing a bacterial suspension to 0.02 mM (filled columns) or 0.03 mM (pattern columns) of CTAB alone (black columns) or in combination with 1 mM of cinnamaldehyde (grey columns), when two different pH 7 or pH 8 were used in the formulation, for 5 min. Values are mean ± SD. The statistical significance is presented (** $p < 0.01$; *** $p < 0.001$).

VIII.2.2. Formulation optimization – with soil load

The next step of this study was to test the combination CTAB/cinnamaldehyde in the presence of soil load. According to EN 1276:2009, the test should be performed in clean conditions (0.3 g L^{-1} of BSA) and dirty conditions (3 g L^{-1} of BSA) (CEN, 2009). In addition, to improve the disinfecting efficacy against the Gram-negative bacterium, EDTA was added to the formulation. For these conditions, another screening with different concentrations of cinnamaldehyde, EDTA and CTAB was performed aimed to improve the efficacy against *E. coli* as well as to produce a formulation that may be efficient enough to pass the standard tests (Table VIII.1; *Appendix A.6.*). Considering the results obtained so far, a formulation containing 1 mM cinnamaldehyde, 25 mM EDTA and 0.5 mM CTAB was selected for further testing and optimization.

Table VIII.1. Concentrations tested for cinnamaldehyde, EDTA and CTAB in combination by using the suspension test against *E. coli* NCTC 10418

Cinnamaldehyde (mM)	EDTA (mM)	CTAB (mM)
1	10	0.02
2	**25**	**0.5**
		1

E. coli and *S. aureus* were exposed to CTAB, CTAB/cinnamaldehyde and CTAB/cinnamaldehyde/EDTA at the previously stablished concentrations in order to understand the importance of each component on the formulation, both under clean (0.3 g L^{-1}) and dirty conditions (3 g L^{-1}) (Figure VIII.4). The efficiency of the formulation against *S. aureus* was confirmed, as a total CFU reduction for clean and dirty conditions was observed. The combination consisting of CTAB/cinnamaldehyde was used in clean and dirty conditions and has shown to promote a decrease in efficacy in *S. aureus*, from 5.78 to 3.20 $\log_{10}$ CFU mL^{-1} reduction and a higher efficacy for *E. coli* under clean conditions. However, the highest efficacy was achieved for CTAB alone and CTAB/cinnamaldehyde/EDTA, causing reductions of 3.50 ± 1.29 $\log_{10}$ CFU mL^{-1} and 3.27 ± 0.54 $\log_{10}$ CFU mL^{-1}, respectively. When the amount of bacteria used was reduced to 1/3 (Figure VIII.4), CTAB/cinnamaldehyde/EDTA showed a higher reduction of 4.69 ± 0.64 $\log_{10}$ CFU mL^{-1} and 4.20 ± 0.89 $\log_{10}$ CFU mL^{-1} under clean and dirty conditions, respectively.

At this point it was decided to use the following formulation: 1 mM cinnamaldehyde, 25 mM EDTA, 0.5 mM CTAB in PB at pH 7 and isopropanol (5 % v v^{-1}).

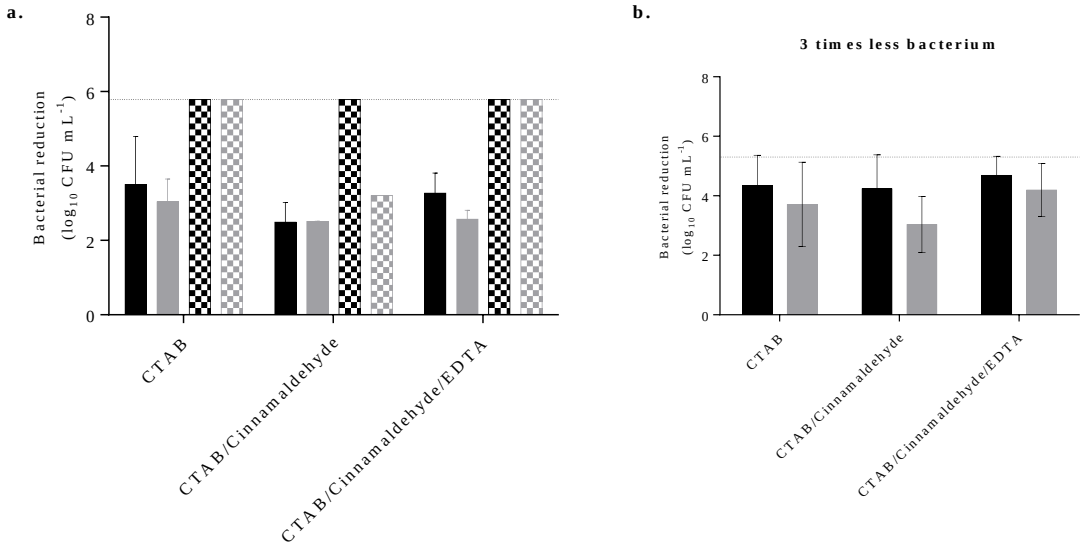


Figure VIII.4. Bacterial reduction in the presence of soil load. a. *E. coli* NCTC 10418 (filled columns) and *S. aureus* NCTC 10788 (pattern columns) CFU reduction after exposing a bacterial suspension for 5 min to CTAB alone, CTAB in combination with cinnamaldehyde or CTAB in combination with cinnamaldehyde and EDTA. The test was performed under clean (black columns) or dirty (grey columns) conditions. b. *E. coli* NCTC 10418 log_{10} CFU reduction after exposing a 3 times less concentrated bacterial suspension for 5 min to CTAB alone, CTAB in combination with cinnamaldehyde or CTAB in combination with cinnamaldehyde and EDTA. The test was performed under clean (0.3 g L^{-1}; grey columns) or dirty (3 g L^{-1}; black columns) conditions. Horizontal dashed line in the figure represents total reduction of bacteria. Values are mean ± SD.

VIII.2.3. Surface wiping – mechanical and formulation efficacy

The main purpose of the development of this formulation was to be used in wipes; more specifically to be impregnated in ready-to-use wipes. In order to understand the effectiveness of the formulation together with the mechanical action of the wipe two different types of wipes were chosen (wipe A and B). With the surface wiping method, it was possible to study the ability to remove and kill bacteria from a contaminated surface (D1) and to evaluate the consecutive transfer of bacteria to clean surfaces (D1.1 and D1.2) following wiping. As it is possible to observe from Figure VIII.5, *E. coli* removal from surfaces (without formulation) was similar for both wipes, with remaining 3.42 ± 0.46 log_{10} CFU or 3.73 ± 0.53 log_{10} CFU, whether wipe A or B was used to wipe D1. However, when no formulation was used, the use of the same wipe to wipe two clean surfaces resulted in high cell contamination. D1.1 had a contamination of 3.18 ± 0.35 log_{10} CFU and 3.61 ± 0.38 log_{10} CFU and D1.2 of 2.80 ± 0.62 log_{10} CFU and 3.52 ± 0.74 log_{10} CFU of *E. coli* when wipe A or B were used, respectively. When the wipe was impregnated

with the formulation a decrease in bacterial load on the surfaces was observed. In fact, for wipe A, the CFU were below the detection limit, 1.49 ± 0.00 log_{10} (total CFU), on D1 ($p < 0.001$), D1.1 ($p < 0.01$) and D1.2 ($p < 0.05$), when compared to the wipe without formulation. When wipe B was impregnated with the formulation 2.05 ± 0.79 log_{10} CFU remained on D1 ($p < 0.01$), and CFU were below the limit of detection on D1.1 ($p < 0.01$) and on D1.2 ($p < 0.01$), when compared to the wipe without the formulation. Both wipes had a similar mechanical effect against *S. aureus.* In the contaminated surface D1 remained 5.38 ± 0.20 log_{10} CFU and 5.19 ± 0.33 log_{10} CFU, after wiping with wipe A or B, respectively. While in D1.1 remained 4.91 ± 0.52 log_{10} CFU and 4.88 ± 0.40 log_{10} CFU and in D1.2 4.65 ± 0.47 log_{10} CFU and 4.68 ± 0.36 log_{10} CFU whether wipe A or B were used, respectively. When the wipes were impregnated with the formulation a reduction on the remaining *S. aureus* was achieved ($p < 0.001$). Therefore, for D1 2.76 ± 0.22 log_{10} CFU (wipe A) and 3.64 ± 0.24 log_{10} CFU (wipe B) remained on the surface while the number of CFU on D1.1 and D1.2 were below the detection limit following the use of either wipes.

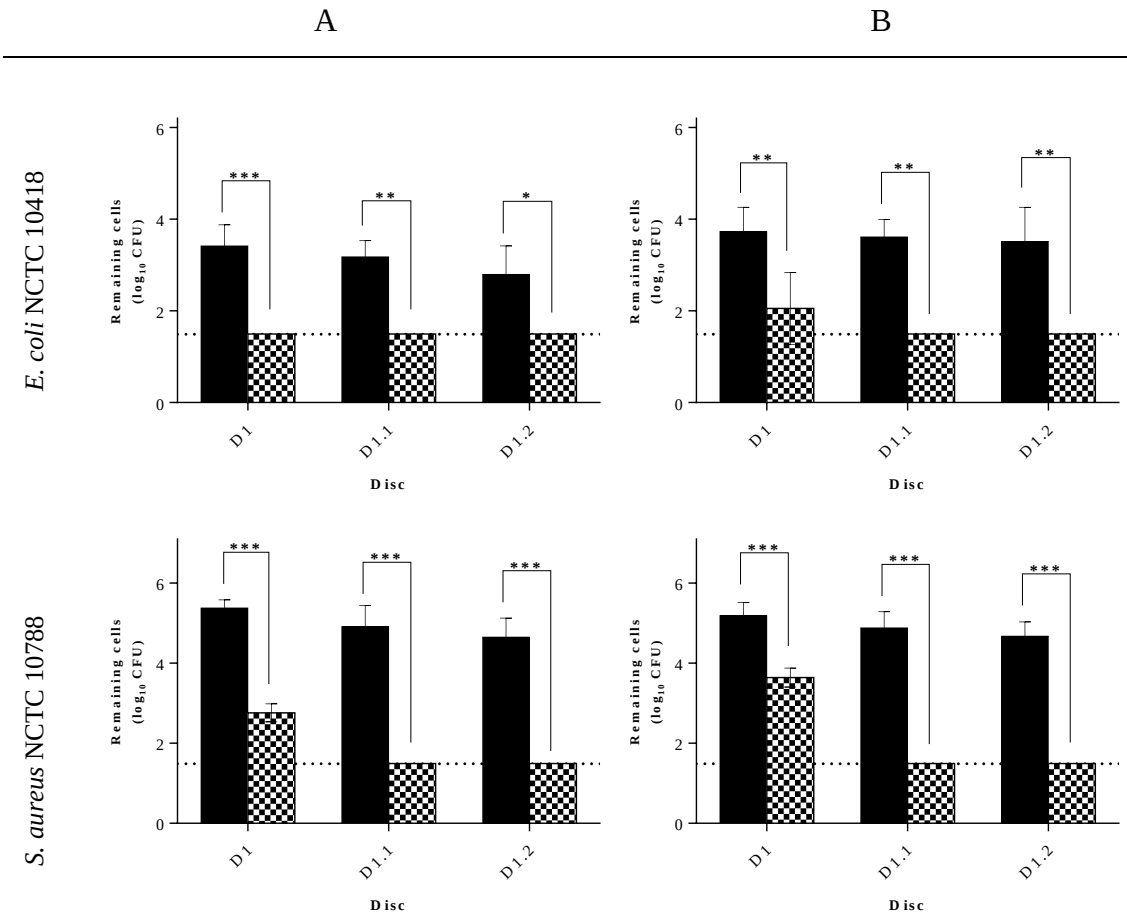


Figure VIII.5. Remaining cultivable cells on the contaminated surface (D1) and on two clean surfaces (D1.1 and D1.2) after being wiped with wipe A or B. *E. coli* NCTC 10418 and *S. aureus* NCTC 10788 were used as surfaces contaminants. Mechanical (filled columns) and biocidal (columns with pattern) action were evaluated. Values are mean ± SD. Horizontal dashed line represents the limit of detection of the method (1.49 $\log_{10}$ CFU). The statistical significance is represented (* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$).

A summary of the effectiveness of wipe A and B to remove bacteria from a contaminated surface as well as the overall cell removal (bacteria that are removed and killed during the process of wiping three surfaces) is shown in Figure VIII.6. The mechanical action of both wipes is able to remove from D1 2.78 ± 0.52 $\log_{10}$ CFU of *E. coli* and 1.71 ± 0.26 $\log_{10}$ CFU of *S. aureus* or 2.47 ± 0.42 $\log_{10}$ CFU of *E. coli* and 1.93 ± 0.30 $\log_{10}$ CFU of *S. aureus* when wipe A or B are used, respectively. In total, wipe A mechanical action removes from the surface 2.45 ± 0.41 $\log_{10}$ CFU of *E. coli* and 1.50 ± 0.35 $\log_{10}$ CFU of *S. aureus*, while wipe B removes 2.06 ± 0.41 $\log_{10}$ CFU of *E. coli* and 1.66 ± 0.31 $\log_{10}$ CFU of *S. aureus*. When impregnated with the formulation the efficacy of the wipe is improved, in fact, with wipe A bacterial removal from D1 achieves 4.83 ± 0.22 $\log_{10}$ CFU of *E. coli* ($p < 0.001$) and 4.36 ± 0.23 $\log_{10}$ CFU of *S.*

aureus ($p < 0.001$) while wipe B achieves 4.27 ± 0.57 $\log_{10}$ CFU of *E. coli* ($p < 0.01$) and 3.48 ± 0.24 $\log_{10}$ CFU of *S. aureus* ($p < 0.001$). Considering the overall cell removal the impregnated wipe A can remove 4.35 ± 0.22 $\log_{10}$ CFU of *E. coli* ($p < 0.001$) and 4.27 ± 0.22 $\log_{10}$ CFU of *S. aureus* ($p < 0.001$), while wipe B removes 4.04 ± 0.46 $\log_{10}$ CFU of *E. coli* ($p < 0.001$) and 3.47 ± 0.23 $\log_{10}$ CFU of *S. aureus* ($p < 0.001$).

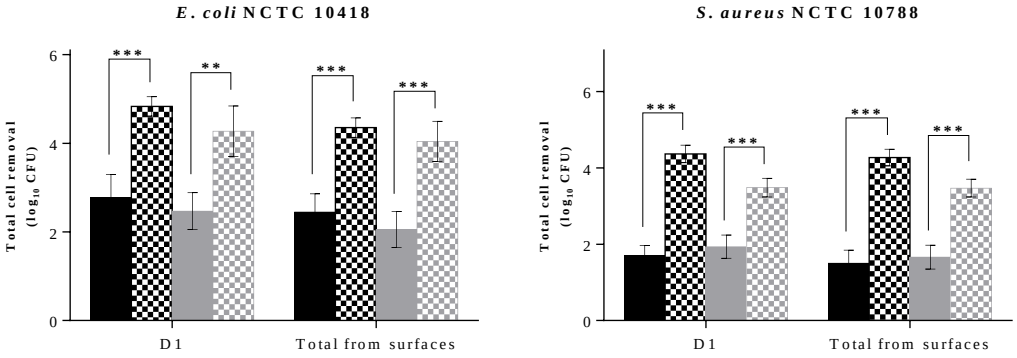


Figure VIII.6. *E. coli* NCTC 10418 and *S. aureus* NCTC 10788 reduction from the inoculated disc (D1) and all the bacteria that are not recovered from the discs (Total from surfaces). The mechanical action (filled columns) and the biocidal effect (columns with pattern) were tested for two types of wipe, A (black) and B (grey). Values are mean ± SD. The statistical significance is represented (** p<0.01; *** p<0.001).

VIII.2.4. Formulation chemical stability

The last step on the development of a formulation to be used in ready-to-use wipes should include a stability study of the components in the mixture as well as a study on the stability of the mixture after a period of time. Therefore, two preliminary studies have been performed, one to check the potential chemical interaction of the phytochemical with the biocide or other components of the formulation, accomplished by ^{1}H NMR, the second aimed to quantify, by HPLC analysis, the content of cinnamaldehyde in the formulation (in the moment of preparation and after one month). From the analysis of the ^{1}H NMR spectra, Figure VIII.7, it was possible to conclude that cinnamaldehyde maintain its chemical integrity as part of a mixture with CTAB/EDTA, whether in water or as a formulation. In fact, the same type of signals was observed in the comparative analysis with the spectrum of cinnamaldehyde. The HPLC quantification analysis of a fresh formulation allowed to conclude that it has a concentration of cinnamaldehyde ranging from 1.15 – 1.24 mM, which is similar to the standard concentration stablished for the

formulation (1 mM). However, after one month of shelf storage of one sample of the formulation, the concentration of cinnamaldehyde was 0.24 mM, a result that pointed out the potential occurrence of chemical degradation processes (*Appendix A.8.*).

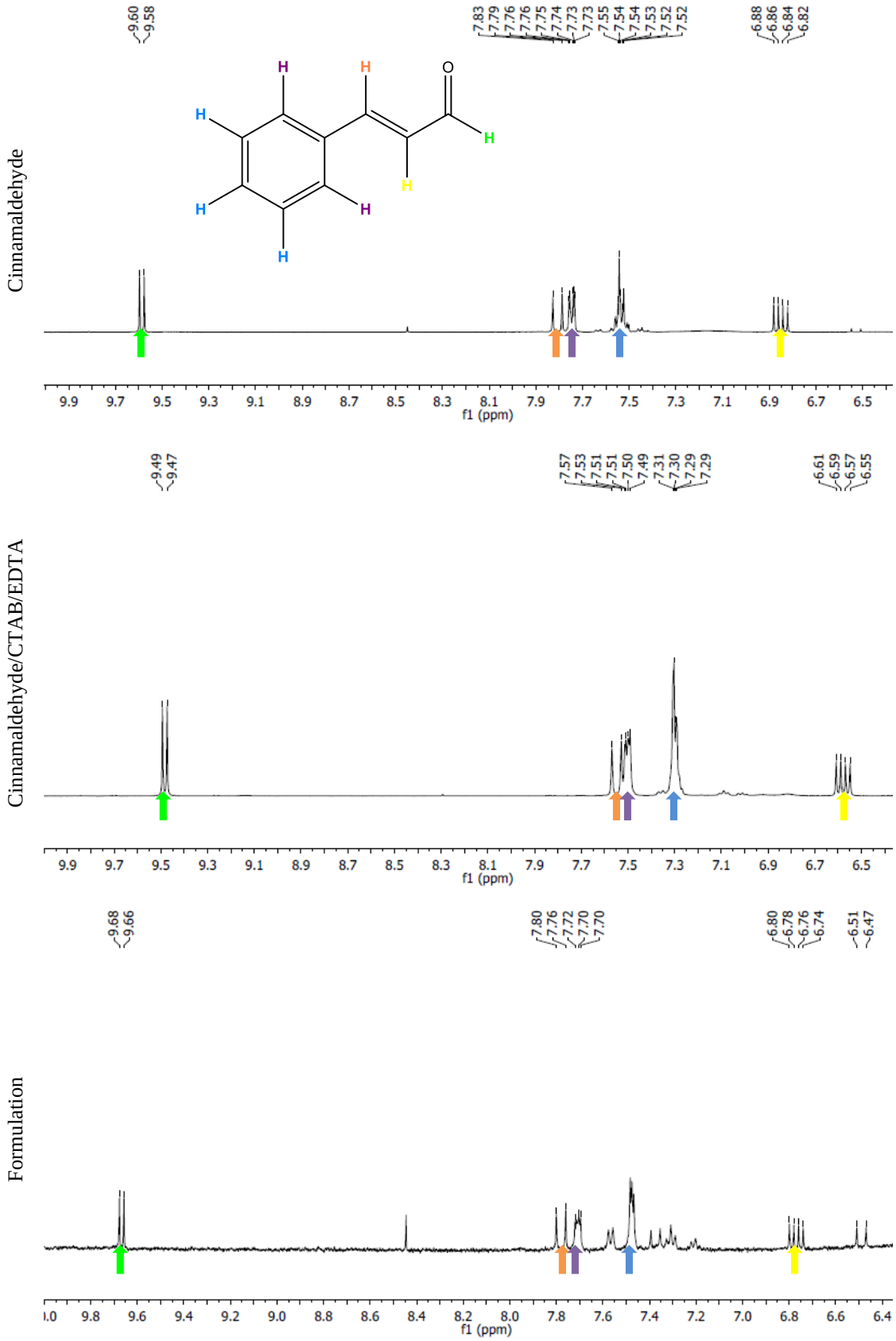


Figure VIII.7. ^{1}H NMR spectra of cinnamaldehyde (top), a mixture of cinnamaldehyde/CTAB/EDTA in water (middle) and the formulation (bottom). All the solutions were prepared in deuterated water.

VIII.3. Discussion

Surface contamination and microbial spreading to clean surfaces is a consequence of improper surface disinfection and it is a major problem since it may contribute to the infection of people and the contamination of objects (Bhatta *et al.*, 2018; Ismaïl *et al.*, 2013). One of the methodologies used to ensure a proper and efficient disinfection includes the use of ready-to-use wipes, impregnated with a disinfectant formulation (Panousi *et al.*, 2009). In the work presented in this chapter all the previously acquired information (Chapter IV, V, VI and VII) was used to develop a formulation consisting in a mixture of a natural product and a biocide. At this point, the most promising biocide was CTAB when combined with cinnamaldehyde or α-methylhydrocinnamic acid.

VIII.3.1. Formulation development

The development of a formulation has some requirements and needs to be in accordance with official standards, such as EN 1276:2009 "Chemical disinfectants and antiseptics". To comply with the test a reduction of 5 $\log_{10}$ CFU mL^{-1} must be achieved (CEN, 2009). Initially, the combination of CTAB with 1 mM of cinnamaldehyde was found to be efficient in killing more than 5 $\log_{10}$ CFU mL^{-1}, which is in accordance with the standard rules. On the other hand, the use of α-methylhydrocinnamic decreased the efficiency of the mixture and, as such the mixture fails the test. CTAB is a cationic surfactant whose mode of action is related to the denaturation of proteins inducing changes in the properties of cell membrane and, therefore, facilitates the entrance of other antimicrobials (Jin *et al.*, 2015). This surfactant is also able to concentrate, solubilise and compartmentalise ions and molecules which can enhance its antimicrobial action and also of other antimicrobials (Ramanaiah *et al.*, 2014). So, the combined effect of CTAB with cinnamaldehyde may be advantageous when compared with the combination with α-methylhydrocinnamic acid, due to its lower MW (132.162 g mol^{-1} cinnamaldehyde, 164.204 g mol^{-1} α-methylhydrocinnamic acid) and higher lipophilicity, which are parameters that can facilitate cinnamaldehyde cross of the bacterial membrane (Di Pasqua *et al.*, 2006; Gill *et al.*, 2004). In fact, cinnamaldehyde is described to be able to interact with membranes, proteins, nucleic acids, lipids and carbohydrates of *E. coli* (Mousavi *et al.*, 2016). It was also hypothesised that low concentrations of cinnamaldehyde act on the cell membrane components and, at a higher dosage, it can diffuse into the bacteria,

modifying the cytoplasm enzymes in the transcriptome, and consequently can promote cell death (Friedman, 2017; Gill *et al.*, 2004; Wang *et al.*, 2017; Wang *et al.*, 2018).

The development of a formulation must also be user safe and environmentally friendly (Jover *et al.*, 1992; Singh *et al.*, 2017; Timm *et al.*, 2013). Therefore, in this study the replacement of DMSO by the less cytotoxic solvent isopropanol was positive. On the other hand, it has also improved the disinfection efficacy of the mixture. The establishment of a working pH of 7 was also relevant in the case of *S. aureus*, against the Gram-negative bacterium the reduction was not significative. Usually, Gram-negative bacteria are more resistant to antimicrobials when compared to Gram-positive (Brown, 2015; Lambert *et al.*, 2004; Vale *et al.*, 2019). In fact, a 10-100 fold increase in QAC concentration is generally required to inhibit *P. aeruginosa* in comparison with *S. aureus* (Lambert *et al.*, 2004). As it was possible to observe, the maintenance of a defined pH is of extreme importance since a change of pH can alter the biocide ionization and consequently its activity. However, contrarily to what was observed in this study, usually an increase in pH enhances the activity of cationic biocides (Maillard, 2005).

In order to improve the efficacy of the mixture against both *S. aureus* and *E. coli*, EDTA was used. EDTA is believed to disrupt the lipopolysaccharide structure in the outer membrane of Gram-negative bacteria, which makes the bacteria more permeable to other agents - potentiating the antimicrobial efficacy (Lambert *et al.*, 2004; Maillard, 2005). In fact, EDTA has already been reported as potentiator of the activity of antimicrobials, preservatives, antibiotics and cationic surfactants such as QACs (Finnegan *et al.*, 2015; Lambert *et al.*, 2004). When combined with a QAC, EDTA has even demonstrated synergy when used against *P. aeruginosa* (Lambert *et al.*, 2004). Some authors hypothesised the potentiation as a result of a loss of barrier function of the outer membrane, such as efflux pumps inhibition, as well as an enhanced uptake mechanism or removal of inactivating factors that are found in the membrane or in the periplasmic space caused by the presence of EDTA (Chaudhary *et al.*, 2012b; Finnegan *et al.*, 2015; Lambert *et al.*, 2004). In addition, Lambert *et al.* (2004) was able to model the two inhibition process of EDTA mode of action. One happens bellow a threshold concentration and according to the literature is a general inhibitory process, such as removing metal ions from the growth medium, while the second that happens at a higher concentration corresponds to the destabilization of the outer membrane that consequently leads to cell lysis. Other authors have also reported enhanced antimicrobial efficacy in the presence of EDTA (Chaudhary *et al.*, 2012b; Finnegan *et al.*, 2015; Sung *et al.*, 2008;

Vale *et al.*, 2019). Furthermore, in terms of formulation safety requirements, the use of EDTA has been considered safe for up to 40 mg/kg/body weight when administered intravenously to Swiss albino mice (Chaudhary *et al.*, 2012b) as well as when administered in combination with ceftriaxone and sulbactam, EDTA was safe until 150 mg/kg/body weight (Chaudhary *et al.*, 2012a). This combination has even been recommended for the treatment of Multidrug Resistant septicaemia (Patil *et al.*, 2015).

The following step was to test the mixture in the presence of soil load, which represents the microbial burden that may exist on the surfaces to be disinfected. The mixture developed in this work was found to be efficient. However, despite passing the Standard test for *S. aureus*, its performance on *E. coli* was not sufficient to obtain the 5 $\log_{10}$ CFU mL^{-1} requirement. However, the presence of EDTA has improved the formulation efficacy against the Gram-negative bacterium possibility by interfering with the bacteria outer membrane as described in the literature (Chaudhary *et al.*, 2012b; Finnegan *et al.*, 2015; Lambert *et al.*, 2004). The presence of organic load was already reported to decrease the disinfection efficacy, since biocides can react with organic matter (Maillard, 2005; Ribeiro *et al.*, 2018).

At this point a formulation was established, 1 mM cinnamaldehyde, 25 mM EDTA, 0.5 mM CTAB in PB pH 7 with isopropanol (5 % v v^{-1}), to be tested in conditions closer to reality that will mimic surface wiping.

VIII.3.2. Formulation is efficient in surface disinfection and prevents microbial transfer to clean surfaces

The bacterial removal from a contaminated surface by the wiping procedure described previously was more efficient in the presence of the formulation when compared with the mechanical action of wiping. In addition, the formulation-containing wipe with the formulation prevented the microbial spreading to clean surfaces. The best surface disinfection was achieved by using wipe A, both for *E. coli* and *S. aureus*. In addition, both impregnated wipes were able to prevent microbial transfer to clean surfaces. The texture of wipe A was different in comparison with wipe B and, in this case, no significant difference was observed when the mechanical action was assessed. The ability of the positive charged CTA^+ to interact with the negatively charged bacteria by non-selective electrostatic interactions may have also helped in preventing the contamination of clean surfaces. In fact, Jin *et al.* (2015) had observed a higher *E. coli*

and *B. subtilis* capture by Fe_3O_4 particles due to the presence of CTAB, even in the presence of natural organic matter.

VIII.3.3. Formulation is unstable when stored for one month

The formulation despite its efficacy in cleaning contaminated surfaces is unstable, after 1 month of shelf storage. In fact, preliminary analytical studies showed that after time, the concentration of cinnamaldehyde in the mixture is four times less. On the other hand, it was also observed by NMR that no chemical interactions occur between the phytochemical and the biocide. The instability of cinnamaldehyde in solution has been observed by Si *et al.* (2006) when cinnamaldehyde was suspended in water. Cinnamaldehyde activity against *Salmonella* serotype Typhimurium DT104 has decreased from 84 % to 61 % at 4 °C and from 94 % to 40 % at 22 °C after 7 days of storage (Si *et al.*, 2006). According to Gholivand *et al.* (2008), pure trans-cinnamaldehyde is decomposed at 70 °C and higher temperature into benzaldehyde. They also hypothesised that when oxygen is present trans-cinnamaldehyde undergoes a heat-induced carbon-carbon bond cleavage and forms glyoxal and benzaldehyde. Benzaldehyde has been used as a flavouring and fragrance in food and cosmetic and it was approved by the U.S. Food and Drug Administration (Gowder, 2014; Opgrande *et al.*, 2000; Report, 2006).

VIII.4. Conclusion

Surface disinfection is of extreme importance both in healthcare and industrial facilities. Therefore, the development of new formulations that ensure a clean and disinfected environment are demanded, especially the ones that can be easily applied such as being used with wipes or even when impregnated in ready-to-use wipes.

In this context, the purpose of this work was the development of a formulation that could be used with wipes for a proper surface disinfection. This formulation was based on the mixture of a biocide (CTAB) a phytochemical (cinnamaldehyde), and a chelator (EDTA), which together had proven high efficacy against surface contamination and also avoiding transfer or microbial burden. At this point, the formulation developed is very promising. In addition, the formulation can be further tested for sporicidal since

CTAB was reported as being sporicidal has it was capable of killing three *Bacillus* species (Dong *et al.*, 2019). The formulation must also be tested to ensure it does not promote resistance however cinnamaldehyde did not promote resistance when tested by Ali *et al.* (2005). More complete tests that mimic the real conditions where wipes are used must still be used to ensure an efficient disinfection in practice (Panousi *et al.*, 2009; Williams *et al.*, 2009; Williams *et al.*, 2007).

Chapter IX. Conclusions

1. Final Remarks
2. Future Work and Perspectives

IX.1. Final Remarks

This PhD thesis was developed taking into consideration the enormous potential of phytochemicals to be used for disinfection, in order to supress the need of new and improved chemicals and formulations to which no resistance has yet been described. Therefore, a wide range of structurally related phytochemicals were selected and tested in combination with several biocides in order to develop a formulation to be incorporated in wipes, as presented in Figure IX.1. In addition, the work was extended to acquire data on phytochemicals mode of action.

Overall, it was possible to notice that cinnamaldehyde and eugenol had MIC's comparable to commonly used biocides, while cinnamic acid at sub-MIC/MBC demonstrated the ability to disperse adhered bacteria. In addition, cinnamic acid and cinnamaldehyde derivatives were able to inhibit the quorum sensing of *C. violaceum*.

The study of the combination of LA or CTAB with phytochemicals and derivatives was successfully accomplished. In general, the growth of *E. coli* and *S. aureus* was inhibited by the combination of LA with phytochemicals and derivatives that possess a carboxylic group in their structure, while the cinnamic acid, allyl cinnamate or hydrocinnamic acid increased LA activity against sessile cells of *E. coli*. CTAB combined with allyl cinnamate with α-methylcinnamic acid had increased efficacy against sessile cells.

Cinnamaldehyde ability to promote EB accumulation in resistant *S. aureus* strains was hypothesised to be a result of an interaction with the cell membrane that affects the proton motive force and consequently inhibits MFS pumps. Other phytochemicals and derivatives that possess a carboxylic group reduced the MIC of EB of some *S. aureus* strains harbouring *norA, tetK* and *smr* possibly by sequestering H^+ and consequently affecting ΔpH.

With all the information gathered along the thesis a formulation based on a mixture of a biocide (CTAB), a phytochemical (cinnamaldehyde) and a chelator (EDTA), was developed and impregnated into ready-to-use wipes for surface disinfection. This formulation has proven efficacy against surface contamination and avoids microbial transference to clean surfaces.

As proven with this thesis, phytochemicals and derivatives have great potential to be used in disinfection, both as antimicrobials per se and as part of a biocidal formulation.

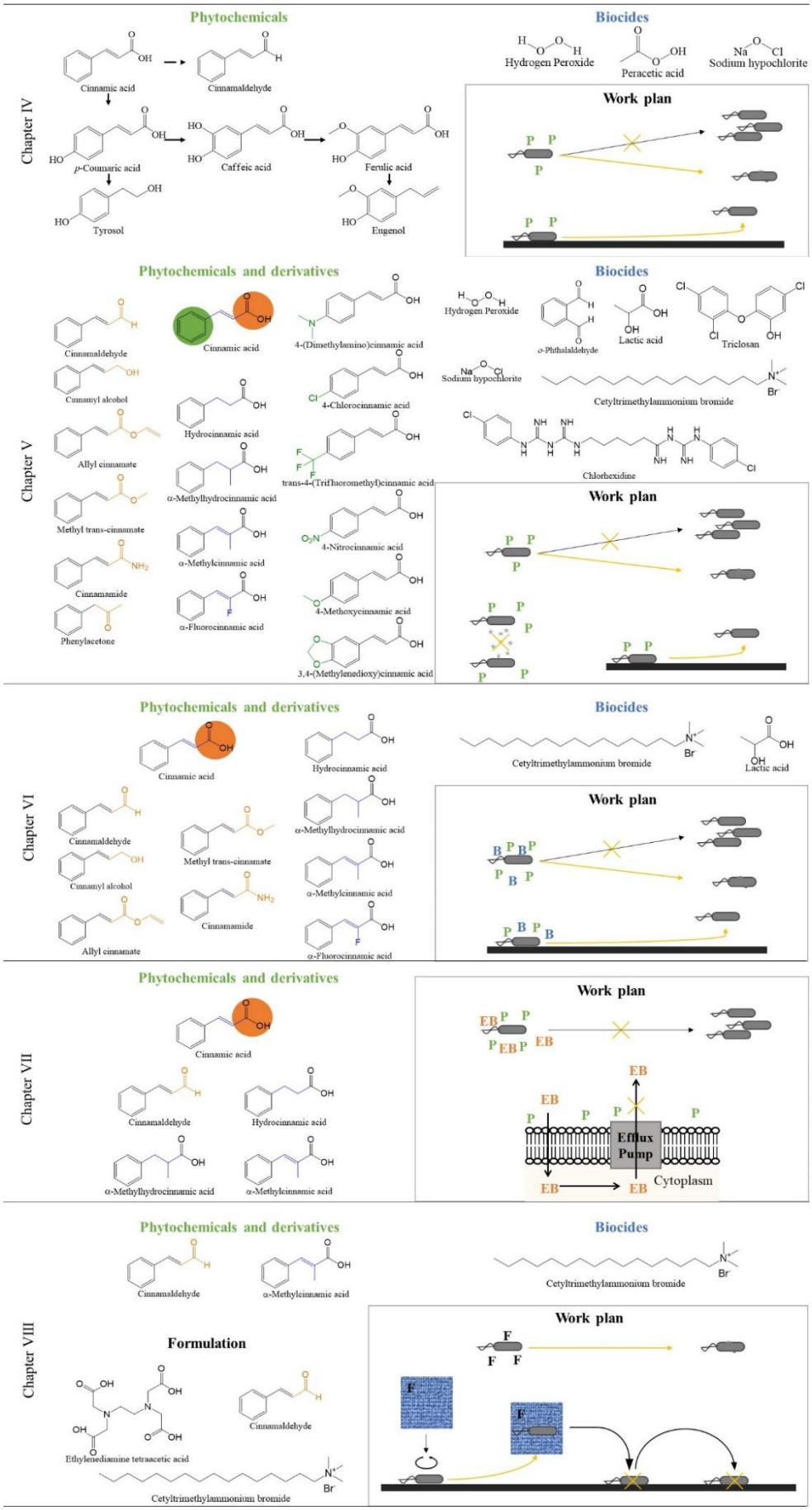


Figure IX.1. Overall schematic project of the thesis.

IX.2. Future Work and Perspectives

The development of this thesis has answered some questions on the potential of phytochemicals to be used in disinfection as biocide formulations. However, it also has raised new questions and new ideas to pursue. In fact, the same line of thinking used in this thesis can be used to study a wider range of phytochemicals, structurally related or not. This selection can be based on each phytochemicals class, previously reported antimicrobial efficacy and already approved by FDA/GRAS.

Phytochemicals mechanism of action should be further assessed by studying bacterial surface properties such as charge (zeta potential), membrane integrity (selective uptake of propidium iodide from Live/Dead BacLight kit, atomic absorption spectroscopy of potassium ions, CryoSEM and SEM). In the case of efflux inhibition, cinnamaldehyde interaction with the different efflux systems should be analysed by docking simulations to evaluate its putative interactions with the membrane.

Regarding *S. aureus* XU212 resistance, subsequent study must be done by exploring its resistance profile towards biocides, antibiotics and phytochemicals as well as the study of this strain membrane properties. The gene/protein expression profile after exposure to the phytochemicals must also be assessed (microarrays, 2D-electrophoresis).

Since the developed formulation, despite being efficient in killing bacteria in suspension (4.20-4.69 $\log_{10}$ CFU ml^{-1} of *E. coli* and 5.78 $\log_{10}$ CFU ml^{-1} for *S. aureus*) as well in removing bacteria from a contaminated surface (4.04-4.35 $\log_{10}$ CFU of *E. coli* and 3.47-4.27 $\log_{10}$ CFU of *S. aureus*), was not stable for shelf storage several additional optimizations need to be performed before being considered a product for commercialization. The process of optimization may include storage at different temperatures, the type of container, type of buffer and solvent, or even the addition of stabilizers to avoid cinnamaldehyde degradation. Additionally, a series of diverse shelf-life assays must be performed. Considering that the optimization is successful and that the formulation is still efficient according to EN 1276 several additional steps must be performed. In fact, an end product always needs to pass European Standard rules accordingly with the area they are intended to be used. Finally, the formulation can be impregnated in wipes to produce ready-to-use wipes, its efficacy can be tested in different surface materials (EN 13697, EN 16615, ASTM E2967 − 15) as well as the possibility of a residual effect of the formulation on bacterial adhesion after wiping must be assessed.

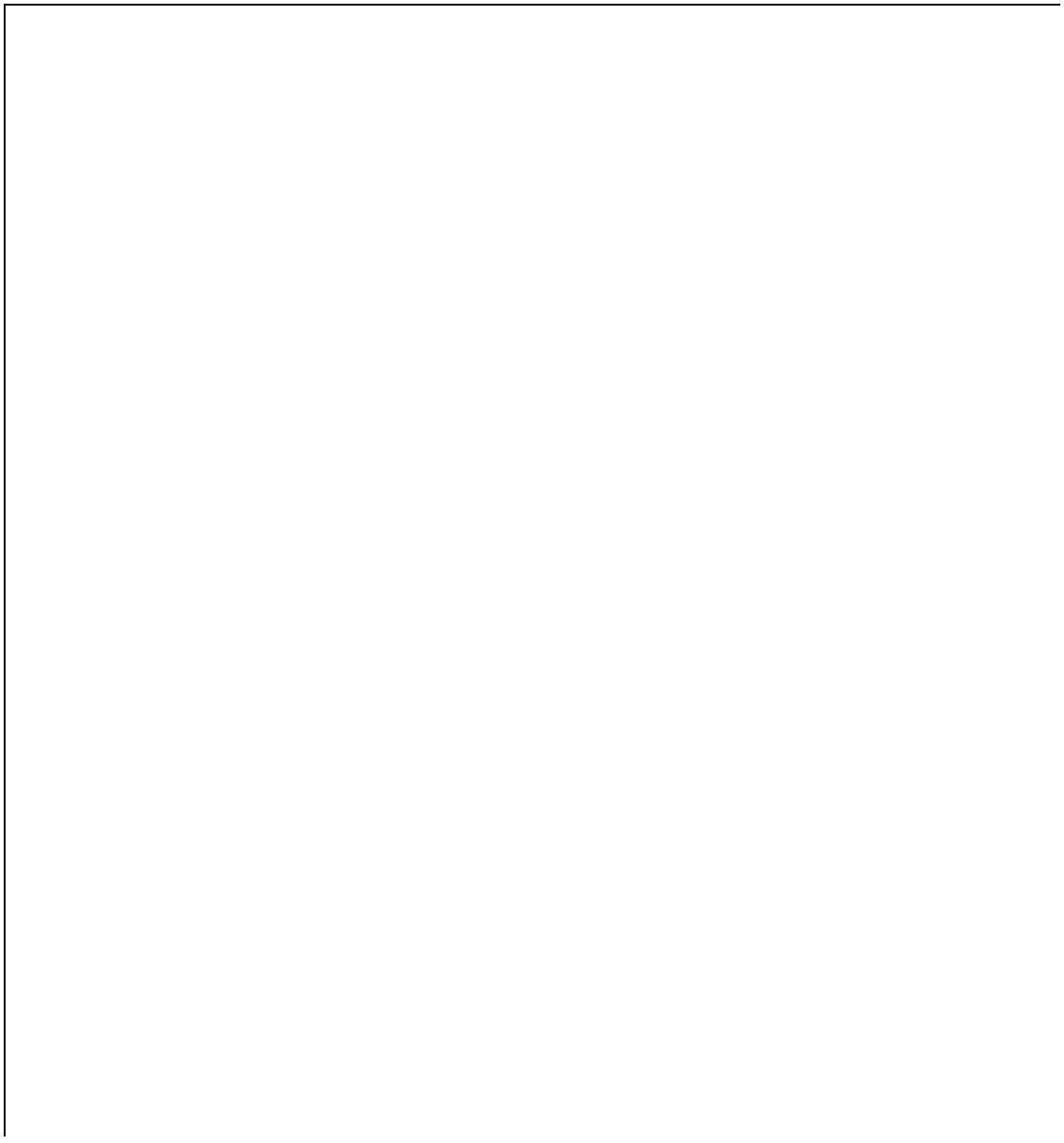
Another important aspect that should be analysed when developing a formulation for disinfection is the possibility of development of biocide resistance and cross-resistance to antibiotics. In this case, the protocol developed by Forbes *et al.* (2014) can be used, where it is possible to measure the propensity of a biocidal product to promote bacterial resistance to the biocide and cross-resistance to unrelated compounds including antibiotics. In addition, the stability of the resistance should also be tested.

Among other possible applications, the knowledge acquired during the development of this thesis will reinforce the possible uses of phytochemicals and derivatives in different areas, such as antimicrobial, resistance modulation, potentiation of biocides and surface disinfection.

Considering the relevancy of these applications, the data obtained along this thesis is expected to impact immediately the scientific community by the publication of the results obtained and possibly, in a near future, the society by the translation of the knowledge in a beneficial product.

> "To raise new questions, new possibilities, to regard old problems from a new angle, requires creative imagination and marks a real advance in science."
>
> *Albert Einstein*

References



Abed, E., Maythamabasmakki, Dhifafjabbarshamran, 2019. Increasing the effectiveness of ethanol against *Staphylococcus aureus* (MRSA) by using *Pulicaria undulata* plant extract from Al Muthanna desert. International Journal of Pharmaceutical Research qq, 505-509.

Abouelhassan, Y., Garrison, A.T., Bai, F., Norwood, V.M., Nguyen, M.T., Jin, S., Huigens, R.W., 2015. A phytochemical–halogenated quinoline combination therapy strategy for the treatment of pathogenic bacteria. ChemMedChem 10, 1157-1162.

Abreu, A.C., Coqueiro, A., Sultan, A.R., Lemmens, N., Kim, H.K., Verpoorte, R., van Wamel, W.J.B., Simões, M., Choi, Y.H., 2017. Looking to nature for a new concept in antimicrobial treatments: isoflavonoids from *Cytisus striatus* as antibiotic adjuvants against MRSA. Scientific Reports 7, 1-16.

Abreu, A.C., McBain, A.J., Simões, M., 2012. Plants as sources of new antimicrobials and resistance-modifying agents. Natural Product Reports 29, 1007-1021.

Abreu, A.C., Paulet, D., Coqueiro, A., Malheiro, J., Borges, A., Saavedra, M.J., Choi, Y.H., Simões, M., 2016a. Antibiotic adjuvants from *Buxus sempervirens* to promote effective treatment of drug-resistant *Staphylococcus aureus* biofilms. RSC Advances 6, 95000-95009.

Abreu, A.C., Saavedra, M.J., Simões, L.C., Simões, M., 2016b. Combinatorial approaches with selected phytochemicals to increase antibiotic efficacy against *Staphylococcus aureus* biofilms. Biofouling 32, 1103-1114.

Abreu, A.C., Serra, S.C., Borges, A., Saavedra, M.J., Salgado, A.J., Simões, M., 2014. Evaluation of the best method to assess antibiotic potentiation by phytochemicals against *Staphylococcus aureus*. Diagnostic Microbiology and Infectious Disease 79, 125-134.

Abreu, A.C., Tavares, R.R., Borges, A., Mergulhão, F., Simões, M., 2013. Current and emergent strategies for disinfection of hospital environments. Journal of Antimicrobial Chemotherapy 68, 2718-2732.

Adams, T.B., Cohen, S.M., Doull, J., Feron, V.J., Goodman, J.I., Marnett, L.J., Munro, I.C., Portoghese, P.S., Smith, R.L., Waddell, W.J., Wagner, B.M., 2004. The FEMA GRAS assessment of cinnamyl derivatives used as flavor ingredients. Food and Chemical Toxicology 42, 157-185.

Adisakwattana, S., 2017. Cinnamic acid and its derivatives: Mechanisms for prevention and management of diabetes and its complications. Nutrients 9, 1-27.

Adonizio, A.L., Downum, K., Bennett, B.C., Mathee, K., 2006. Anti-quorum sensing activity of medicinal plants in southern Florida. Journal of Ethnopharmacology 105, 427-435.

Ahn, Y., Kim, J.M., Kweon, O., Kim, S.J., Jones, R.C., Woodling, K., Gamboa da Costa, G., LiPuma, J.J., Hussong, D., Marasa, B.S., Cerniglia, C.E., 2016. Intrinsic resistance of *Burkholderia cepacia* complex to benzalkonium chloride. mBio 7, 1-11.

Al-Adham, I., Haddadin, R., Collier, P., 2013. Types of microbicidal and microbistatic agents, In: Fraise, A.P., Maillard, J.-Y., Sattar, S.A. (Eds.), Russell, Hugo & Ayliffe's: Principles and Practice of Disinfection, Preservation and Sterilization, 5th ed. Wiley-Blackwell, Oxford, UK, pp. 5-70.

Al-Adham, I., Haddadin, R., Collier, P., 2012. Types of microbicidal and microbistatic agents, In: Fraise, A., Maillard, J.Y., Sattar, S. (Eds.), Russell, Hugo and Ayliffe's: Principles and Practice of Disinfection, Preservation and Sterilization. Blackwell Publishing Ltd, pp. 5-70.

Alabdullatif, M., Boujezza, I., Mekni, M., Taha, M., Kumaran, D., Yi, Q.L., Landoulsi, A., Ramirez-Arcos, S., 2017. Enhancing blood donor skin disinfection using natural oils. Transfusion 57, 2920-2927.

Alakomi, H.L., Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., Helander, I.M., 2000. Lactic acid permeabilizes Gram-negative bacteria by disrupting the outer membrane. Applied and Environmental Microbiology 66, 2001-2005.

Alam, M.A., Subhan, N., Hossain, H., Hossain, M., Reza, H.M., Rahman, M.M., Ullah, M.O., 2016. Hydroxycinnamic acid derivatives: a potential class of natural compounds for the management of lipid metabolism and obesity. Nutrition & metabolism 13, 1-13.

Aldulaimi, O.A., 2017. General overview of phenolics from plant to laboratory, good antibacterials or not. Pharmacognosy Reviews 11, 123-127.

Ali, S.M., Khan, A.A., Ahmed, I., Musaddiq, M., Ahmed, K.S., Polasa, H., Rao, L.V., Habibullah, C.M., Sechi, L.A., Ahmed, N., 2005. Antimicrobial activities of eugenol and cinnamaldehyde against the human gastric pathogen *Helicobacter pylori*. Annals of Clinical Microbiology and Antimicrobials 4, 1-7.

Allen, M.J., White, G.F., Morby, A.P., 2006. The response of *Escherichia coli* to exposure to the biocide polyhexamethylene biguanide. Microbiology 152, 989-1000.

Altemimi, A., Lakhssassi, N., Baharlouei, A., Watson, D.G., Lightfoot, D.A., 2017. Phytochemicals: Extraction, isolation, and identification of bioactive compounds from plant extracts. Plants 6, 1-23.

Alves, M.J., Ferreira, I.C., Froufe, H.J., Abreu, R.M., Martins, A., Pintado, M., 2013. Antimicrobial activity of phenolic compounds identified in wild mushrooms, SAR analysis and docking studies. Journal of Applied Microbiology 115, 346-357.

Amaral, L., 2015. Thioridazine: A therapeutic panacea for efflux pump mediated multidrug bacterial infections. Biomolecular Research and Therapeutics 4, 1-3.

Amaral, L., Spengler, G., Martins, A., Armada, A., Handzlik, J., Kiec-Kononowicz, K., Molnar, J., 2012. Inhibitors of bacterial efflux pumps that also inhibit efflux pumps of cancer cells. Anticancer Res 32, 2947-2957.

Amaya, S., Pereira, J.A., Borkosky, S.A., Valdez, J.C., Bardón, A., Arena, M.E., 2012. Inhibition of quorum sensing in *Pseudomonas aeruginosa* by sesquiterpene lactones. Phytomedicine 19, 1173-1177.

Amison, R.T., Faure, M.E., O'Shaughnessy, B.G., Bruce, K.D., Hu, Y., Coates, A., Page, C.P., 2020. The small quinolone derived compound HT61 enhances the effect of tobramycin against *Pseudomonas aeruginosa in vitro* and *in vivo*. Pulmonary Pharmacology & Therapeutics 61, 1-9.

Anantharaju, P.G., Gowda, P.C., Vimalambike, M.G., Madhunapantula, S.V., 2016. An overview on the role of dietary phenolics for the treatment of cancers. Nutrition Journal 15, 1-16.

Andersen, J.L., He, G.X., Kakarla, P., K, C.R., Kumar, S., Lakra, W.S., Mukherjee, M.M., Ranaweera, I., Shrestha, U., Tran, T., Varela, M.F., 2015. Multidrug efflux pumps from Enterobacteriaceae, *Vibrio cholerae* and *Staphylococcus aureus* bacterial food pathogens. International journal of environmental research and public health 12, 1487-1547.

Andrade, M., Benfeito, S., Soares, P., Magalhães e Silva, D., Loureiro, J., Borges, A., Borges, F., Simões, M., 2015. Fine-tuning of the hydrophobicity of caffeic acid: studies on the antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*. RSC Advances 5, 53915-53925.

Anthonisen, I.-L., Sunde, M., Steinum, T.M., Sidhu, M.S., Sørum, H., 2002. Organization of the antiseptic resistance gene *qacA* and Tn*552*-related -lactamase genes in multidrug- resistant *Staphylococcus haemolyticus* strains of animal and human origins. Antimicrobial Agents and Chemotherapy 46, 3606-3612.

Antolak, H., Mizerska, U., Berlowska, J., Otlewska, A., Kregiel, D., 2018. *Quillaja saponaria* saponins with potential to enhance the effectiveness of disinfection processes in the beverage industry. Applied Sciences 8, 1-12.

Anwar, A., Siddiqui, R., Shah, M.R., Khan, N.A., 2018. Gold nanoparticle-conjugated cinnamic acid exhibits antiacanthamoebic and antibacterial properties. Antimicrobial Agents and Chemotherapy 62, 1-7.

Araujo, A.C.J., Freitas, P.R., Rodrigues Dos Santos Barbosa, C., Muniz, D.F., Esmeraldo Rocha, J., Neto, J.B.A., M., C.d.S.M., Moura, T.F., Pereira, R.L.S., Ribeiro-Filho, J., Silva, L.E.D., Amaral, W.D., Deschamps, C., Tintino, S.R., Iriti, M., Vitalini, S., Coutinho, H.D.M., 2020. Essential oil of *Croton ceanothifolius* Baill. Potentiates the effect of antibiotics against multiresistant bacteria. Antibiotics 9, 1-8.

Araya-Cloutier, C., Vincken, J.-P., van de Schans, M.G.M., Hageman, J., Schaftenaar, G., den Besten, H.M.W., Gruppen, H., 2018. QSAR-based molecular signatures of prenylated (iso)flavonoids underlying antimicrobial potency against and membrane-disruption in Gram positive and Gram negative bacteria. Scientific Reports 8, 1-14.

ASTM, 2016. E2967 − 15 Standard test method for assessing the ability of pre-wetted towelettes to remove and transfer bacterial contamination on hard, non-porous environmental surfaces using the Wiperator.

Ayaz, M., Ullah, F., Sadiq, A., Ullah, F., Ovais, M., Ahmed, J., Devkota, H.P., 2019. Synergistic interactions of phytochemicals with antimicrobial agents: Potential strategy to counteract drug resistance. Chemico-Biological Interactions 308, 294-303.

Azeredo, L., Pacheco, A.P., Lopes, I., Oliveira, R., Vieira, M.J., 2003. Monitoring cell detachment by surfactants in a parallel plate flow chamber. Water Science and Technology 47, 77-82.

Baiju, N., Modak, S.M., 2007. Broad-spectrum disinfectant composition containing a synergistic combination of cinnamon oil and citric acid. International Journal of Essential Oil Therapeutics 1, 117-121.

Barbieri, R., Coppo, E., Marchese, A., Daglia, M., Sobarzo-Sánchez, E., Nabavi, S.F., Nabavi, S.M., 2017. Phytochemicals for human disease: an update on plant-derived compounds antibacterial activity. Microbiological Research 196, 44-68.

Basri, D.F., Khairon, R., 2012. Pharmacodynamic interaction of *Quercus infectoria* galls extract in combination with vancomycin against MRSA using microdilution

checkerboard and time-kill assay. Evidence-Based Complementary and Alternative Medicine 2012, 493156-493162.

Bassyouni, R.H., Kamel, Z., Abdelfattah, M.M., Mostafa, E., 2016. Cinnamon oil: A possible alternative for contact lens disinfection. Contact Lens and Anterior Eye 39, 277-283.

Bello, F., Babandi, A., Ya'u, M., 2016. Phytochemicals as potential alternatives to counteract bacterial antibiotic resistance: A mini-review. Journal of Biomedical Science 1, 124-141.

Bhatta, D.R., Hamal, D., Shrestha, R., Hosuru Subramanya, S., Baral, N., Singh, R.K., Nayak, N., Gokhale, S., 2018. Bacterial contamination of frequently touched objects in a tertiary care hospital of Pokhara, Nepal: how safe are our hands? Antimicrobial Resistance & Infection Control 7, 1-6.

Bintsis, T., 2018. Microbial pollution and food safety. AIMS microbiology 4, 377-396.

Bjorland, J., Steinum, T., Kvitle, B., Waage, S., Sunde, M., Heir, E., 2005. Widespread distribution of disinfectant resistance genes among Staphylococci of bovine and caprine origin in Norway. Journal of Clinical Microbiology 43, 4363-4368.

Bjorland, J., Steinum, T., Sunde, M., Waage, S., Heir, E., 2003. Novel plasmid-borne gene qacJ mediates resistance to quaternary ammonium compounds in equine *Staphylococcus aureus*, *Staphylococcus simulans*, and *Staphylococcus intermedius*. Antimicrobial Agents and Chemotherapy 47, 3046-3052.

Bjorland, J., Sunde, M., Waage, S., 2001. Plasmid-borne *smr* gene causes resistance to quaternary ammonium compounds in bovine *Staphylococcus aureus*. Journal of Clinical Microbiology 39, 3999-4004.

Blair, J.M., Webber, M.A., Baylay, A.J., Ogbolu, D.O., Piddock, L.J., 2015. Molecular mechanisms of antibiotic resistance. Nature Reviews. Microbiology 13, 42-51.

Blair, J.M.A., Piddock, L.J.V., 2016. How to measure export via bacterial multidrug resistance efflux pumps. mBio 7, 1-6.

Bock, L.J., 2019. Bacterial biocide resistance: A new scourge of the infectious disease world? Archives of Disease in Childhood 104, 1029-1033.

Bodini, S.F., Manfredini, S., Epp, M., Valentini, S., Santori, F., 2009. Quorum sensing inhibition activity of garlic extract and *p*-coumaric acid. Letters in Applied Microbiology 49, 551-555.

Boomsma, B., Bikker, E., Lansdaal, E., Stuut, P., 2015. L-Lactic acid – A safe antimicrobial for home- and personal care formulations. sofw Journal 141, 1-5.

Borges, A., Abreu, A.C., Dias, C., Saavedra, M.J., Borges, F., Simões, M., 2016. New perspectives on the use of phytochemicals as an emergent strategy to control bacterial infections including biofilms. Molecules 21, 1-41.

Borges, A., Ferreira, C., Saavedra, M.J., Simões, M., 2013. Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. Microbial drug resistance 19, 256-265.

Borges, A., Serra, S., Abreu, A.C., Saavedra, M.J., Salgado, A., Simões, M., 2014a. Evaluation of the effects of selected phytochemicals on quorum sensing inhibition and *in vitro* cytotoxicity. Biofouling 30, 183-195.

Borges, A., Simões, L.C., Saavedra, M.J., Simões, M., 2014b. The action of selected isothiocyanates on bacterial biofilm prevention and control. International Biodeterioration & Biodegradation 86, Part A, 25-33.

Borges, A., Sousa, P., Gaspar, A., Vilar, S., Borges, F., Simões, M., 2017. Furvina inhibits the 3-oxo-C12-HSL-based quorum sensing system of *Pseudomonas aeruginosa* and QS-dependent phenotypes. Biofouling 33, 156-168.

Bouarab-Chibane, L., Forquet, V., Lanteri, P., Clement, Y., Leonard-Akkari, L., Oulahal, N., Degraeve, P., Bordes, C., 2019. Antibacterial properties of polyphenols: Characterization and QSAR (quantitative structure-activity relationship) models. Frontiers in Microbiology 10, 1-23.

Brackman, G., Celen, S., Hillaert, U., Van Calenbergh, S., Cos, P., Maes, L., Nelis, H.J., Coenye, T., 2011a. Structure-activity relationship of cinnamaldehyde analogs as inhibitors of AI-2 based quorum sensing and their effect on virulence of *Vibrio* spp. PLoS ONE 6, 1-10.

Brackman, G., Cos, P., Maes, L., Nelis, H.J., Coenye, T., 2011b. Quorum sensing inhibitors increase the susceptibility of bacterial biofilms to antibiotics *in vitro* and i*n vivo*. Antimicrobial Agents and Chemotherapy 55, 2655-2661.

Braoudaki, M., Hilton, A.C., 2005. Mechanisms of resistance in Salmonella enterica adapted to erythromycin, benzalkonium chloride and triclosan. International Journal of Antimicrobial Agents 25, 31-37.

Bremer, P.J., Monk, I., Butler, R., 2002. Inactivation of *Listeria monocytogenes*/*Flavobacterium* spp. biofilms using chlorine: impact of substrate, pH, time and concentration. Letters in Applied Microbiology 35, 321-325.

Brennan, P.J., Nikaido, H., 1995. The envelope of mycobacteria. Annual review of biochemistry 64, 29-63.

Bridier, A., Briandet, R., Thomas, V., Dubois-Brissonnet, F., 2011. Resistance of bacterial biofilms to disinfectants: A review. Biofouling 27, 1017-1032.

Brioukhanov, A.L., Netrusov, A.I., 2004. Catalase and superoxide dismutase: Distribution, properties, and physiological role in cells of strict anaerobes. Biochemistry 69, 949-962.

Brooks, S.E., Walczak, M.A., Malcolm, S., Hameed, R., 2004. Intrinsic *Klebsiella pneumoniae* contamination of liquid germicidal hand soap containing chlorhexidine. Infection Control and Hospital Epidemiology 25, 883-885.

Brooks, S.J., Doyle, E.M., O'Connor, K.E., 2006. Tyrosol to hydroxytyrosol biotransformation by immobilised cell extracts of *Pseudomonas putida* F6. Enzyme and Microbial Technology 39, 191-196.

Brown, E.D., 2015. Gram-negative resistance. ACS Infectious Diseases 1, 507-507.

Buffet-Bataillon, S., Tattevin, P., Bonnaure-Mallet, M., Jolivet-Gougeon, A., 2012. Emergence of resistance to antibacterial agents: The role of quaternary ammonium compounds — A critical review. International Journal of Antimicrobial Agents 39, 381-389.

Busscher, H.J., Weerkamp, A.H., van der Mei, H.C., van Pelt, A.W., de Jong, H.P., Arends, J., 1984. Measurement of the surface free energy of bacterial cell surfaces and its relevance for adhesion. Applied and Environmental Microbiology 48, 980-983.

Butaye, P., Cloeckaert, A., Schwarz, S., 2003. Mobile genes coding for efflux-mediated antimicrobial resistance in Gram-positive and Gram-negative bacteria. International Journal of Antimicrobial Agents 22, 205-210.

Campana, R., Casettari, L., Fagioli, L., Cespi, M., Bonacucina, G., Baffone, W., 2017. Activity of essential oil-based microemulsions against *Staphylococcus aureus* biofilms developed on stainless steel surface in different culture media and growth conditions. International Journal of Food Microbiology 241, 132-140.

Campos, F.M., Couto, J.A., Figueiredo, A.R., Tóth, I.V., Rangel, A.O.S.S., Hogg, T.A., 2009. Cell membrane damage induced by phenolic acids on wine lactic acid bacteria. International Journal of Food Microbiology 135, 144-151.

CEN, 2009. EN 1276:2009 Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas - Test method and requirements (phase 2, step 1).

CEN, 2015a. EN 13727:2012+A2 Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of bactericidal activity in the medical area - Test method and requirements (phase 2, step 1).

CEN, 2015b. EN 16615:2015 Chemical disinfectants and antiseptics - Quantitative test method for the evaluation of bactericidal and yeasticidal activity on nonporous surfaces with mechanical action employing wipes in the medical area (4- field test) - Test method and requirements (phase 2, step 2).

CEN, 2018. EN 14885:2015 Chemical disinfectants and antiseptics - Aplication of European Standards for chemical disinfectants and antiseptics.

CEN, 2019. EN 13697:2015 + A1 Chemical disinfectants and antiseptics - Quantitative non-porous surface test for the evaluation of bactericidal and/or fungicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas - Test method and requirements without mechanical action (phase 2, step 2).

Chambel, A., Viegas, C.A., Sá-Correia, I., 1999. Effect of cinnamic acid on the growth and on plasma membrane H^+–ATPase activity of *Saccharomyces cerevisiae*. International Journal of Food Microbiology 50, 173-179.

Chan, A.C., Ager, D., Thompson, I.P., 2013. Resolving the mechanism of bacterial inhibition by plant secondary metabolites employing a combination of whole-cell biosensors. Journal of Microbiological Methods 93, 209-217.

Chan, B.C., Ip, M., Lau, C.B., Lui, S.L., Jolivalt, C., Ganem-Elbaz, C., Litaudon, M., Reiner, N.E., Gong, H., See, R.H., Fung, K.P., Leung, P.C., 2011. Synergistic effects of baicalein with ciprofloxacin against NorA over-expressed methicillin-resistant *Staphylococcus aureus* (MRSA) and inhibition of MRSA pyruvate kinase. Journal of Ethnopharmacology 137, 767-773.

Chapman, J.S., 2003. Biocide resistance mechanisms. International Biodeterioration & Biodegradation 51, 133-138.

Chatterjee, M., Robson, C.N., Harris, A.L., 1990. Reversal of multidrug resistance by verapamil and modulation by alpha 1-acid glycoprotein in wild-type and multidrug-resistant Chinese hamster ovary cell lines. Cancer Res 50, 2818-2822.

Chaudhary, M., Krishnaraju, V., 2012a. Acute toxicity studies of fixed dose combination of ceftriaxone+sulbactam+ethylenediaminetetraacetic acid in Swiss Albino mice and Sprague Dawley rats. International Journal of Pharma and Bio Sciences 3, 110-122.

Chaudhary, M., Kumar, S., Payasi, A., 2012b. A novel approach to combat acquired multiple resistance in *Escherichia coli* by using EDTA as efflux pump inhibitor. Journal of Microbial & Biochemical Technology 04, 126-130.

Chen, J., Kuroda, T., Huda, M.N., Mizushima, T., Tsuchiya, T., 2003. An RND-type multidrug efflux pump SdeXY from *Serratia marcescens*. Journal of Antimicrobial Chemotherapy 52, 176-179.

Chen, J., Morita, Y., Huda, M.N., Kuroda, T., Mizushima, T., Tsuchiya, T., 2002. VmrA, a member of a novel class of Na(+)-coupled multidrug efflux pumps from *Vibrio parahaemolyticus*. Journal of Bacteriology 184, 572-576.

Chen, Y., Pi, B., Zhou, H., Yu, Y., Li, L., 2009. Triclosan resistance in clinical isolates of *Acinetobacter baumannii*. Journal of Medical Microbiology 58, 1086-1091.

Cho, P., Shi, G., Yap, M., Boost, M.V., 2016. Effects of lead phytochemicals of *Radix scutellariae* on *Acanthamoeba*. Investigative Ophthalmology & Visual Science 57, 6591-6595.

Chotigarpa, R., Na Lampang, K., Pikulkaew, S., Okonogi, S., Ajariyakhajorn, K., Mektrirat, R., 2018. Inhibitory effects and killing kinetics of lactic acid rice gel against pathogenic bacteria causing bovine mastitis. Scientia Pharmaceutica 86, 1-10.

Cieplik, F., Jakubovics, N.S., Buchalla, W., Maisch, T., Hellwig, E., Al-Ahmad, A., 2019. Resistance toward chlorhexidine in oral bacteria – Is there cause for concern? Frontiers in Microbiology 10, 1-11.

CLSI, 2007. Performande standards for antimicrobial susceptibility testing; seventeenth informational supplement M100-S17. NCCLS.

CLSI, 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: Approved standard - ninth edition M07- A09. NCCLS.

Cohen, S.M., Eisenbrand, G., Fukushima, S., Gooderham, N.J., Guenderich, F.P., Hecht, S.S., Rietjens, I.M.C.M., Harman, C., Taylor, S.V., 2018. GRAS Flovouring Substances. Food Technology, 62-77.

Commission, Commission Implementing Regulation (EU) No 872/2012, 2012. List of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council. https://eur-lex.europa.eu/eli/reg_impl/2012/872/oj

Corry, J.E.L., James, C., James, S.J., Hinton, M., 1995. *Salmonella*, *Campylobacter* and *Escherichia coli* 0157:H7 decontamination techniques for the future. International Journal of Food Microbiology 28, 187-196.

Costa, S.S., Falcão, C., Viveiros, M., Machado, D., Martins, M., Melo-Cristino, J., Amaral, L., Couto, I., 2011. Exploring the contribution of efflux on the resistance to fluoroquinolones in clinical isolates of *Staphylococcus aureus*. BMC Microbiology 11, 1-12.

Costa, S.S., Junqueira, E., Palma, C., Viveiros, M., Melo-Cristino, J., Amaral, L., Couto, I., 2013a. Resistance to antimicrobials mediated by efflux pumps in *Staphylococcus aureus*. Antibiotics (Basel, Switzerland) 2, 83-99.

Costa, S.S., Mourato, C., Viveiros, M., Melo-Cristino, J., Amaral, L., Couto, I., 2013b. Description of plasmid pSM52, harbouring the gene for the Smr efflux pump, and its involvement in resistance to biocides in a meticillin-resistant *Staphylococcus aureus strain*. International Journal of Antimicrobial Agents 41, 490-492.

Costa, S.S., Ntokou, E., Martins, A., Viveiros, M., Pournaras, S., Couto, I., Amaral, L., 2010. Identification of the plasmid-encoded *qacA* efflux pump gene in meticillin-resistant *Staphylococcus aureus* (MRSA) strain HPV107, a representative of the MRSA Iberian clone. International Journal of Antimicrobial Agents 36, 557-561.

Costa, S.S., Viveiros, M., Amaral, L., Couto, I., 2013c. Multidrug efflux pumps in *Staphylococcus aureus*: An update. Open Microbiol J 7, 59-71.

Costa, S.S., Viveiros, M., Rosato, A.E., Melo-Cristino, J., Couto, I., 2015. Impact of efflux in the development of multidrug resistance phenotypes in *Staphylococcus aureus*. BMC Microbiology 15, 1-16.

Coulon, C., Collignon, A., McDonnell, G., Thomas, V., 2010. Resistance of *Acanthamoeba* cysts to disinfection treatments used in health care settings. Journal of Clinical Microbiology 48, 2689-2697.

Dambolena, J.S., Zygadlo, J.A., Rubinstein, H.R., 2011. Antifumonisin activity of natural phenolic compounds: A structure–property–activity relationship study. International Journal of Food Microbiology 145, 140-146.

Dancer, S.J., 2004. How do we assess hospital cleaning? A proposal for microbiological standards for surface hygiene in hospitals. Journal of Hospital Infection 56, 10-15.

de Cassia da Silveira e Sá, R., Andrade, L.N., dos Reis Barreto de Oliveira, R., de Sousa, D.P., 2014. A review on anti-inflammatory activity of phenylpropanoids found in essential oils. Molecules 19, 1459-1480.

de Paula, S.B., Bartelli, T.F., Di Raimo, V., Santos, J.P., Morey, A.T., Bosini, M.A., Nakamura, C.V., Yamauchi, L.M., Yamada-Ogatta, S.F., 2014. Effect of eugenol on cell surface hydrophobicity, adhesion, and biofilm of *Candida tropicalis* and

Candida dubliniensis isolated from oral cavity of HIV-infected patients. Evidence-Based Complementary and Alternative Medicine 2014, 1-8.

Dellanno, C., Vega, Q., Boesenberg, D., 2009. The antiviral action of common household disinfectants and antiseptics against murine hepatitis virus, a potential surrogate for SARS coronavirus. American Journal of Infection Control 37, 649-652.

Di Pasqua, R., Hoskins, N., Betts, G., Mauriello, G., 2006. Changes in membrane fatty acids composition of microbial cells induced by addiction of thymol, carvacrol, limonene, cinnamaldehyde, and eugenol in the growing media. Journal of Agricultural and Food Chemistry 54, 2745-2749.

Ding, W., Jin, W., Cao, S., Zhou, X., Wang, C., Jiang, Q., Huang, H., Tu, R., Han, S.-F., Wang, Q., 2019. Ozone disinfection of chlorine-resistant bacteria in drinking water. Water Research 160, 339-349.

Dir.98/8/EC, 2012. Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998 concerning the placing of biocidal products on the market. Official Journal of the European Union

Donaghy, J.A., Jagadeesan, B., Goodburn, K., Grunwald, L., Jensen, O.N., Jespers, A., Kanagachandran, K., Lafforgue, H., Seefelder, W., Quentin, M.-C., 2019. Relationship of sanitizers, disinfectants, and cleaning agents with antimicrobial resistance. Journal of Food Protection 82, 889-902.

Dong, W., Green, J., Korza, G., Setlow, P., 2019. Killing of spores of *Bacillus* species by cetyltrimethylammonium bromide. Journal of Applied Microbiology 126, 1391-1401.

Doyle, A.A., Stephens, J.C., 2019. A review of cinnamaldehyde and its derivatives as antibacterial agents. Fitoterapia 139, 1-49.

Dukan, S., Touati, D., 1996. Hypochlorous acid stress in *Escherichia coli*: resistance, DNA damage, and comparison with hydrogen peroxide stress. Journal of Bacteriology 178, 6145-6150.

Dundar, D., Otkun, M., 2010. *In-vitro* efficacy of synergistic antibiotic combinations in multidrug resistant *Pseudomonas aeruginosa* strains. Yonsei Medical Journal 51, 111-116.

EC, 2020. Regulation on the supply and use of biocidal products. https://ec.europa.eu/health/biocides/regulation_en

Edis, Z., Haj Bloukh, S., Ibrahim, M.R., Abu Sara, H., 2020. "Smart" antimicrobial nanocomplexes with potential to decrease surgical site infections (SSI). Pharmaceutics 12, 1-36.

Eilerman, R.G., 2014. Cinnamic acid, cinnamaldehyde, and cinnamyl alcohol, Kirk-Othmer Encyclopedia of Chemical Technology, pp. 1-11.

Eissa, M.E., Abd El Naby, M., Beshir, M.M., 2014. Bacterial vs. fungal spore resistance to peroxygen biocide on inanimate surfaces. Bulletin of Faculty of Pharmacy, Cairo University 52, 219-224.

Ejim, L., Farha, M.A., Falconer, S.B., Wildenhain, J., Coombes, B.K., Tyers, M., Brown, E.D., Wright, G.D., 2011. Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficacy. Nature Chemical Biology 7, 348-350.

Estrela, C., Estrela, C.R.A., Bardin, E.L., Spanó, J.C., Marchesan, M.A., 2002. Mechanisms of action of sodium hypochlorite. Brazilian Dental Journal 13, 113-117.

Falco-Silva, V.S., Silva, D.A., Souza Mde, F., Siqueira-Junior, J.P., 2009. Modulation of drug resistance in *Staphylococcus aureus* by a kaempferol glycoside from *Herissantia tiubae* (Malvaceae). Phytotherapy Research 23, 1367-1370.

Fang, C.-T., Chen, H.-C., Chuang, Y.-P., Chang, S.-C., Wang, J.-T., 2002. Cloning of a cation efflux pump gene associated with chlorhexidine resistance in *Klebsiella pneumoniae*. Antimicrobial Agents and Chemotherapy 46, 2024-2028.

Farhadi, F., Khameneh, B., Iranshahi, M., Iranshahy, M., 2019. Antibacterial activity of flavonoids and their structure–activity relationship: An update review. Phytotherapy Research 33, 13-40.

Fernández Fuentes, M.A., Ortega Morente, E., Abriouel, H., Pérez Pulido, R., Gálvez, A., 2014. Antimicrobial resistance determinants in antibiotic and biocide-resistant Gram-negative bacteria from organic foods. Food Control 37, 9-14.

Fernandez, L., Hancock, R.E., 2012. Adaptive and mutational resistance: Role of porins and efflux pumps in drug resistance. Clinical Microbiology Reviews 25, 661-681.

Ferraris, M., Chiesara, E., Radice, S., Giovara, A., Frigerio, S., Fumagalli, R., Marabini, L., 2005. Study of potential toxic effects on rainbow trout hepatocytes of surface water treated with chlorine or alternative disinfectants. Chemosphere 60, 65-73.

Ferreira, C., Pereira, A.M., Pereira, M.C., Melo, L.F., Simões, M., 2011. Physiological changes induced by the quaternary ammonium compound

benzyldimethyldodecylammonium chloride on *Pseudomonas fluorescens*. Journal of Antimicrobial Chemotherapy 66, 1036-1043.

Ferreira, V., Wiedmann, M., Teixeira, P., Stasiewicz, M.J., 2014. *Listeria monocytogenes* persistence in food-associated environments: Epidemiology, strain characteristics, and implications for public health. Journal of Food Protection 77, 150-170.

Finnegan, M., Linley, E., Denyer, S.P., McDonnell, G., Simons, C., Maillard, J.Y., 2010. Mode of action of hydrogen peroxide and other oxidizing agents: differences between liquid and gas forms. Journal of Antimicrobial Chemotherapy 65, 2108-2115.

Finnegan, S., Percival, S.L., 2015. EDTA: An antimicrobial and antibiofilm agent for use in wound care. Advances in wound care 4, 415-421.

Fischbach, M.A., 2011. Combination therapies for combating antimicrobial resistance. Current Opinion in Microbiology 14, 519-523.

Fisher, J., 2003. Cleaning procedures in the factory - Types of Disinfectant, In: Caballero, B. (Ed.), Encyclopedia of Food Sciences and Nutrition (Second Edition). Academic Press, Oxford, pp. 1382-1385.

Floyd, J.L., Smith, K.P., Kumar, S.H., Floyd, J.T., Varela, M.F., 2010. Lmrs is a multidrug efflux pump of the major facilitator superfamily from *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 54, 5406-5412.

Forbes, S., Dobson, C.B., Humphreys, G.J., McBain, A.J., 2014. Transient and sustained bacterial adaptation following repeated sublethal exposure to microbicides and a novel human antimicrobial peptide. Antimicrobial Agents and Chemotherapy 58, 5809-5817.

Fraise, A.P., 1999. Choosing disinfectants. Journal of Hospital Infection 43, 255-264.

Fraise, A.P., 2002. Biocide abuse and antimicrobial resistance--a cause for concern? Journal of Antimicrobial Chemotherapy 49, 11-12.

Fraise, A.P., 2013. Decontamination of the environment and medical equipment in hospitals, In: Russell, H.A.s. (Ed.), Principles and Practice of Disinfection, Preservation and Sterilization, 5th ed. Blackwell Publishing Ltd, pp. 445-458.

Fresco, P., Borges, F., Diniz, C., Marques, M.P., 2006. New insights on the anticancer properties of dietary polyphenols. Medicinal Research Reviews 26, 747-766.

Friedman, M., 2017. Chemistry, antimicrobial mechanisms, and antibiotic activities of cinnamaldehyde against pathogenic bacteria in animal feeds and human foods. Journal of Agricultural and Food Chemistry 65, 10406-10423.

Fukuzaki, S., 2006. Mechanisms of actions of sodium hypochlorite in cleaning and disinfection processes. Biocontrol Science 11, 147-157.

Furi, L., Ciusa, M.L., Knight, D., Di Lorenzo, V., Tocci, N., Cirasola, D., Aragones, L., Coelho, J.R., Freitas, A.T., Marchi, E., Moce, L., Visa, P., Northwood, J.B., Viti, C., Borghi, E., Orefici, G., C., B., Morrissey, I., Oggioni, M.R., 2013. Evaluation of reduced susceptibility to quaternary ammonium compounds and bisbiguanides in clinical isolates and laboratory-generated mutants of *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 57, 3488-3497.

Galié, S., García-Gutiérrez, C., Miguélez, E.M., Villar, C.J., Lombó, F., 2018. Biofilms in the food industry: Health aspects and control methods. Frontiers in Microbiology 9, 1-18.

Ganesh, V., Hettiarachchy, N.S., Griffis, C.L., Martin, E.M., Ricke, S.C., 2012. Electrostatic spraying of food-grade organic and inorganic acids and plant extracts to decontaminate *Escherichia coli* o157:H7 on spinach and iceberg lettuce. Journal of Food Science 77, M391-M396.

Garcia-Salinas, S., Elizondo-Castillo, H., Arruebo, M., Mendoza, G., Irusta, S., 2018. Evaluation of the antimicrobial activity and a cytotoxicity of different components of natural origin present in essential oils. Molecules 23, 1-18.

Garrido, J., Borges, F., 2013. Wine and grape polyphenols — A chemical perspective. Food Research International 54, 1844-1858.

Garvey, M.I., Piddock, L.J., 2008. The efflux pump inhibitor reserpine selects multidrug-resistant *Streptococcus pneumoniae* strains that overexpress the ABC transporters PatA and PatB. Antimicrobial Agents and Chemotherapy 52, 1677-1685.

Gerometta, E., Grondin, I., Smadja, J., Frederich, M., Gauvin-Bialecki, A., 2020. A review of traditional uses, phytochemistry and pharmacology of the genus *Indigofera*. Journal of Ethnopharmacology 253, 1-77.

Gholivand, M.B., Ahmadi, F., 2008. Simultaneous determination of trans-cinnamaldehyde and benzaldehyde in different real samples by differential pulse polarography and study of heat stability of trans-cinnamaldehyde. Analytical Letters 41, 3324-3341.

Ghosh, I.N., Patil, S.D., Sharma, T.K., Srivastava, S.K., Pathania, R., Navani, N.K., 2013. Synergistic action of cinnamaldehyde with silver nanoparticles against spore-forming bacteria: A case for judicious use of silver nanoparticles for antibacterial applications. International Journal of Nanomedicine 8, 4721-4731.

Gibbons, S., 2004. Anti-staphylococcal plant natural products. Natural Product Reports 21, 263-277.

Gibbons, S., Oluwatuyi, M., Kaatz, G.W., 2003. A novel inhibitor of multidrug efflux pumps in *Staphylococcus aureus*. Journal of Antimicrobial Chemotherapy 51, 13-17.

Gibbons, S., Udo, E.E., 2000. The effect of reserpine, a modulator of multidrug efflux pumps, on the *in vitro* activity of tetracycline against clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA) possessing the *tet(K)* determinant. Phytotherapy Research 14, 139-140.

Gilbert, P., McBain, A.J., 2003a. Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. Clinical Microbiology Reviews 16, 189-208.

Gilbert, P., McBain, A.J., Rickard, A.H., 2003b. Formation of microbial biofilm in hygienic situations: A problem of control. International Biodeterioration & Biodegradation 51, 245-248.

Gilbert, P., Moore, L.E., 2005. Cationic antiseptics: diversity of action under a common epithet. Journal of AppliedMicrobiology 99, 703-715.

Gill, A.O., Holley, R.A., 2004. Mechanisms of bactericidal action of cinnamaldehyde against *Listeria monocytogenes* and of eugenol against *L. monocytogenes* and *Lactobacillus sakei*. Applied and Environmental Microbiology 70, 5750-5755.

Gold, K.M., Hitchins, V.M., 2013. Cleaning assessment of disinfectant cleaning wipes on an external surface of a medical device contaminated with artificial blood or *Streptococcus pneumoniae*. American Journal of Infection Control 41, 901-907.

Gomes, F., Rodrigues, M.E., Martins, N., Ferreira, I.C.F.R., Henriques, M., 2019. Phenolic plant extracts versus penicillin G: *In vitro* susceptibility of *Staphylococcus aureus* isolated from bovine mastitis. Pharmaceuticals 12, 1-8.

Górniak, I., Bartoszewski, R., Króliczewski, J., 2019. Comprehensive review of antimicrobial activities of plant flavonoids. Phytochemistry Reviews 18, 241-272.

Gottardi, W., Nagl, M., 2005. Chlorine covers on living bacteria: the initial step in antimicrobial action of active chlorine compounds. Journal of Antimicrobial Chemotherapy 55, 475-482.

Gowder, S.J.T., 2014. Safety assessment of food flavor - Cinnamaldehyde. Biosafety 3, 1-2.

Gray, M.J., Wholey, W.-Y., Jakob, U., 2013. Bacterial responses to reactive chlorine species. Annual review of microbiology 67, 141-160.

Grinius, L.L., Goldberg, E.B., 1994. Bacterial multidrug resistance is due to a single membrane protein which functions as a drug pump. The Journal of Biological Chemistry 269, 29998-30004.

Guerin-Mechin, L., Dubois-Brissonnet, F., Heyd, B., Leveau, J.Y., 2000. Quaternary ammonium compound stresses induce specific variations in fatty acid composition of *Pseudomonas aeruginosa*. International Journal of Food Microbiology 55, 157-159.

Guillen, J., Bernabeu, A., Shapiro, S., Villalain, J., 2004. Location and orientation of triclosan in phospholipid model membranes. European Biophysics Journal 33, 448-453.

Guzman, J.D., 2014. Natural cinnamic acids, synthetic derivatives and hybrids with antimicrobial activity. Molecules 19, 19292-19349.

Gverzdys, T., Kramer, G., Nodwell, J.R., 2016. Tetrodecamycin: an unusual and interesting tetronate antibiotic. Bioorganic & Medicinal Chemistry 24, 6269-6275.

Gyawali, R., Ibrahim, S.A., 2012. Synergistic effect of copper and lactic acid against *Salmonella* and *Escherichia coli* O157:H7: A review. Emirates Journal of Food and Agriculture 24, 1-11.

Habicht, J., Brune, K., 1983. Inhibition of prostaglandin E2 release by salicylates, benzoates and phenols: a quantitative structure-activity study. Journal of Pharmacy and Pharmacology 35, 718-723.

Hall, B.G., Acar, H., Nandipati, A., Barlow, M., 2014. Growth rates made easy. Molecular Biology and Evolution 31, 232-238.

Hansch, C., Steinmetz, W.E., Leo, A.J., Mekapati, S.B., Kurup, A., Hoekman, D., 2003. On the role of polarizability in chemical−biological interactions. Journal of Chemical Information and Computer Sciences 43, 120-125.

Hansen, L.H., Jensen, L.B., Sorensen, H.I., Sorensen, S.J., 2007. Substrate specificity of the OqxAB multidrug resistance pump in *Escherichia coli* and selected enteric bacteria. Journal of Antimicrobial Chemotherapy 60, 145-147.

Hassan, K.A., Liu, Q., Henderson, P.J.F., Paulsen, I.T., 2015. Homologs of the A*cinetobacter baumannii* aceI transporter represent a new family of bacterial multidrug efflux systems. mBio 6, 1-5.

Hassan, M.Z., Sturm-Ramirez, K., Rahman, M.Z., Hossain, K., Aleem, M.A., Bhuiyan, M.U., Islam, M.M., Rahman, M., Gurley, E.S., 2019. Contamination of hospital surfaces with respiratory pathogens in Bangladesh. PLoS ONE 14, 1-10.

He, G.-X., Kuroda, T., Mima, T., Morita, Y., Mizushima, T., Tsuchiya, T., 2004. An H(+)-coupled multidrug efflux pump, pmpM, a member of the MATE family of transporters, from *Pseudomonas aeruginosa*. Journal of Bacteriology 186, 262-265.

Heath, R.J., Rubin, J.R., Holland, D.R., Zhang, E., Snow, M.E., Rock, C.O., 1999. Mechanism of triclosan inhibition of bacterial fatty acid synthesis. The Journal of Biological Chemistry 274, 11110-11114.

Hegstad, K., Langsrud, S., Lunestad, B., Scheie, A., Sunde, M., Yazdankhah, S., 2010. Does the wide use of quaternary ammonium compounds enhance the selection and spread of antimicrobial resistance and thus threaten our health? Microbial drug resistance 16, 91-104.

Hemaiswarya, S., Kruthiventi, A.K., Doble, M., 2008. Synergism between natural products and antibiotics against infectious diseases. Phytomedicine 15, 639-652.

Henly, E.L., Dowling, J.A.R., Maingay, J.B., Lacey, M.M., Smith, T.J., Forbes, S., 2019. Biocide exposure induces changes in susceptibility, pathogenicity, and biofilm formation in uropathogenic *Escherichia coli*. Antimicrobial Agents and Chemotherapy 63, 1-15.

Herruzo, R., Vizcaino, M.J., Herruzo, I., 2017. An exception to the rule “no association between antibiotic resistance and decreased microbicidal efficacy of disinfectants”: Orthophthalaldehyde (OPA) and *Pseudomonas aeruginosa* isolated from ICU patients. Journal of Preventive Medicine and Hygiene 58, e42-e47.

Hirshfield, I.N., Terzulli, S., O'Byrne, C., 2003. Weak organic acids: a panoply of effects on bacteria. Sci Prog 86, 245-269.

Holley, R.A., Patel, D., 2005. Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. Food Microbiology 22, 273-292.

Hopkins, J.A., 2013. Legislation affecting disinfectant products in europe: The biocidal products directive and the registration, evaluation and authorization of chemicals regulations, In: Russell, H.A.s. (Ed.), Principles and Practice of Disinfection, Preservation and Sterilization, 5th ed. Blackwell Publishing Ltd, pp. 445-458.

Hsieh, P., Siegel, S.A., Rogers, B., Davis, D., Lewis, K., 1998. Bacteria lacking a multidrug pump: A sensitive tool for drug discovery. Proceedings of the National Academy of Sciences of the United States of America 95, 6602-6606.

Huang, Q.-S., Zhu, Y.-J., Li, H.-L., Zhuang, J.-X., Zhang, C.-L., Zhou, J.-J., Li, W.-G., Chen, Q.-X., 2009. Inhibitory effects of methyl trans-cinnamate on mushroom tyrosinase and its antimicrobial activities. Journal of Agricultural and Food Chemistry 57, 2565-2569.

Huang, Y.-H., Lin, J.-S., Ma, J.-C., Wang, H.-H., 2016. Functional characterization of triclosan-resistant enoyl-acyl-carrier protein reductase (fabV) in *Pseudomonas aeruginosa*. Frontiers in Microbiology 7, 1-13.

Humayoun, S.B., Hiott, L.M., Gupta, S.K., Barrett, J.B., Woodley, T.A., Johnston, J.J., Jackson, C.R., Frye, J.G., 2018. An assay for determining the susceptibility of *Salmonella* isolates to commercial and household biocides. PLoS ONE 13, 1-24.

Humphreys, P.N., 2011. Testing standards for sporicides. Journal of Hospital Infection 77, 193-198.

Hussain, G., Rasul, A., Anwar, H., Aziz, N., Razzaq, A., Wei, W., Ali, M., Li, J., Li, X., 2018. Role of plant derived alkaloids and their mechanism in neurodegenerative disorders. International journal of biological sciences 14, 341-357.

Imlay, J.A., Linn, S., 1986. Bimodal pattern of killing of DNA-repair-defective or anoxically grown *Escherichia coli* by hydrogen peroxide. Journal of Bacteriology 166, 519-527.

Ioannou, C.J., Hanlon, G.W., Denyer, S.P., 2007. Action of disinfectant quaternary ammonium compounds against *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 51, 296-306.

Ismaïl, R., Aviat, F., Michel, V., Le Bayon, I., Gay-Perret, P., Kutnik, M., Fédérighi, M., 2013. Methods for recovering microorganisms from solid surfaces used in the food industry: A review of the literature. International journal of environmental research and public health 10, 6169-6183.

Jackson, P.A., Widen, J.C., Harki, D.A., Brummond, K.M., 2017. Covalent modifiers: a chemical perspective on the reactivity of α,β-unsaturated carbonyls with thiols via hetero-Michael addition reactions. Journal of Medicinal Chemistry 60, 839-885.

Janczuk, B., Chibowski, E., Bruque, J.M., Kerkeb, M.L., Caballero, F.G., 1993. On the consistency of surface free energy components as calculated from contact angles of different liquids: An application to the cholesterol surface. Journal of Colloid and Interface Science 159, 421-428.

Jin, Y., Deng, J., Liang, J., Shan, C., Tong, M., 2015. Efficient bacteria capture and inactivation by cetyltrimethylammonium bromide modified magnetic nanoparticles. Colloids and Surfaces B: Biointerfaces 136, 659-665.

Johnston, M.D., Hanlon, G.W., Denyer, S.P., Lambert, R.J., 2003. Membrane damage to bacteria caused by single and combined biocides. Journal of Applied Microbiology 94, 1015-1023.

Johnston, M.D., Lambert, R.J.W., Hanlon, G.W., Denyer, S.P., 2002. A rapid method for assessing the suitability of quenching agents for individual biocides as well as combinations. Journal of Applied Microbiology 92, 784-789.

Joshi, J.R., Burdman, S., Lipsky, A., Yariv, S., Yedidia, I., 2016. Plant phenolic acids affect the virulence of *Pectobacterium aroidearum* and *P. carotovorum* ssp. *brasiliense* via quorum sensing regulation. Molecular Plant Pathology 17, 487-500.

Jover, R., Ponsoda, X., Castell, J.V., Gomez-Lechon, M.J., 1992. Evaluation of the cytotoxicity of ten chemicals on human cultured hepatocytes: Predictability of human toxicity and comparison with rodent cell culture systems. Toxicology in Vitro 6, 47-52.

Jung, P.P., Christian, N., Kay, D.P., Skupin, A., Linster, C.L., 2015. Protocols and programs for high-throughput growth and aging phenotyping in yeast. PLoS ONE 10, 1-20.

Kaatz, G.W., Moudgal, V.V., Seo, S.M., Kristiansen, J.E., 2003. Phenothiazines and thioxanthenes inhibit multidrug efflux pump activity in *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 47, 719-726.

Kampf, G., 2018. Biocidal agents used for disinfection can enhance antibiotic resistance in Gram-negative species. Antibiotics 7, 1-24.

Karmakar, S., Abraham, T.J., Kumar, S., Kumar, S., Shukla, S.P., Roy, U., Kumar, K., 2019. Triclosan exposure induces varying extent of reversible antimicrobial resistance in *Aeromonas hydrophila* and *Edwardsiella tarda*. Ecotoxicology and Environmental Safety 180, 309-316.

Karpiński, T.M., Adamczak, A., 2019. Fucoxanthin-An antibacterial carotenoid. Antioxidants 8, 1-8.

Karsa, D.R., 2007. Biocides, In: Johansson, I., Somasundaran, P. (Eds.), Handbook for Cleaning/Decontamination of Surfaces. Elsevier Science B.V., Amsterdam, pp. 593-623.

Keeton, J.T., Eddy, S.M., 2008. Chemical methods for decontamination of meat and poultry, In: Beier, R.C., Pillai, S.D., Phillips, T.D. (Eds.), Preharvest and Postharvest Food Safety: Contemporary Issues and Future Directions. Blackwell Publishing and the Institute of Food Technologists, pp. 317-336.

Kępa, M., Miklasińska-Majdanik, M., Wojtyczka, R., Idzik, D., Korzeniowski, K., Smoleń-Dzirba, J., Wąsik, T., 2018. Antimicrobial potential of caffeic acid against *Staphylococcus aureus* clinical strains. BioMed Research International 2018, 1-9.

Kerns, E.H., Di, L., 2008a. Chapter 1 - Introduction, Drug-like Properties: Concepts, Structure Design and Methods. Academic Press, San Diego, pp. 3-5.

Kerns, E.H., Di, L., 2008b. Chapter 4 - Rules for rapid property profiling from structure, Drug-like Properties: Concepts, Structure Design and Methods. Academic Press, San Diego, pp. 37-42.

Khatri, S., Kumar, M., Phougat, N., Chaudhary, R., Chhillar, A.K., 2016. Perspectives on phytochemicals as antibacterial agents: An outstanding contribution to modern therapeutics. Mini-Reviews in Medicinal Chemistry 16, 290-308.

Khorasani, S., Azizi, M.H., Barzegar, M., Hamidi-Esfahani, Z., Kalbasi-Ashtari, A., 2017. Inhibitory effects of cinnamon, clove and celak extracts on growth of *Aspergillus flavus* and its aflatoxins after spraying on pistachio nuts before cold storage. Journal of Food Safety 37, 1-10.

Kim, S.A., Rhee, M.S., 2013. Marked synergistic bactericidal effects and mode of action of medium-chain fatty acids in combination with organic acids against *Escherichia coli* O157:H7. Applied and Environmental Microbiology 79, 6552-6560.

Kishk, R., Mandour, M., Hessam, W., Nmer, N., 2014. Efflux pump genes and chlorhexidine resistance: Clue for *Klebsiella pneumoniae* infections in intensive care units, Egypt. African Journal of Microbiology Research 8, 2162-2167.

Kitis, M., 2004. Disinfection of wastewater with peracetic acid: A review. Environment International 30, 47-55.

Kollanoor Johny, A.K., Darre, M., Donoghue, A.M., Donoghue, D.J., Venkitanarayanan, K., 2010. Antibacterial effect of trans-cinnamaldehyde, eugenol, carvacrol, and thymol on *Salmonella Enteritidis* and *Campylobacter jejuni* in chicken cecal contents *in vitro*. The Journal of Applied Poultry Research 19, 237-244.

Korošec, B., Sova, M., Turk, S., Kraševec, N., Novak, M., Lah, L., Stojan, J., Podobnik, B., Berne, S., Zupanec, N., Bunc, M., Gobec, S., Komel, R., 2014. Antifungal

activity of cinnamic acid derivatives involves inhibition of benzoate 4-hydroxylase (CYP53). Journal of Applied Microbiology 116, 955-966.

Krulwich, T.A., Jin, J.J., Guffanti, A.A., Bechhofer, D.H., 2001. Functions of tetracycline efflux proteins that do not involve tetracycline. Journal of molecular microbiology and biotechnology 3, 237-246.

Kumar, N., Goel, N., 2019. Phenolic acids: Natural versatile molecules with promising therapeutic applications. Biotechnology Reports 24, 1-10.

Kummerle, N., Feucht, H.H., Kaulfers, P.M., 1996. Plasmid-mediated formaldehyde resistance in *Escherichia coli*: Characterization of resistance gene. Antimicrobial Agents and Chemotherapy 40, 2276-2279.

Kweyamba, P.A., Zofou, D., Efange, N., Assob, J.N., Kitau, J., Nyindo, M., 2019. *In vitro* and *in vivo* studies on anti-malarial activity of *Commiphora africana* and *Dichrostachys cinerea* used by the Maasai in Arusha region, Tanzania. Malaria Journal 18, 1-6.

Lambert, P.A., 2002. Cellular impermeability and uptake of biocides and antibiotics in Gram-positive bacteria and mycobacteria. Journal of Applied Microbiology 92 Suppl, 46S-54S.

Lambert, R.J.W., Hanlon, G.W., Denyer, S.P., 2004. The synergistic effect of EDTA/antimicrobial combinations on *Pseudomonas aeruginosa*. Journal of Applied Microbiology 96, 244-253.

Langeveld, W.T., Veldhuizen, E.J.A., Burt, S.A., 2014. Synergy between essential oil components and antibiotics: A review. Critical Reviews in Microbiology 40, 76-94.

Lappi, V.R., Thimothe, J., Nightingale, K.K., Gall, K., Scott, V.N., Wiedmann, M., 2004. Longitudinal studies on *Listeria* in smoked fish plants: Impact of intervention strategies on contamination patterns. Journal of Food Protection 67, 2500-2514.

Lavilla Lerma, L., Benomar, N., Gálvez, A., Abriouel, H., 2013. Prevalence of bacteria resistant to antibiotics and/or biocides on meat processing plant surfaces throughout meat chain production. International Journal of Food Microbiology 161, 97-106.

Lee, N., Yuen, K.-Y., Kumana, C.R., 2003. Clinical role of β-lactam/β-lactamase inhibitor bombinations. Drugs 63, 1511-1524.

Lei, H., Li, Y., Xiao, S., Yang, X., Lin, C., Norris, S.L., Wei, D., Hu, Z., Ji, S., 2017. Logistic growth of a surface contamination network and its role in disease spread. Scientific Reports 7, 1-10.

Leite, G.C., Neto, L.V.P., Gaudereto, J.J., Carrilho, C.M.D.M., Rossi, F., Levin, A.S., Costa, S., 2015. Effect of antibiotics combination and comparison of methods for detection of synergism in multiresistant Gram-negative bacteria. Infectious Diseases and Therapy 3, 1-9.

Leriche, V., Briandet, R., Carpentier, B., 2003. Ecology of mixed biofilms subjected daily to a chlorinated alkaline solution: spatial distribution of bacterial species suggests a protective effect of one species to another. Environmental Microbiology 5, 64-71.

Lewis, K., 2008. Multidrug tolerance of biofilms and persister cells, Curr Top Microbiol Immunol, pp. 107-131.

Li, D., Zeng, S., Gu, A.Z., He, M., Shi, H., 2013. Inactivation, reactivation and regrowth of indigenous bacteria in reclaimed water after chlorine disinfection of a municipal wastewater treatment plant. Journal of Environmental Sciences 25, 1319-1325.

Liebgott, P.P., Labat, M., Amouric, A., Tholozan, J.L., Lorquin, J., 2008. Tyrosol degradation via the homogentisic acid pathway in a newly isolated *Halomonas* strain from olive processing effluents. Journal of Applied Microbiology 105, 2084-2095.

Liebgott, P.P., Labat, M., Casalot, L., Amouric, A., Lorquin, J., 2007. Bioconversion of tyrosol into hydroxytyrosol and 3,4-dihydroxyphenylacetic acid under hypersaline conditions by the new *Halomonas* sp. strain HTB24. FEMS Microbiology Letters 276, 26-33.

Linley, E., Denyer, S.P., McDonnell, G., Simons, C., Maillard, J.Y., 2012. Use of hydrogen peroxide as a biocide: new consideration of its mechanisms of biocidal action. Journal of Antimicrobial Chemotherapy 67, 1589-1596.

Lipinski, C.A., 2004. Lead- and drug-like compounds: the rule-of-five revolution. Drug Discovery Today: Technologies 1, 337-341.

Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 1997. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Advanced Drug Delivery Reviews 23, 3-25.

Liu, R.H., 2004. Potential synergy of phytochemicals in cancer prevention: Mechanism of action. The Journal of Nutrition 134, 3479s-3485s.

Livermore, D.M., 2012. Current epidemiology and growing resistance of Gram-negative pathogens. The Korean Journal of Internal Medicine 27, 128-142.

Lou, Z., Wang, H., Rao, S., Sun, J., Ma, C., Li, J., 2012. *p*-Coumaric acid kills bacteria through dual damage mechanisms. Food Control 25, 550-554.

Magesh, H., Kumar, A., Alam, A., Priyam, Sekar, U., Sumantran, V.N., Vaidyanathan, R., 2013. Identification of natural compounds which inhibit biofilm formation in clinical isolates of *Klebsiella pneumoniae*. Indian Journal of Experimental Biology 51, 764-772.

Mah, T.-F.C., O'Toole, G.A., 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends in Microbiology 9, 34-39.

Maillard, J.-Y., 2005. Antimicrobial biocides in the healthcare environment: efficacy, usage, policies, and perceived problems. Therapeutics and Clinical Risk Management 1, 307-320.

Maillard, J.-Y., McDonnell, G., 2012. Selection and use of disinfectants. In Practice 34, 292-299.

Maillard, J.Y., 2002. Bacterial target sites for biocide action. Journal of Applied Microbiology 92 Suppl, 16s-27s.

Maillard, J.Y., 2007. Bacterial resistance to biocides in the healthcare environment: should it be of genuine concern? Journal of Hospital Infection 65, Supplement 2, 60-72.

Manzoor, S.E., Lambert, P.A., Griffiths, P.A., Gill, M.J., Fraise, A.P., 1999. Reduced glutaraldehyde susceptibility in *Mycobacterium chelonae* associated with altered cell wall polysaccharides. Journal of Antimicrobial Chemotherapy 43, 759-765.

Marchi, E., Furi, L., Arioli, S., Morrissey, I., Di Lorenzo, V., Mora, D., Giovannetti, L., Oggioni, M.R., Viti, C., 2015. Novel insight into antimicrobial resistance and sensitivity phenotypes associated to *qac* and *norA* genotypes in *Staphylococcus aureus*. Microbiological Research 170, 184-194.

María Ferrer-Luque, C., Teresa Arias-Moliz, M., Ruíz-Linares, M., Elena Martínez García, M., Baca, P., 2014. Residual activity of cetrimide and chlorhexidine on *Enterococcus faecalis*-infected root canals. International Journal of Oral Science 6, 46-49.

Martinez, K., Mackert, J., McIntosh, M., 2017. Polyphenols and intestinal health, In: Watson, R.R. (Ed.), Nutrition and Functional Foods for Healthy Aging. Academic Press, pp. 191-210.

Mavri, A., Smole Mozina, S., 2012. Involvement of efflux mechanisms in biocide resistance of *Campylobacter jejuni* and *Campylobacter coli*. Journal of Medical Microbiology 61, 800-808.

McDonnell, G., Russell, A.D., 1999. Antiseptics and disinfectants: Activity, action, and resistance. Clinical Microbiology Reviews 12, 147-179.

McLean, R.J.C., Pierson, L.S., Fuqua, C., 2004. A simple screening protocol for the identification of quorum signal antagonists. Journal of Microbiological Methods 58, 351-360.

Meireles, A., Machado, I., Fulgêncio, R., Mergulhão, F., Melo, L., Simões, M., 2015. Efficacy of antimicrobial combinations to reduce the use of sodium hypochlorite in the control of planktonic and sessile *Escherichia coli*. Biochemical Engineering Journal 104, 115-122.

Mendes, F.S.F., Garcia, L.M., Moraes, T.S., Casemiro, L.A., Alcântara, C.B., Ambrósio, S.R., Veneziani, R.C.S., Miranda, M.L.D., Martins, C.H.G., 2020. Antibacterial activity of *Salvia officinalis* L. against periodontopathogens: An in vitro study. Anaerobe 63, 1-8.

Minatel, I.O., Borges, C.V., Ferreira, M.I., Gomez, H.A.G., Chen, C.-Y.O., Lima, G.P.P., 2017. Phenolic compounds: Functional properties, impact of processing and bioavailability, In: Soto-Hernandez, M., Palma-Tenango, M., Garcia-Mateos, M.R. (Eds.), Phenolic Compounds - Biological Activity. IntechOpen.

Mitchell, B.A., Paulsen, I.T., Brown, M.H., Skurray, R.A., 1999. Bioenergetics of the staphylococcal multidrug export protein QacA. Identification of distinct binding sites for monovalent and divalent cations. The Journal of Biological Chemistry 274, 3541-3548.

Mizozoe, M., Otaki, M., Aikawa, K., 2019. The mechanism of chlorine damage using enhanced green fluorescent protein-expressing *Escherichia coli*. Water 11, 1-11.

Molin, S., Tolker-Nielsen, T., 2003. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. Current Opinion in Biotechnology 14, 255-261.

Monte, J., Abreu, A.C., Borges, A., Simões, L.C., Simões, M., 2014. Antimicrobial activity of selected phytochemicals against *Escherichia coli* and *Staphylococcus aureus* and their biofilms. Pathogens 3, 473-498.

Mourato, C.A., 2012. A contribuição das bombas de efluxo QacA e Smr para a multirresistência em *Staphylococcus aureus*, Master Thesis, Universidade Nova de Lisboa, Universidade Nova de Lisboa.

Mousavi, F., Bojko, B., Bessonneau, V., Pawliszyn, J., 2016. Cinnamaldehyde characterization as an antibacterial agent toward *E. coli* metabolic profile using 96-

blade solid-phase microextraction coupled to liquid chromatography-mass spectrometry. Journal of Proteome Research 15, 963-975.

Mulberrry, G., Snyder, A.T., Heilman, J., Pyrek, J., Stahl, J., Evaluation of a waterless, scrubless chlorhexidine gluconate/ethanol surgical scrub for antimicrobial efficacy. American Journal of Infection Control 29, 377-382.

Myszka, K., Schmidt, M.T., Majcher, M., Juzwa, W., Olkowicz, M., Czaczyk, K., 2016. Inhibition of quorum sensing-related biofilm of *Pseudomonas fluorescens* KM121 by *Thymus vulgare* essential oil and its major bioactive compounds. International Biodeterioration & Biodegradation 114, 252-259.

Nair, A.V., Singh, H., Raturi, S., Neuberger, A., Tong, Z., Ding, N., Agboh, K., van Veen, H.W., 2016. Relocation of active site carboxylates in major facilitator superfamily multidrug transporter LmrP reveals plasticity in proton interactions. Scientific Reports 6, 1-12.

Nakata, K., Tsuchido, T., Matsumura, Y., 2011. Antimicrobial cationic surfactant, cetyltrimethylammonium bromide, induces superoxide stress in *Escherichia coli* cells. Journal of Applied Microbiology 110, 568-579.

Narui, K., Noguchi, N., Wakasugi, K., Sasatsu, M., 2002. Cloning and characterization of a novel chromosomal drug efflux gene in *Staphylococcus aureus*. Biological and Pharmaceutical Bulletin 25, 1533-1536.

Nayim, P., Mbaveng, A.T., Wamba, B.E.N., Fankam, A.G., Dzotam, J.K., Kuete, V., 2018. Antibacterial and antibiotic-potentiating activities of thirteen Cameroonian edible plants against Gram-negative resistant phenotypes. The Scientific World Journal 2018, 1-14.

Ng, E.Y., Trucksis, M., Hooper, D.C., 1994. Quinolone resistance mediated by *norA*: physiologic characterization and relationship to *flqB*, a quinolone resistance locus on the *Staphylococcus aureus* chromosome. Antimicrobial Agents and Chemotherapy 38, 1345-1355.

Nishino, K., Yamaguchi, A., 2001. Analysis of a complete library of putative drug transporter genes in *Escherichia coli*. Journal of Bacteriology 183, 5803-5812.

Niu, C., Afre, S., Gilbert, E.S., 2006. Subinhibitory concentrations of cinnamaldehyde interfere with quorum sensing. Letters in Applied Microbiology 43, 489-494.

Niu, C., Gilbert, E.S., 2004. Colorimetric method for identifying plant essential oil components that affect biofilm formation and structure. Applied and Environmental Microbiology 70, 6951-6956.

Ntreh, A.T., Weeks, J.W., Nickels, L.M., Zgurskaya, H.I., 2016. Opening the channel: The two functional interfaces of *Pseudomonas aeruginosa* Opmh with the triclosan efflux pump triABC. Journal of Bacteriology 198, 3176-3185.

Ohene-Agyei, T., Mowla, R., Rahman, T., Venter, H., 2014. Phytochemicals increase the antibacterial activity of antibiotics by acting on a drug efflux pump. Microbiologyopen 3, 885-896.

Oniciuc, E.-A., Likotrafiti, E., Alvarez-Molina, A., Prieto, M., López, M., Alvarez-Ordóñez, A., 2019. Food processing as a risk factor for antimicrobial resistance spread along the food chain. Current Opinion in Food Science 30, 21-26.

Opgrande, J.L., Dobratz, C.J., Brown, E., Liang, J., Conn, G.S., Shelton, F.J., With, J., 2000. Benzaldehyde, Kirk-Othmer Encyclopedia of Chemical Technology.

Otter, J.A., Vickery, K., Walker, J.T., deLancey Pulcini, E., Stoodley, P., Goldenberg, S.D., Salkeld, J.A.G., Chewins, J., Yezli, S., Edgeworth, J.D., 2015. Surface-attached cells, biofilms and biocide susceptibility: Implications for hospital cleaning and disinfection. Journal of Hospital Infection 89, 16-27.

Padrón, J.A., Carrasco, R., Pellón, R.F., 2002. Molecular descriptor based on a molar refractivity partition using Randic-type graph-theoretical invariant. Journal of Pharmacy & Pharmaceutical Sciences 5, 258-266.

Panousi, M.N., Williams, G.J., Girdlestone, S., Hiom, S.J., Maillard, J.Y., 2009. Evaluation of alcohol wipes used during aseptic manufacturing. Letters in Applied Microbiology 48, 648-651.

Park, B., Nizet, V., Liu, G.Y., 2008. Role of *Staphylococcus aureus* catalase in niche competition against *Streptococcus pneumoniae*. Journal of Bacteriology 190, 2275-2278.

Patel, D., Kosmidis, C., Seo, S.M., Kaatz, G.W., 2010. Ethidium bromide MIC screening for enhanced efflux pump gene expression or efflux activity in *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 54, 5070-5073.

Patel, R., 2005. Biofilms and antimicrobial resistance. Clinical Orthopaedics and Related Research, 41-47.

Patil, U.N., Jambulingappa, K.L., 2015. A combination strategy of ceftriaxone, sulbactam and disodium edetate for the treatment of multi-drug resistant (MDR) septicaemia: A retrospective, observational study in indian tertiary care hospital. Journal of Clinical and Diagnostic Research 9, 29-32.

Paul, D., Chakraborty, R., Mandal, S.M., 2019. Biocides and health-care agents are more than just antibiotics: Inducing cross to co-resistance in microbes. Ecotoxicology and Environmental Safety 174, 601-610.

Paulsen, I.T., Skurray, R.A., 1993. Topology, structure and evolution of two families of proteins involved in antibiotic and antiseptic resistance in eukaryotes and prokaryotes -An analysis. Gene 124, 1-11.

Penna, T.C.V., Mazzola, P.G., Martins, A.M.S., 2001. The efficacy of chemical agents in cleaning and disinfection programs. BMC Infectious Diseases 1, 16-24.

Peperidou, A., Pontiki, E., Hadjipavlou-Litina, D., Voulgari, E., Avgoustakis, K., 2017. Multifunctional cinnamic acid derivatives. Molecules 22, 1-17.

Pericone, C.D., Overweg, K., Hermans, P.W., Weiser, J.N., 2000. Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract. Infection and Immunity 68, 3990-3997.

Pidot, S.J., Gao, W., Buultjens, A.H., Monk, I.R., Guerillot, R., Carter, G.P., Lee, J.Y.H., Lam, M.M.C., Grayson, M.L., Ballard, S.A., Mahony, A.A., Grabsch, E.A., Kotsanas, D., Korman, T.M., Coombs, G.W., Robinson, J.O., Goncalves da Silva, A., Seemann, T., Howden, B.P., Johnson, P.D.R., Stinear, T.P., 2018. Increasing tolerance of hospital *Enterococcus faecium* to handwash alcohols. Science Translational Medicine 10, 1-10.

Pieren, M., Tigges, M., 2012. Adjuvant strategies for potentiation of antibiotics to overcome antimicrobial resistance. Current Opinion in Pharmacology 12, 551-555.

Pontiki, E., Hadjipavlou-Litina, D., Litinas, K., Geromichalos, G., 2014. Novel cinnamic acid derivatives as antioxidant and anticancer agents: Design, synthesis and modeling studies. Molecules 19, 9655-9674.

Poole, K., 2002. Outer membranes and efflux: the path to multidrug resistance in Gram-negative bacteria. Current Pharmaceutical Biotechnology 3, 77-98.

Poole, K., 2005. Efflux-mediated antimicrobial resistance. Journal of Antimicrobial Chemotherapy 56, 20-51.

Poole, K., 2007. Efflux pumps as antimicrobial resistance mechanisms. Ann Med 39, 162-176.

Poole, S.K., Poole, C.F., 1994. Thin-layer chromatographic method for the determination of the principal polar aromatic flavour compounds of the cinnamons of commerce. Analyst 119, 113-120.

Priya, S., Kumar, N.S., Hemalatha, S., 2018. Antiviral phytocompounds target envelop protein to control Zika virus. Computational Biology and Chemistry 77, 402-412.

Prorok, T., Jana, M., Patel, D., Pahan, K., 2019. Cinnamic acid protects the nigrostriatum in a mouse model of Parkinson's disease via peroxisome proliferator-activated receptor alpha. Neurochemical Research 44, 751-762.

Putman, M., van Veen, H.W., Konings, W.N., 2000. Molecular properties of bacterial multidrug transporters. Microbiology and Molecular Biology Reviews 64, 672-693.

Rajagopal, K., Singh, P.K., Kumar, R., Siddiqui, K.F., 2014. CTAB-mediated, single-step preparation of competent *Escherichia coli*, *Bifidobacterium* sp. and *Kluyveromyces lactis* cells. Meta Gene 2, 807-818.

Rajamohan, G., Srinivasan, V.B., Gebreyes, W.A., 2010. Novel role of *Acinetobacter baumannii* RND efflux transporters in mediating decreased susceptibility to biocides. Journal of Antimicrobial Chemotherapy 65, 228-232.

Ramanaiah, M., Sailaja, B.B.V., 2014. Protonation equilibria of L-phenylalanine and maleic acid in cationic micellar media. Chemical Speciation & Bioavailability 26, 119-125.

Ramm, L., Siani, H., Wesgate, R., Maillard, J.-Y., 2015. Pathogen transfer and high variability in pathogen removal by detergent wipes. American Journal of Infection Control 43, 724-728.

Ramos-Nino, M.E., Clifford, M.N., Adams, M.R., 1996. Quantitative structure activity relationship for the effect of benzoic acids, cinnamic acids and benzaldehydes on *Listeria monocytogenes*. Journal of Applied Bacteriology 80, 303-310.

Rao, P.V., Gan, S.H., 2014. Cinnamon: A multifaceted medicinal plant. Evidence-Based Complementary and Alternative Medicine 2014, 1-12.

Rasmussen, L.H., Kjeldgaard, J., Christensen, J.P., Ingmer, H., 2013. Multilocus sequence typing and biocide tolerance of *Arcobacter butzleri* from Danish broiler carcasses. BMC Research Notes 6, 322-329.

Rastija, V., Nikolić, S., Medić-Šarić, M., 2009. Molecular modeling of wine polyphenols. Journal of Mathematical Chemistry 46, 820-833.

Report, 1997. Final report on the safety assessment of cetrimonium chloride, cetrimonium bromide, and steartrimonium chloride. International Journal of Toxicology 16, 195-220.

Report, 2006. Final report on the safety assessment of benzaldehyde, International Journal of Toxicology, pp. 11-27.

Reybrouck, G., 1998. The testing of disinfectants. International Biodeterioration & Biodegradation 41, 269-272.

Ribeiro, M., Simões, L.C., Simões, M., 2018. Biocides, In: Schmidt, T.M. (Ed.), Encyclopedia of Microbiology (Fourth Edition). Academic Press, Oxford, pp. 478-490.

Ridgway, H.F., Olson, B.H., 1982. Chlorine resistance patterns of bacteria from two drinking water distribution systems. Applied and Environmental Microbiology 44, 972-987.

Rizzotti, L., Rossi, F., Torriani, S., 2016. Biocide and antibiotic resistance of *Enterococcus faecalis* and *Enterococcus faecium* isolated from the swine meat chain. Food Microbiology 60, 160-164.

Rodrigues, A., Nogueira, R., Melo, L.F., Brito, A.G., 2013. Effect of low concentrations of synthetic surfactants on polycyclic aromatic hydrocarbons (PAH) biodegradation. International Biodeterioration & Biodegradation 83, 48-55.

Roedel, A., Dieckmann, R., Brendebach, H., Hammerl, J.A., Kleta, S., Noll, M., Al Dahouk, S., Vincze, S., 2019. Biocide-tolerant *Listeria monocytogenes* isolates from german food production plants do not show cross-resistance to clinically relevant antibiotics. Applied and Environmental Microbiology 85, 1-15.

Rotter, M.L., 2008. Special problems in hospital antisepsis, In: Adam P Fraise, Peter A Lambert, Maillard, J.-Y. (Eds.), Russell, Hugo & Ayliffe's Principles and Practice of Disinfection, Preservation & Sterilization. Blackwell Publishing Ltd, pp. 540-562.

Roy, A., 2017. A review on the alkaloids an important therapeutic compound from plants. International Journal of Plant Biotechnology 3, 1-9.

Russell, A.D., 1990. Bacterial spores and chemical sporicidal agents. Clinical Microbiology Reviews 3, 99-119.

Russell, A.D., 1997. Plasmids and bacterial resistance to biocides. Journal of Applied Microbiology 83, 155-165.

Russell, A.D., 2002. Introduction of biocides into clinical practice and the impact on antibiotic-resistant bacteria. Journal of Applied Microbiology 92, 121S-135S.

Russell, A.D., 2003. Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. The Lancet Infectious Diseases 3, 794-803.

Russell, A.D., 2004. Bacterial adaptation and resistance to antiseptics, disinfectants and preservatives is not a new phenomenon. Journal of Hospital Infection 57, 97-104.

Russell, A.D., Suller, M.T., Maillard, J.-Y., 1999. Do antiseptics and disinfectants select for antibiotic resistance? Journal of Medical Microbiology 48, 613-615.

Russotto, V., Cortegiani, A., Raineri, S.M., Giarratano, A., 2015. Bacterial contamination of inanimate surfaces and equipment in the intensive care unit. Journal of Intensive Care 3, 1-8.

Rutala, W.A., Weber, D.J., 1997. Uses of inorganic hypochlorite (bleach) in health-care facilities. Clinical Microbiology Reviews 10, 597-610.

Rutala, W.A., Weber, D.J., 2004. Disinfection and sterilization in health care facilities: what clinicians need to know. Clinical Infectious Diseases 39, 702-709.

Rutala, W.A., Weber, D.J., Committee, H.I.C.P.A., 2008, updated 2019. Guideline for disinfection and sterilization in healthcare facilities. Centers for Disease Control and Prevention.

Salem, M.Z.M., Mansour, M.M.A., Elansary, H.O., 2019. Evaluation of the effect of inner and outer bark extracts of sugar maple (*Acer saccharum* var. *saccharum*) in combination with citric acid against the growth of three common molds. Journal of Wood Chemistry and Technology 39, 136-147.

Sánchez-Maldonado, A.F., Schieber, A., Gänzle, M.G., 2011. Structure–function relationships of the antibacterial activity of phenolic acids and their metabolism by lactic acid bacteria. Journal of Applied Microbiology 111, 1176-1184.

Sarker, U., Hossain, M.M., Oba, S., 2020. Nutritional and antioxidant components and antioxidant capacity in green morph *Amaranthus* leafy vegetable. Scientific Reports 10, 1-10.

Scalbert, A., Andres-Lacueva, C., Arita, M., Kroon, P., Manach, C., Urpi-Sarda, M., Wishart, D., 2011. Databases on food phytochemicals and their health-promoting effects. Journal of Agricultural and Food Chemistry 59, 4331-4348.

Schwaiger, K., Harms, K.S., Bischoff, M., Preikschat, P., Mölle, G., Bauer-Unkauf, I., Lindorfer, S., Thalhammer, S., Bauer, J., Hölzel, C.S., 2014. Insusceptibility to disinfectants in bacteria from animals, food and humans-is there a link to antimicrobial resistance? Frontiers in Microbiology 5, 1-12.

Sharma, P., 2011. Cinnamic acid derivatives: A new chapter of various pharmacological activities. Journal of Chemical and Pharmaceutical Research 3, 403-423.

Shepherd, M.J., Moore, G., Wand, M.E., Sutton, J.M., Bock, L.J., 2018. *Pseudomonas aeruginosa* adapts to octenidine in the laboratory and a simulated clinical setting, leading to increased tolerance to chlorhexidine and other biocides. Journal of Hospital Infection 100, e23-e29.

Shirmohammadlou, N., Zeighami, H., Haghi, F., Kashefieh, M., 2018. Resistance pattern and distribution of carbapenemase and antiseptic resistance genes among multidrug-resistant *Acinetobacter baumannii* isolated from intensive care unit patients. Journal of Medical Microbiology 67, 1467-1473.

Si, W., Gong, J., Chanas, C., Cui, S., Yu, H., Caballero, C., Friendship, R.M., 2006. *In vitro* assessment of antimicrobial activity of carvacrol, thymol and cinnamaldehyde towards *Salmonella* serotype Typhimurium DT104: Effects of pig diets and emulsification in hydrocolloids. Journal of Applied Microbiology 101, 1282-1291.

Si, X., Quan, X., 2017. Prevention of multi-species wastewater biofilm formation using vanillin and EPS disruptors through non-microbicidal mechanisms. International Biodeterioration & Biodegradation 116, 211-218.

Siddiqua, S., Anusha, B.A., Ashwini, L.S., Negi, P.S., 2015. Antibacterial activity of cinnamaldehyde and clove oil: Effect on selected foodborne pathogens in model food systems and watermelon juice. Journal of Food Science and Technology 52, 5834-5841.

Sidhu, M.S., Heir, E., Leegaard, T., Wiger, K., Holck, A., 2002. Frequency of disinfectant resistance genes and genetic linkage with beta-lactamase transposon Tn*552* among clinical Staphylococci. Antimicrobial Agents and Chemotherapy 46, 2797-2803.

Sikkema, J., de Bont, J.A.M., Poolman, B., 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiological Reviews 59, 201-222.

Silva, S., Costa, E.M., Horta, B., Calhau, C., Morais, R.M., Pintado, M.M., 2016. Anti-biofilm potential of phenolic acids: The influence of environmental pH and intrinsic physico-chemical properties. Biofouling 32, 853-860.

Simões, L.C., Lemos, M., Pereira, A.M., Abreu, A.C., Saavedra, M.J., Simões, M., 2011. Persister cells in a biofilm treated with a biocide. Biofouling 27, 403-411.

Simões, L.C., Simões, M., Oliveira, R., Vieira, M.J., 2007. Potential of the adhesion of bacteria isolated from drinking water to materials. Journal of Basic Microbiology 47, 174-183.

Simões, M., 2011. Antimicrobial strategies effective against infectious bacterial biofilms. Current Medicinal Chemistry 18, 2129-2145.

Simões, M., Bennett, R.N., Rosa, E.A., 2009. Understanding antimicrobial activities of phytochemicals against multidrug resistant bacteria and biofilms. Natural Product Reports 26, 746-757.

Simões, M., Pereira, M.O., Vieira, M.J., 2005. Action of a cationic surfactant on the activity and removal of bacterial biofilms formed under different flow regimes. Water Research 39, 478-486.

Simões, M., Rocha, S., Coimbra, M.A., Vieira, M.J., 2008. Enhancement of *Escherichia coli* and *Staphylococcus aureus* antibiotic susceptibility using sesquiterpenoids. Medicinal Chemistry 4, 616-623.

Singh, I., 2017. Antimicrobials in higher plants: Classification, mode of action and bioactivities. Chemical Biology Letters 4, 48-62.

Singh, M., McKenzie, K., Ma, X., 2017. Effect of dimethyl sulfoxide on *in vitro* proliferation of skin fibroblast cells. Journal of Biotech Research 8, 78-82.

Sivasankar, C., Jha, N.K., Ghosh, R., Shetty, P.H., 2020. Anti quorum sensing and anti virulence activity of tannic acid and it's potential to breach resistance in *Salmonella enterica* Typhi / Paratyphi A clinical isolates. Microbial Pathogenesis 138, 1-12.

Slipski, C.J., Zhanel, G.G., Bay, D.C., 2018. Biocide selective tolC-independent efflux pumps in Enterobacteriaceae. The Journal of membrane biology 251, 15-33.

Smith, E.C., Kaatz, G.W., Seo, S.M., Wareham, N., Williamson, E.M., Gibbons, S., 2007. The phenolic diterpene totarol inhibits multidrug efflux pump activity in *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 51, 4480-4483.

Smith, K., Hunter, I.S., 2008. Efficacy of common hospital biocides with biofilms of multi-drug resistant clinical isolates. Journal of Medical Microbiology 57, 966-973.

Smith, R.L., Cohen, S.M., Doull, J., Feron, V.I., Goodman, J.I., Marnett, L.J., Portoghese, P.S., Waddell, W.J., Wagner, B.M., Adams, T.B., 2003. GRAS Flovouring Substances. Food Technology 57, 46 - 59.

Sogawa, Y., Kobayashi, H., Kajiura, T., Nishihara, Y., 2010. Comparison of residual antimicrobial activity of chlorhexidine-containing antiseptics: An express report. Journal of Healthcare-associated Infection 2, 32-36.

Song, X., Vossebein, L., Zille, A., 2019. Efficacy of disinfectant-impregnated wipes used for surface disinfection in hospitals: A review. Antimicrobial Resistance and Infection Control 8, 1-14.

Spoering, A.L., Lewis, K., 2001. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. Journal of Bacteriology 183, 6746-6751.

Srinivasan, V.B., Rajamohan, G., 2013. KpnEF, a new member of the *Klebsiella pneumoniae* cell envelope stress response regulon, is an SMR-type efflux pump involved in broad-spectrum antimicrobial resistance. Antimicrobial Agents and Chemotherapy 57, 4449-4462.

Srinivasan, V.B., Rajamohan, G., Gebreyes, W.A., 2009. Role of AbeS, a novel efflux pump of the SMR family of transporters, in resistance to antimicrobial agents in *Acinetobacter baumannii*. Antimicrobial Agents and Chemotherapy 53, 5312-5316.

Srinivasan, V.B., Singh, B.B., Priyadarshi, N., Chauhan, N.K., Rajamohan, G., 2014. Role of novel multidrug efflux pump involved in drug resistance in *Klebsiella pneumoniae*. PLoS ONE 9, 1-10.

Stewart, P.S., 2015. Antimicrobial tolerance in biofilms. Microbiology Spectrum 3, 1-30.

Strommenger, B., Kettlitz, C., Werner, G., Witte, W., 2003. Multiplex PCR assay for simultaneous detection of nine clinically relevant antibiotic resistance genes in *Staphylococcus aureus*. Journal of Clinical Microbiology 41, 4089-4094.

Su, X.Z., Chen, J., Mizushima, T., Kuroda, T., Tsuchiya, T., 2005. AbeM, an H+-coupled *Acinetobacter baumannii* multidrug efflux pump belonging to the MATE family of transporters. Antimicrobial Agents and Chemotherapy 49, 4362-4364.

Sung, W.S., Lee, D.G., 2008. Mechanism of decreased susceptibility for Gram-negative bacteria and synergistic effect with ampicillin of indole-3-carbinol. Biological & Pharmaceutical Bulletin 31, 1798-1801.

Suzuki, N., Rivero, R.M., Shulaev, V., Blumwald, E., Mittler, R., 2014. Abiotic and biotic stress combinations. New Phytologist 203, 32-43.

Szomolay, B., Klapper, I., Dockery, J., Stewart, P.S., 2005. Adaptive responses to antimicrobial agents in biofilms. Environmental Microbiology 7, 1186-1191.

Tan, S.M., Lee, S.M., Dykes, G.A., 2015. Acetic acid induces pH-independent cellular energy depletion in *Salmonella enterica*. Foodborne Pathogens and Disease 12, 183-189.

Tattawasart, U., Hann, A.C., Maillard, J.Y., Furr, J.R., Russell, A.D., 2000. Cytological changes in chlorhexidine-resistant isolates of *Pseudomonas stutzeri*. Journal of Antimicrobial Chemotherapy 45, 145-152.

Tehrani, K.H.M.E., Martin, N.I., 2018. β-lactam/β-lactamase inhibitor combinations: An update. MedChemComm 9, 1439-1456.

Timm, M., Saaby, L., Moesby, L., Hansen, E.W., 2013. Considerations regarding use of solvents *in in vitro* cell based assays. Cytotechnology 65, 887-894.

Todd, E.C., Greig, J.D., Bartleson, C.A., Michaels, B.S., 2009. Outbreaks where food workers have been implicated in the spread of foodborne disease. Part 6. Transmission and survival of pathogens in the food processing and preparation environment. Journal of Food Protection 72, 202-219.

Torres, S., Pandey, A., Castro, G.R., 2011. Organic solvent adaptation of Gram positive bacteria: Applications and biotechnological potentials. Biotechnology Advances 29, 442-452.

Tuladhar, E., Hazeleger, W.C., Koopmans, M., Zwietering, M.H., Beumer, R.R., Duizer, E., 2012. Residual viral and bacterial contamination of surfaces after cleaning and disinfection. Applied and Environmental Microbiology 78, 7769-7775.

Tyan, K., Jin, K., Kang, J., 2019. Novel colour additive for bleach disinfectant wipes reduces corrosive damage on stainless steel. Journal of Hospital Infection 103, 227-230.

Upadhyay, A., Upadhyaya, I., Mooyottu, S., Kollanoor-Johny, A., Venkitanarayanan, K., 2014. Efficacy of plant-derived compounds combined with hydrogen peroxide as antimicrobial wash and coating treatment for reducing *Listeria monocytogenes* on cantaloupes. Food Microbiology 44, 47-53.

Vale, J., Ribeiro, M., Abreu, A.C., Soares-Silva, I., Simões, M., 2019. The use of selected phytochemicals with EDTA against *Escherichia coli* and *Staphylococcus epidermidis* single- and dual-species biofilms. Letters in Applied Microbiology 68, 313-320.

van Oss, C.J., Chaudhury, M.K., Good, R.J., 1987. Monopolar surfaces. Advances in Colloid and Interface Science 28, 35-64.

van Oss, C.J., Good, R.J., Chaudhury, M.K., 1988. Additive and nonadditive surface tension components and the interpretation of contact angles. Langmuir 4, 884-891.

van Oss, C.J., Ju, L., Chaudhury, M.K., Good, R.J., 1989. Estimation of the polar parameters of the surface tension of liquids by contact angle measurements on gels. Journal of Colloid and Interface Science 128, 313-319.

Vázquez-Sánchez, D., Galvão, J.A., Mazine, M.R., Gloria, E.M., Oetterer, M., 2018. Control of *Staphylococcus aureus* biofilms by the application of single and

combined treatments based in plant essential oils. International Journal of Food Microbiology 286, 128-138.

Vieira, R.G.L., Moraes, T.D.S., Silva, L.O., Bianchi, T.C., Veneziani, R.C.S., Ambrosio, S.R., Bastos, J.K., Pires, R.H., Martins, C.H.G., 2018. In vitro studies of the antibacterial activity of *Copaifera* spp. oleoresins, sodium hypochlorite, and peracetic acid against clinical and environmental isolates recovered from a hemodialysis unit. Antimicrobial Resistance and Infection Control 7, 1-13.

Vila, J., Martí, S., Sánchez-Céspedes, J., 2007. Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*. Journal of Antimicrobial Chemotherapy 59, 1210-1215.

Virto, R., Mañas, P., Alvarez, I., Condon, S., Raso, J., 2005. Membrane damage and microbial inactivation by chlorine in the absence and presence of a chlorine-demanding substrate. Applied and Environmental Microbiology 71, 5022-5028.

Virto, R., Sanz, D., Álvarez, I., Condón, S., Raso, J., 2006. Application of the Weibull model to describe inactivation of *Listeria monocytogenes* and *Escherichia coli* by citric and lactic acid at different temperatures. Journal of the Science of Food and Agriculture 86, 865-870.

Wagle, B.R., Upadhyay, A., Upadhyaya, I., Shrestha, S., Arsi, K., Liyanage, R., Venkitanarayanan, K., Donoghue, D.J., Donoghue, A.M., 2019. Trans-cinnamaldehyde, eugenol and carvacrol reduce *Campylobacter jejuni* biofilms and modulate expression of select genes and proteins. Frontiers in Microbiology 10, 1-16.

Wand, M.E., 2017. Bacterial resistance to hospital disinfection, In: Hurst, C.J. (Ed.), Modeling the Transmission and Prevention of Infectious Disease. Springer International Publishing, Cham, pp. 19-54.

Wang, C., Chang, T., Yang, H., Cui, M., 2015. Antibacterial mechanism of lactic acid on physiological and morphological properties of *Salmonella* Enteritidis, *Escherichia coli* and *Listeria monocytogenes*. Food Control 47, 231-236.

Wang, L.-H., Wang, M.-S., Zeng, X.-A., Gong, D.-M., Huang, Y.-B., 2017. An *in vitro* investigation of the inhibitory mechanism of β-galactosidase by cinnamaldehyde alone and in combination with carvacrol and thymol. Biochimica et Biophysica Acta (BBA) - General Subjects 1861, 3189-3198.

Wang, Y., Feng, K., Yang, H., Zhang, Z., Yuan, Y., Yue, T., 2018. Effect of cinnamaldehyde and citral combination on transcriptional profile, growth, oxidative

damage and patulin biosynthesis of *Penicillium expansum*. Frontiers in Microbiology 9, 597-597.

Wang, Y., Kheir, M.M., Chai, Y., Hu, J., Xing, D., Lei, F., Du, L., 2011. Comprehensive study in the inhibitory effect of berberine on gene transcription, including TATA box. PLoS ONE 6, 1-11.

Wang, Y., Li, H., Xie, X., Wu, X., Li, X., Zhao, Z., Luo, S., Wan, Z., Liu, J., Fu, L., Li, X., 2020. *In vitro* and *in vivo* assessment of the antibacterial activity of colistin alone and in combination with other antibiotics against *Acinetobacter baumannii* and *Escherichia coli*. Journal of Global Antimicrobial Resistance 20, 351-359.

Webber, M.A., Randall, L.P., Cooles, S., Woodward, M.J., Piddock, L.J., 2008. Triclosan resistance in *Salmonella enterica* serovar Typhimurium. Journal of Antimicrobial Chemotherapy 62, 83-91.

Weber, D.J., Rutala, W.A., Sickbert-Bennett, E.E., 2007. Outbreaks associated with contaminated antiseptics and disinfectants. Antimicrobial Agents and Chemotherapy 51, 4217-4224.

Wesgate, R., Robertson, A., Barrell, M., Teska, P., Maillard, J.Y., 2019. Impact of test protocols and material binding on the efficacy of antimicrobial wipes. Journal of Hospital Infection 103, e25-e32.

Williams, G.J., Denyer, S.P., Hosein, I.K., Hill, D.W., Maillard, J.-Y., 2009. Limitations of the efficacy of surface disinfection in the healthcare setting. Infection Control and Hospital Epidemiology 30, 570-573.

Williams, G.J., Denyer, S.P., Hosein, I.K., Hill, D.W., Maillard, J.Y., 2007. The development of a new three-step protocol to determine the efficacy of disinfectant wipes on surfaces contaminated with *Staphylococcus aureus*. Journal of Hospital Infection 67, 329-335.

World, World Health Organization and Pan American Health Organization, 2016. Decontamination and Reprocessing of Medical Devices for Health-care Facilities. WHO Document Production Services, 1-119.

Wren, M.W.D., Rollins, M.S.M., Jeanes, A., Hall, T.J., Coën, P.G., Gant, V.A., 2008. Removing bacteria from hospital surfaces: a laboratory comparison of ultramicrofibre and standard cloths. Journal of Hospital Infection 70, 265-271.

Yakabe, L.E., Parker, S.R., Kluepfel, D.A., 2011. Cationic surfactants: Potential surface disinfectants to manage *Agrobacterium tumefaciens* biovar 1 contamination of grafting tools. Plant Disease 96, 409-415.

Yang, H., Yu, Y., Fu, C., Chen, F., 2019. Bacterial acid resistance toward organic weak acid revealed by RNA-seq transcriptomic analysis in *Acetobacter pasteurianus*. Frontiers in Microbiology 10, 1-14.

Yao, J., Rock, C.s.O., 2016. Resistance mechanisms and the future of bacterial enoyl-acyl carrier protein reductase (FabI) antibiotics. Cold Spring Harbor Perspectives in Medicine 6, 1-11.

Yilmaz, S., Sova, M., Ergun, S., 2018. Antimicrobial activity of trans-cinnamic acid and commonly used antibiotics against important fish pathogens and nonpathogenic isolates. Journal of Applied Microbiology 125, 1714-1727.

Young, A.J., Lowe, G.L., 2018. Carotenoids-Antioxidant properties. Antioxidants 7, 1-4.

Young, S.B., Setlow, P., 2003. Mechanisms of killing of *Bacillus subtilis* spores by hypochlorite and chlorine dioxide. Journal of Applied Microbiology 95, 54-67.

Young, S.B., Setlow, P., 2004. Mechanisms of killing of *Bacillus subtilis* spores by Decon and Oxone, two general decontaminants for biological agents. Journal of Applied Microbiology 96, 289-301.

Zhang, J., Xiao, A., Wang, T., Liang, X., Gao, J., Li, P., Shi, T., 2014. Effect and mechanism of action of cinnamic acid on the proliferation and apoptosis of leukaemia cells. Biomedical Research 25, 405-408.

Zhang, Y., Wei, J., Qiu, Y., Niu, C., Song, Z., Yuan, Y., Yue, T., 2019. Structure-dependent inhibition of *Stenotrophomonas maltophilia* by polyphenol and its impact on cell membrane. Frontiers in Microbiology 10, 1-11.

Zhou, L., Zheng, H., Tang, Y., Yu, W., Gong, Q., 2013. Eugenol inhibits quorum sensing at sub-inhibitory concentrations. Biotechnology Letters 35, 631-637.

Zhu, L., Lin, J., Ma, J., Cronan, J.E., Wang, H., 2010. Triclosan resistance of *Pseudomonas aeruginosa* PAO1 is due to fabV, a triclosan-resistant enoyl-acyl carrier protein reductase. Antimicrobial Agents and Chemotherapy 54, 689-698.

Appendix

A. Supplementary Information

B. Publications

C. Communications in Scientific Meetings

A. Supplementary information

A.1. Culture medium modification and determination of the best washing solution

In order to maintain a work pH of 7, it was determined that PB 0.02 M was necessary to preserve the pH of MHB as well as for the washing steps. Otherwise, the pH would vary when the biocides and the phytochemicals or derivatives were added to both MHB or washing solution (Figure A.1).

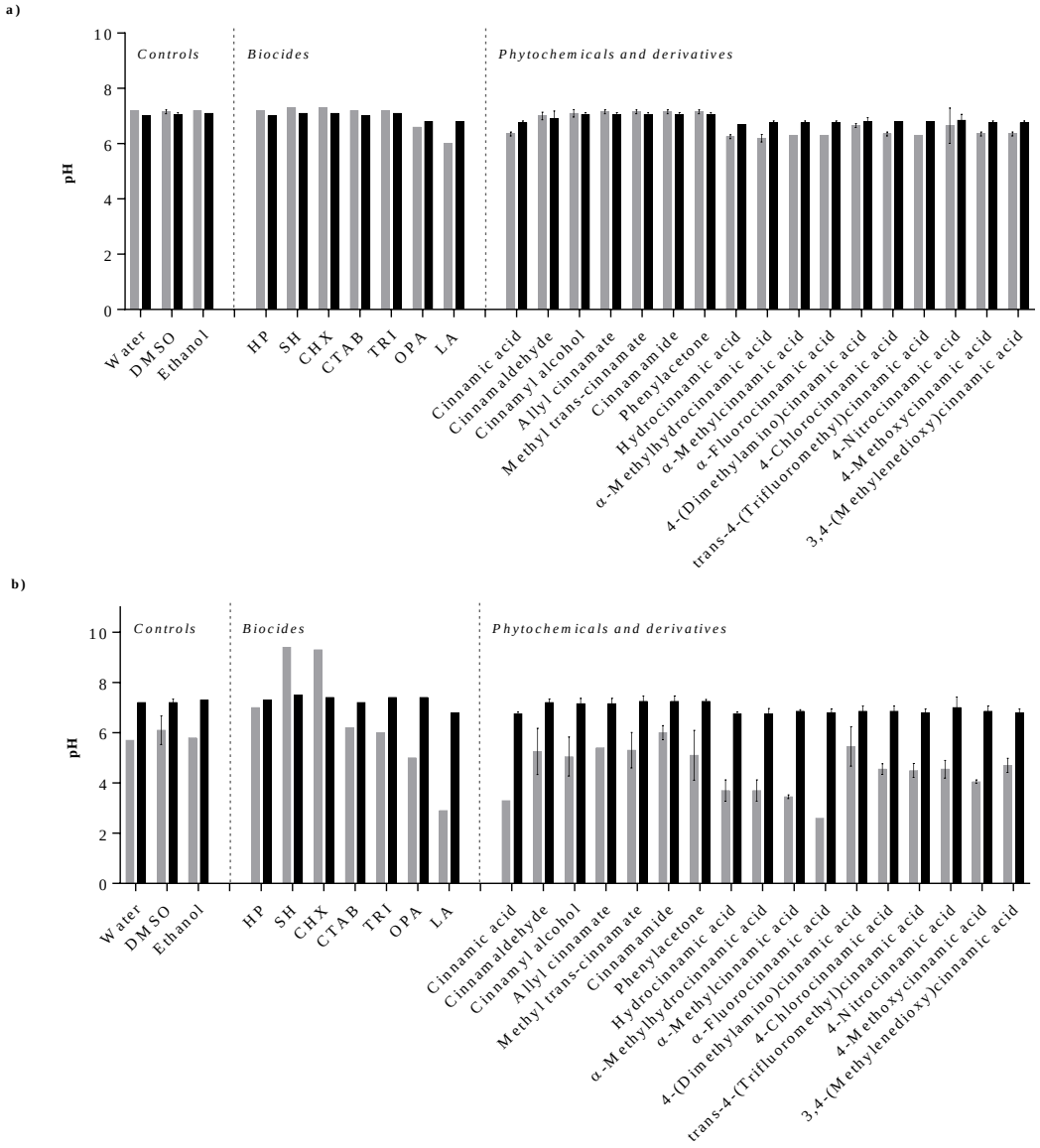


Figure A.1. pH variation after the addition of biocides, phytochemicals and derivatives: a) when the growth medium was not controlled (grey bars) or controlled (black bars) with PB pH 7; b) when the NaCl 8.5 g L^{-1} (grey bars) or PB pH 7 (black bars) were used. The controls are the solvents used to solubilize the biocides and phytochemicals and derivatives.

A.2. Determination of the maximum volume of solvent that does not influence bacterial visible growth

The data obtained when assessing growth curves of *E. coli* CECT 434, *E. coli* NCTC 10418, *S. aureus* CECT 976, *S. aureus* NCTC10799 and *E. hirae* 13383 (Figure A.2) allowed to stablish a maximum volume of 5% of solvent. This maximum of 5% was determined taking into consideration the effect of the growth curves of the bacteria and the solubility of all the phytochemicals and derivatives to be tested.

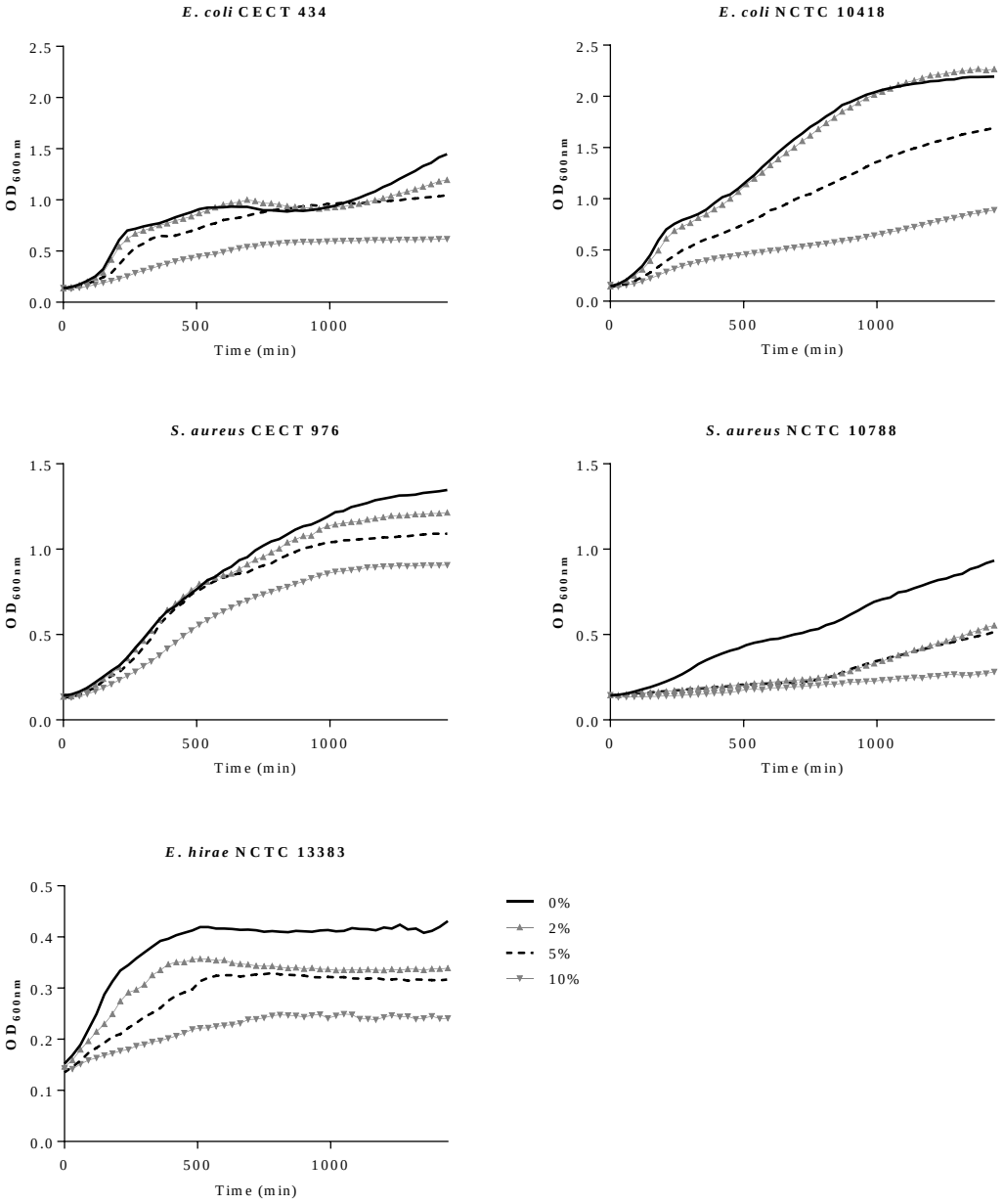


Figure A.2. Growth curves of *E. coli* CECT 434 and NCTC 10418, *S. aureus* CECT 976 and NCTC10799 and *E. hirae* 13383 in the presence of 0%, 2%, 5% and 10% of DMSO (legend in the figure).

A.3. Reproducibility of 96-well plates CFU recovering method

Sessile cells were scraped with a pipette tip for 1 min and re-suspended in NaCl 8.5 g L^{-1} (Chapter IV) or PB pH7 (Chapter V, VI) the contact time and method was prior established to give the best reproducibility (four repeats) in results for the selected bacteria as follows: *E. coli* CECT 434 4.75 ± 0.49 $\log_{10}$ CFU cm^{-2}, *E. coli* NCTC 10418 5.77 ± 0.18 $\log_{10}$ CFU cm^{-2}, *S. aureus* CECT 976 4.97 ± 0.64 $\log_{10}$ CFU cm^{-2}, *S. aureus* NCTC 10788 5.87 ± 0.13 $\log_{10}$ CFU cm^{-2}, *E. hirae* NCTC 13383 5.76 ± 0.12 $\log_{10}$ CFU cm^{-2}.

A.4. Determination of quorum sensing inhibition by disc diffusion assay using *Chromobacterium violaceum*

C. violaceum ATCC 12472 was grown overnight in Luria-Bertani broth (LB, Liofilchem, Italy) at 30 ± 3°C, under 150 rpm of agitation. Standard disc diffusion assay was performed for 2 µmol of each chemical (correspondent to the 10 mM of chemicals applied in bacterial adhesion assay). Briefly, bacterial suspension ($\approx 10^8$ CFU ml^{-1}) was poured with a sterilized swab over LB agar plates. Next, sterile paper discs (6 mm diameter) were placed over the LB agar plates and 20 µl of each solution was added. Antimicrobial and quorum sensing inhibition (a ring of colorless but viable cells) halos were measured after 18-24 hours of incubation at 30 ± 3°C (Adonizio *et al.*, 2006; McLean *et al.*, 2004).

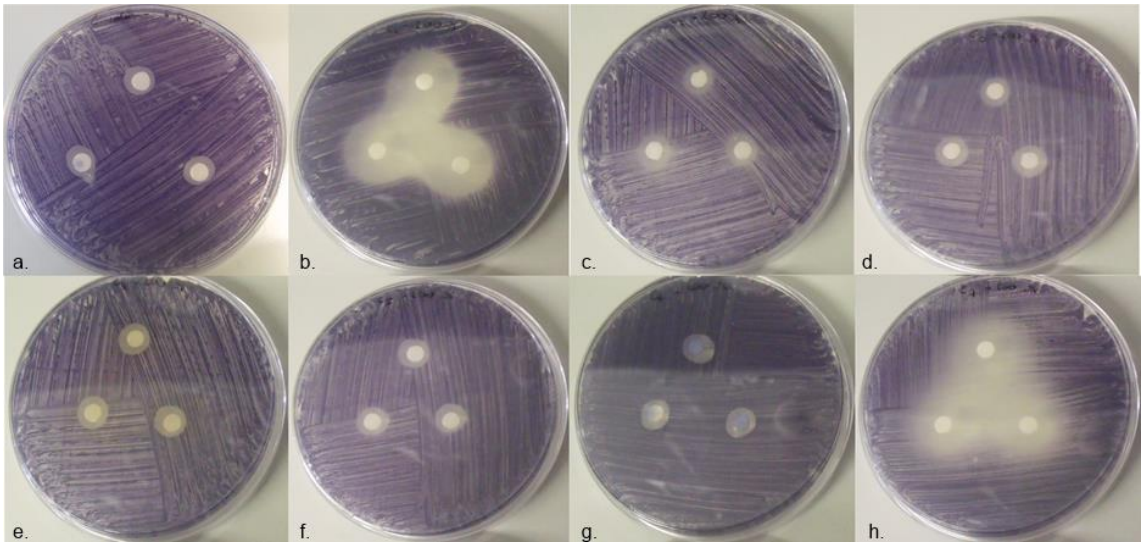


Figure A.3. Quorum sensing and growth inhibition halos (absence of purple coloration and/or absence of bacteria, respectively) when exposed to DMSO (a.) or 2 µmol of cinnamaldehyde (b.), trans-cinnamic acid (c.), p-coumaric acid (d.), caffeic acid (e.), trans ferulic acid (f.), tyrosol (g.) and eugenol (h.).

A.5. Determination of the best CTAB concentration

Table A.1. CTAB concentrations able to reduce 5 log_{10} CFU mL^{-1} bacterial counts, after 5 min of exposure. ND: No CFU was detected (> 6 log_{10} CFU mL^{-1} reduction)

CTAB (mM)	**Bacteria reduction (log_{10} CFU mL^{-1})**
0.005	< 4
0.0075	< 4
0.01	< 4
0.02	< 4
0.03	< 4
0.04	4.41
0.05	5.17
0.06	ND
0.07	ND
0.08	ND
0.09	ND
0.1	ND
0.25	ND
0.5	ND
1	ND

A.6. Determination of the best concentration of CTAB, cinnamaldehyde and EDTA in combination

Table A.2. Cinnamaldehyde, EDTA and CTAB concentrations able to reduce 5 log_{10} CFU mL^{-1} bacterial counts after 5 min of exposure. ND: No CFU was detected (limit of detection 6 log_{10} CFU mL^{-1} reduction)

Cinnamaldehyde (mM)	**EDTA (mM)**	**CTAB (mM)**	**Bacteria reduction (log_{10} CFU mL^{-1})**	
			Clean conditions	Dirty Conditions
1	10	0.02	< 4	< 4
		0.05	< 4	< 4
		0.1	< 4	< 4
		0.25	< 4	< 4
		0.5	< 4 – 5.6	< 4
		0.75	< 4 - ND	< 4
		1	5.9 - ND	< 4
	25	0.02	< 4	< 4
		0.05	< 4	< 4
		0.1	< 4 – 5.6	< 4
		0.25	4.9 – 5.1	< 4 - 5
		0.5	5.2 - ND	< 4 - 6
		0.75	5.7 – ND	< 4
		1	ND	< 4
2	10	0.02	< 4	< 4
		0.05	< 4	< 4
		0.1	< 4	< 4
		0.25	< 4	< 4
		0.5	< 4	< 4
		0.75	< 4	< 4
		1	< 4	< 4
	25	0.02	< 4	< 4
		0.05	< 4	< 4
		0.1	< 4	< 4
		0.25	< 4	< 4
		0.5	< 4	< 4
		0.75	< 4	< 4
		1	< 4	< 4

A.7. High Performance Liquid Chromatography (HPLC) chromatograms

A.7.1. Cinnamaldehyde calibration curve

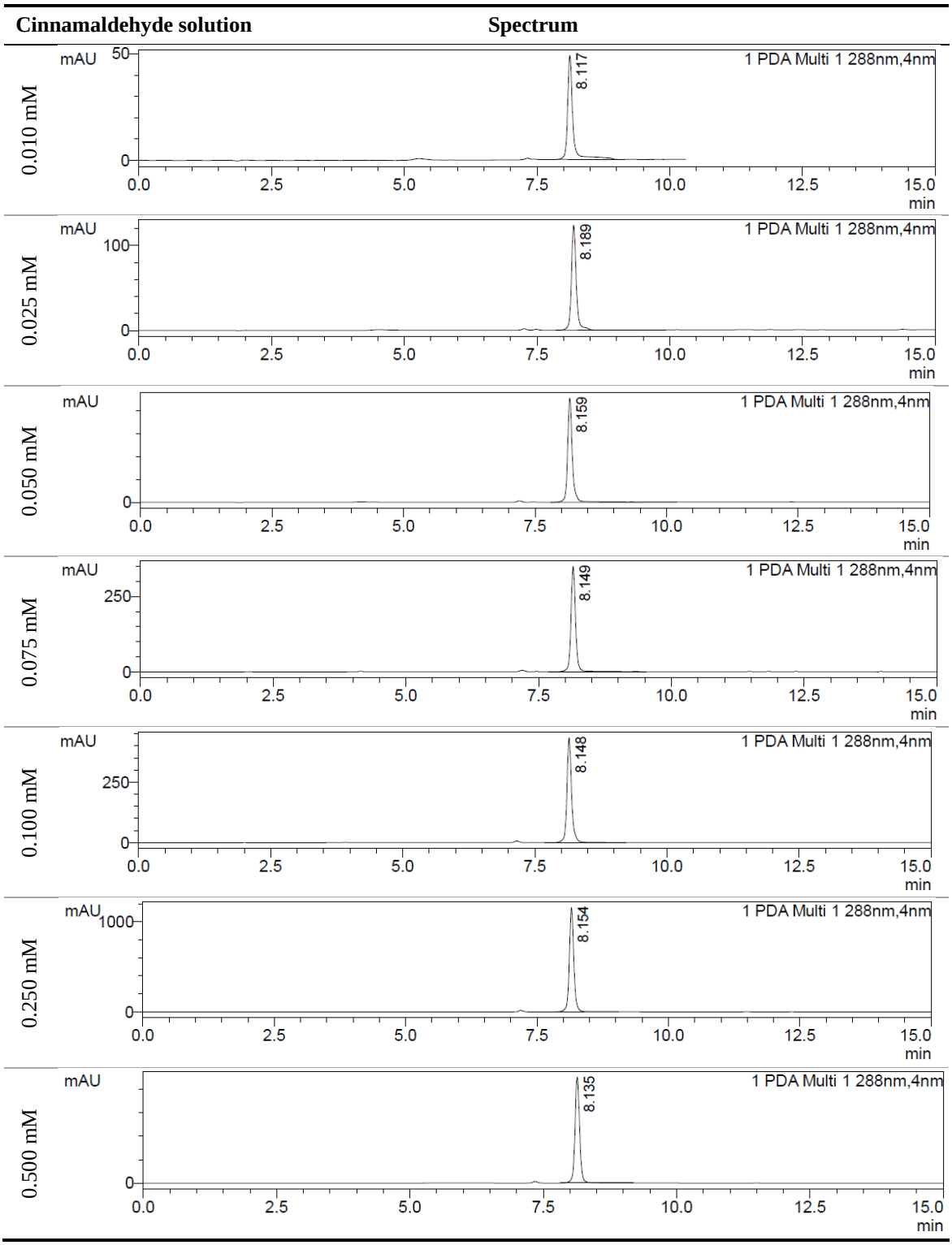


Figure A.4. HPLC chromatograms of cinnamaldehyde solutions of different concentrations.

Table A.3. Cinnamaldehyde solutions parameters obtained by the analysis of HPLC chromatograms (Figure A.4)

Standard Concentration (mM)	Retention Time (min)	Area (mAU min^{-1})	Height (mAU)
0.010	8.117	344372	48623
0.025	8.189	780332	122936
0.050	8.159	1434718	225592
0.075	8.149	2107190	348251
0.100	8.148	2669536	431753
0.250	8.154	6932379	1151639
0.500	8.135	13800878	2247732

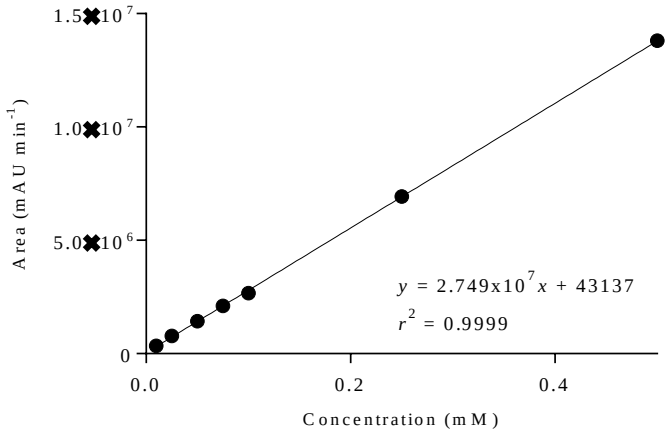


Figure A.5. Calibration curve obtained by plotting the area of the peaks *versus* cinnamaldehyde standard concentrations, Table A.3.

A.7.2. Determination of cinnamaldehyde concentration on the formulation by HPLC

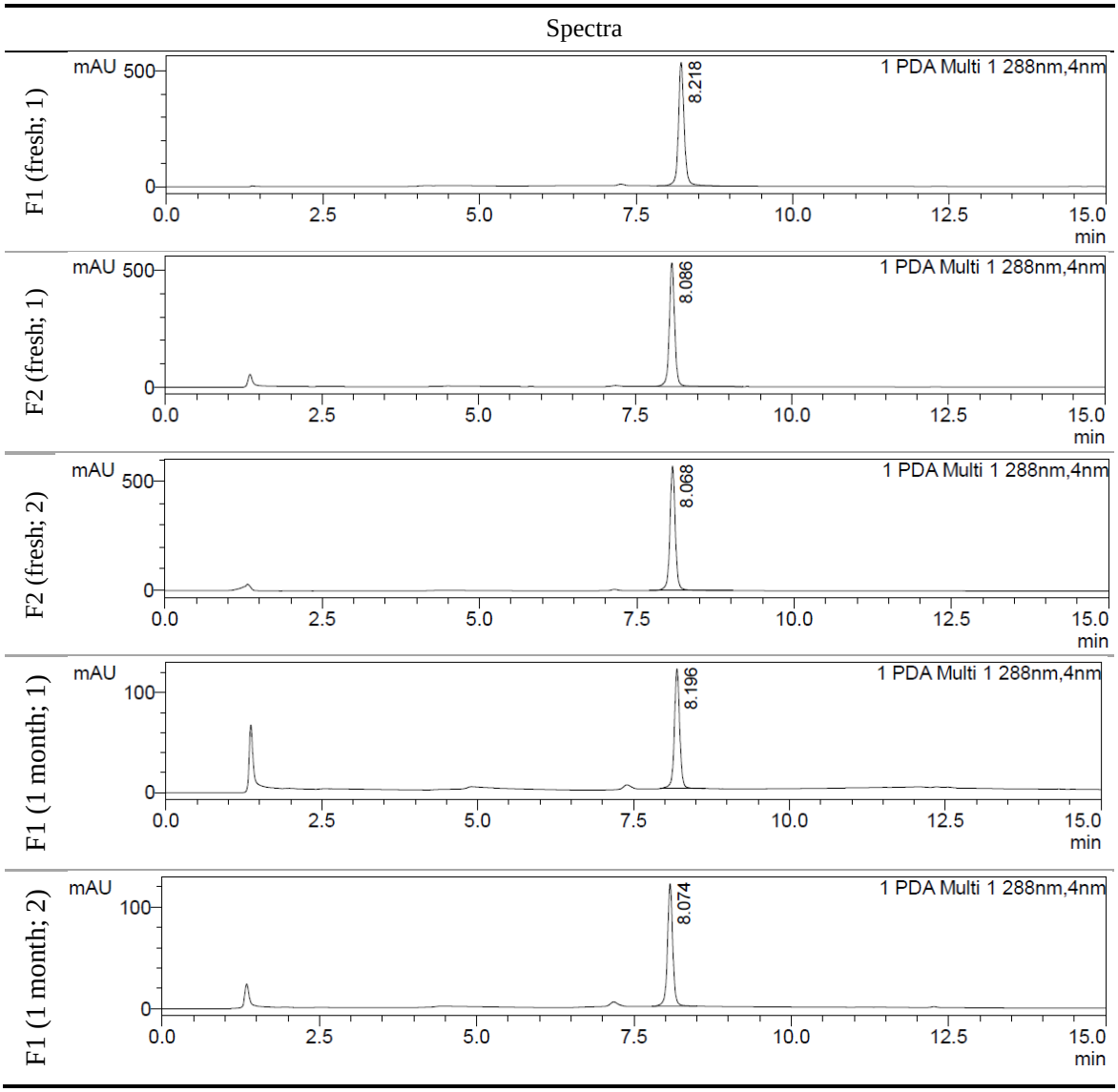


Figure A.6. HPLC chromatograms obtained for two freshly made formulations (F1 and F2) and after 1 month of shelf storage of F1.

Table A.4. Cinnamaldehyde concentration in fresh formulation and after 1 month storage. Parameters obtained by the analysis of HPLC chromatograms are also presented (Figure A.6)

Sample	Retention Time (min)	Area (mAU min^{-1})	Height (mAU)	Calculated Concentration (mM)
F1 (fresh)	8.218	3421794	530104	0.123
F2 (fresh; 1)	8.086	3193603	528627	0.115
F2 (fresh; 2)	8.068	3439400	570388	0.124
F1 (1 month; 1)	8.196	716357	119418	0.024
F1 (1 month; 2)	8.074	708283	120121	0.024

B. Publications

B.1. Papers published in peer reviewed journals

Abreu A.C., Paulet D., Coqueiro A., Malheiro J., Borges A., Saavedra M.J., Choi Y.H., Simões M., 2016. Antibiotic adjuvants from *Buxus sempervirens* to promote effective treatment of drug-resistant *Staphylococcus aureus* biofilms. RSC Advances, 6 (97):95000–95009. Doi: 10.1039/C6RA21137B. (https://pubs.rsc.org/en/content/articlelanding/2016/ra/c6ra21137b#!divAbstract)

Gomes I., Malheiro J., Simões M., 2017. Controlo da formação de biofilmes em superfícies hospitalares. TecnoHospital, 79:12-15. (http://www.tecnohospital.pt/noticias/revista-n79-janeiro-fevereiro-2017/)

Malheiro J., Araújo P., Machado I., Lemos M., Mergulhão F., Melo L., Simões M., 2015. The effects of selected brominated and chlorinated chemicals on *Pseudomonas fluorescens* planktonic cells and flow-generated biofilms. Journal of Food Processing and Preservation, 40(2):316–328. Doi: 10.1111/jfpp.12609. (https://onlinelibrary.wiley.com/doi/abs/10.1111/jfpp.12609)

Malheiro J., Gomes I., Borges A., Bastos M.M., Maillard J.Y., Borges F., Simões M., 2016. Phytochemical profiling as a solution to palliate disinfectant limitations. Biofouling, 32(9):1007–1016. Doi: 10.1080/08927014.2016.1220550. (https://www.tandfonline.com/doi/abs/10.1080/08927014.2016.1220550?journalCode=gbif20)

Malheiro J.F., Maillard J.-Y., Borges F., Simões M., 2018. Evaluation of cinnamaldehyde and cinnamic acid derivatives in microbial growth control. International Biodeterioration & Biodegradation, 141:71-78. Doi: 10.1016/j.ibiod.2018.06.003. (https://www.tandfonline.com/doi/abs/10.1080/08927014.2016.1220550?journalCode=gbif20)

Malheiro J.F., Maillard J.-Y., Borges F., Simões M., 2019. Biocide potentiation using cinnamic phytochemicals and derivatives. Molecules, 24(21):1-15. Doi: 10.3390/molecules24213918. (https://www.mdpi.com/1420-3049/24/21/3918)

Malheiro J. F., Oliveira C., Cagide F., Borges F., Simões M., Maillard J-Y., 2020. Surface wiping test to study biocide -cinnamaldehyde combination to improve efficiency in surface disinfection. International Journal of Molecular Science, 21(21):1-14.

Doi: 10.3390/ijms21217852. (https://www.mdpi.com/1422-0067/21/21/7852/htm)

B.2. Papers in conference proceedings

Malheiro J, Gomes I, Borges A, Abreu A, Loureiro J, Mergulhão F, Simões M. 2015. Cinnamic acid in the control of planktonic and sessile cells of *Escherichia coli* and *Staphylococcus aureus*. In: Proceedings book of the III International Conference on Antimicrobial Research – ICAR2014 (Madrid, Spain, 1st-3rd October 2014). Book title: "Multidisplinary approaches for studying and combating microbial pathogens". Eds. A. Mendez-Vilas (Brown Walker Press) Boca Raton, FL, USA, pp. 68-72. ISBN–13: 978-1-62734-544-6

B.3 Book Chapters

Abreu A. C., Borges A., Malheiro J., Saavedra M. J., Simões M., 2013. Resurgence of the interest in plants as sources of medicines and resistance-modifying agents. In: Microbial pathogens and strategies for combating them: science, technology and education. Publisher: Formatex Research Center; Editor: A. Méndez-Vilas; Microbiology book series - 2013 editions.

Andrade M., Malheiro J., Borges F., Saavedra M. J., Simões M. The potential of phytochemical products in biofilm control. In: Recent Trends in Biofilm Science and Technology. Publisher: Academic Press is an imprint of Elsevier; Editor: M. Simões, A. Borges, L. Simões; Cap. 12; pp. 273-293. ISBN: 978-0-12-819497-3.

Borges A., Abreu A. C., Malheiro J., Saavedra M. J., Simões M., 2013. Biofilm prevention and control by dietary phytochemicals. In: Microbial pathogens and strategies for combating them: science, technology and education. Publisher: Formatex Research Center; Editor: A. Méndez-Vilas; Microbiology book series - 2013 editions.

Borges A, Malheiro J, Simões M. 2015. *Bacillus cereus* a foodborne pathogen involved in biofilm infections. In: Impact of biofilms in health: a transcriptomic perspective. Section II - Transcriptomes of relevant medical biofilms. Publisher: University of Minho - CEB, Braga, Portugal; Editor(s): N. Cerca; Cap. 12; pp. 245-262. ISBN: 978-989-97478-6-9 (1st edition: January 2015).

Malheiro J., Simões M., 2017. Antimicrobial resistance of biofilms in medical devices. In: Biofilms and Implantable Medical Devices - Infection and Control. Publisher: Woodhead Publishing Series in Biomaterials; Editor: Ying Deng, Wei Lv. Pp. 97-113. ISBN: 978-0-08-100382-4 Doi: 10.1016/B978-0-08-100382-4.01001-7.

C. Communications in scientific meetings

C.1. Oral presentations

Gomes I., Lemos M., Malheiro J., Simões L.C., Simões M., 2015. Sodium hypochlorite on drinking water biofilm treatment. 4th European Congress on Microbial Biofilms (Eurobiofilms 2015). Brno, Czech Republic.

Gomes I., Malheiro J., Mergulhão F., Simões M., 2015. Efficacy of current and natural-based biocides on the disinfection of silicone and stainless steel surfaces. 1st Doctoral Congress In Engineering (DCE). Porto, Portugal.

Malheiro J., Gomes I., Borges A., Abreu A., Mergulhão F. and Simões M., 2014. Cinnamic acid in the control of planktonic and sessile cells of *Escherichia coli* and *Staphylococcus aureus*. International Conference on Antimicrobial Research - ICAR2014. Madrid, Spain.

Malheiro J., Gomes I.B., Borges A., Maillard J.Y., Borges F., Simões M., 2015. The effects of selected phytochemical compounds on the control of planktonic and sessile bacteria. 1st Doctoral Congress In Engineering (DCE). Porto, Portugal.

Malheiro J., Maillard J.Y., Borges F., Simões M., 2017. Microbial growth control with phytochemicals and derivatives. The 17th Triennial International Biodeterioration and Biodegradation Symposium. Manchester, UK.

Malheiro J. F., Borges F., Maillard J-Y., Simões M., 2019. Phytochemicals - a new strategy to improve disinfection efficiency. 3rd International Caparica Congress In Antibiotic Resistance. Caparica, Portugal

C.2. Poster presentations

Abreu A.C., Gomes I., Malheiro J., Simões M., 2014. The use of natural alkaloids and flavonoids with combination with antibiotics in the prevention and control of *Staphylococcus aureus* biofilms. 24th ECCMID. Barcelona, Spain.

Gomes I., Malheiro J., Abreu A., Borges A., Mergulhão F., Simões M., 2014. Use of eugenol and cuminaldehyde for disinfection of stainless steel surfaces. 62nd International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA2014). Guimarães, Portugal.

Gomes I., Malheiro J., Abreu A., Mergulhão F., Simões M., 2014. Use of different disinfectants, phytochemicals and antibiotics to inactivate resistant *S. aureus* strains adhered to different materials. 24th ECCMID. Barcelona, Spain.

Gomes I., Malheiro J., Borges A.P., Mergulhão F., Simões M., 2015. Biocides on the disinfection of silicone and stainless steel surfaces. 4th European Congress on Microbial Biofilms (Eurobiofilms 2015). Brno, Czech Republic.

Malheiro J., Abreu A., Borges A., Simões M., 2013. High-efficiency disinfectants – synergistic effects. 1st Symposium on Medicinal Chemistry. Braga, Portugal.

Malheiro J., Gomes I., Borges A., Abreu A., Mergulhão F., Simões M., 2014. Phytochemicals efficacy against planktonic bacteria and biofilms. 62nd International Congress and Annual Meeting of the Society for medicinal Plant and natural Products Research - GA2014. Guimarães, Portugal.

Malheiro J., Gomes I.B., Borges A.P., Simões M., 2015. Cinnamic acid modifies bacterial surface and induces biofilm removal. 4th European Congress on Microbial Biofilms (Eurobiofilms 2015). Brno, Czech Republic.

Malheiro J., Gomes I.B., Mergulhão F., Melo L., Simões M., 2015. The role of biocide adaptation on biofilm formation and susceptibility. 4th European Congress on Microbial Biofilms (Eurobiofilms 2015). Brno, Czech Republic.

Malheiro J., Gomes I., Borges A., Bastos M.M.S.M., Maillard J-Y., Borges F., Simões M., 2016. Looking for a green solution to surpass disinfectant drawbacks. Biofilms7. Porto, Portugal.

Malheiro J. F., Costa S. S., Couto I., Borges F., Maillard J-Y., Simões M., 2019. Phytochemical derivatives as *Staphylococcus aureus* efflux pump modulators. 3rd International Caparica Congress In Antibiotic Resistance. Caparica, Portugal

Malheiro J. F., Costa S. S., Couto I., Borges F., Maillard J-Y., Simões M., 2019. Cinnamaldehyde modulates efflux of antimicrobial resistant *Staphylococcus aureus*. Microbiotec'19. Coimbra, Portugal.

Malheiro J. F., Borges F., Simões M., Maillard J-Y., 2019. Surface wiping test to study biocide and cinnamaldehyde combination to improve surface disinfection efficiency. Microbiotec'19. Coimbra, Portugal.

Malheiro J. F., Oliveira C., Cagide F., Borges F., Simões M., Maillard J-Y., 2020. A new surface wiping test to study surface disinfection by a novel chemical combination. Biofilms9, online conference.