INTEGRATED MASTER IN BIOENGINEERING

# Effects of caffeic acid alkyl esters in the control of planktonic and sessile cells

Dissertation for Master Degree in Bioengineering – Specialization in Biological Engineering

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“Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.”

*Marie Curie*

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# ABSTRACT

Bacterial multidrug resistance to the commonly used antibiotics is a global concern, especially when coupled with biofilm formation, a phenomenon that causes increased resistance. In fact, biofilms are much more resistant to the action of current antimicrobial agents than planktonic cells and, thus, harder to eliminate. This issue led to a quest for the development of potential new active products and new improved alternative strategies for biofilm control. Some phytochemical products, which are produced by plants as part of their chemical strategies for stress response, including microbial attacks, are regarded as new alternative antimicrobial agents that are not as vulnerable as current drugs to bacterial resistance mechanisms.

In this view, the effects of a phytochemical (caffeic acid) and a series of alkyl ester derivatives in the control of planktonic bacterial growth and in biofilm inhibition of *Staphylococcus aureus* and *Escherichia coli* have been studied, with the intention of analyzing the influence of the alkyl ester side chain length in their activity. The overall results are a contribution to the rational design and development of new effective antimicrobial agents.

Caffeic acid esters were found to be effective antimicrobial agents for both the planktonic and sessile states in both bacteria. Their activity was directly dependent on their lipophilicity, i.e., on the length of their alkyl side chain, which affected bacterial susceptibility, the physicochemical properties of the bacteria and their ability to adhere to different surface materials. The compounds did not have any apparent quorum sensing inhibition activity. Gram-negative bacteria were less susceptible than Gram-positive bacteria to the action of these compounds in both planktonic and sessile forms, rendering lower susceptibilities, minor effects upon their physicochemical surface properties and higher adhesion levels. The compounds were proposed to act as membrane permeabilizers, inducing membrane alterations and causing membrane rupture and consequent cell death.

However, the influence of the alkyl side chain length is not yet fully understood, once no obvious pattern was observed apart from the fact that longer alkyl side chain compounds had better results in inhibiting bacterial growth and bacterial adhesion for the Gram-positive bacterium, while medium length alkyl side chain compounds were more effective for the Gram-negative bacterium.

***Keywords:*** adhesion; antimicrobial activity; bacterial resistance; biofilm; biofilm prevention; phytochemical; phenolics; caffeic acid; caffeic acid alkyl esters; quorum sensing.

# RESUMO

As multirresistências bacterianas aos antibióticos comummente utilizados são uma preocupação global, especialmente quando acopladas à formação de biofilmes e ao subsequente aumento da resistência. De facto, os biofilmes são bastante mais resistentes à ação dos agentes antimicrobianos atualmente utilizados do que as células em estado planctónico e, desta forma, muito mais difíceis de eliminar. Esta problemática desencadeou a procura pelo desenvolvimento de potenciais novos produtos e estratégias melhoradas para o controlo de biofilmes. Alguns produtos fitoquímicos, os quais são produzidos por plantas como parte integrante das suas estratégias de defesa contra ataques microbianos, são considerados como potenciais e alternativos agentes antimicrobianos, não sendo tão vulneráveis aos mecanismos de resistência bacterianos como os agentes atualmente em uso.

Desta forma, os efeitos de um fitoquímico (ácido cafeico) e de uma série de derivados alquil éster no controlo do crescimento bacteriano no estado planctónico, bem como na inibição da formação de biofilmes de *Staphylococcus aureus* e *Escherichia coli* foram estudados, com a intenção de analisar a influência do comprimento da cadeia éster na atividade dos compostos. Os resultados obtidos contribuem para o *design* e desenvolvimento de novos e eficazes agentes antimicrobianos.

Os ésteres de ácido cafeico revelaram-se eficazes como agentes antimicrobianos, tanto para o estado planctónico como séssil, para ambas as bactérias. A sua atividade é diretamente dependente da lipofilicidade dos compostos, isto é, do comprimento da cadeia éster, a qual afetou a suscetibilidade bacteriana, as propriedades físico-químicas das bactérias e a sua capacidade de aderir a superfícies de diferentes materiais. Os compostos não apresentaram qualquer inibição aparente do *quorum sensing*. As bactérias Gram-negativas demonstraram ser menos suscetíveis do que as bactérias Gram-positivas à ação destes compostos (tanto no estado planctónico como séssil), apresentando suscetibilidades mais baixas. Estas também apresentam efeitos menos notórios nas suas propriedades físico-químicas e maiores níveis de adesão. Desta forma propõe-se que o mecanismo de ação destes compostos passe pela permeabilização, induzindo alterações nas membranas das bactérias e provocando rotura das mesmas e, consequentemente, morte celular.

No entanto, a influência da cadeia éster não ficou completamente clara. Nenhum padrão óbvio foi observado, à exceção do facto de que compostos com cadeias éster mais longas apresentaram melhores resultados na inibição do crescimento bacteriano e da adesão para as bactérias Gram-positivas, enquanto compostos com cadeias éster médias se revelaram mais eficazes para as bactérias Gram-negativas.

***Palavras-chave:*** ácido cafeico; adesão; alquil ésteres de ácido cafeico; atividade antimicrobiana; biofilme; compostos fenólicos; prevenção da formação de biofilmes; fitoquímicos; *quorum sensing;* resistência bacteriana.

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## GLOSSARY AND NOMENCLATURE

$\gamma^-$ – Electron donor parameter of the Lewis acid-base component of the surface free energy (mJ/m$^2$)

$\gamma^+$ – Electron acceptor parameter of the Lewis acid-base component of the surface free energy (mJ/m$^2$)

$\gamma^{AB}$ – Lewis acid-base component of the surface free energy (mJ/m$^2$)

$\gamma^{LW}$ – Lifshitz-van der Waals component of the surface free energy (mJ/m$^2$)

$\Delta G_{1w2}^{TOT}$ – Total free energy of interaction between two surfaces and water (mJ/m$^2$)

$\Delta G_{sws}$ – Free energy of interaction between two entities of a surface and water (mJ/m$^2$)

$\theta$ – Contact angle (°)

CAF – Caffeic acid

CAFC*n* – Caffeic acid ester with a *n*-carbon alkyl side chain

CFU – Colony-forming units

DLVO – Derjaguin-Landau-Verwey-Overbeek theoretical approach to adhesion

DMSO – Dimethyl sulfoxide

EPS – Extracellular polymeric substances

LB – Luria-Bertani broth

LBA – Luria-Bertani agar

MBC – Minimum Bactericidal Concentration (mM)

MHB – Mueller-Hinton broth

MIC – Minimum Inhibitory Concentration (mM)

O.D.$_{XXX\ nm}$ – Optical density at XXX nm

PCA – Plate count agar

PS – Polystyrene

QS – Quorum sensing

QSI – Quorum sensing inhibition

RMAs – Resistance-modifying agents

SAR – Structure-activity relationship

SS – AISI316 Stainless steel

XDLVO – Extenden Derjaguin-Landau-Verwey-Overbeek theoretical approach to adhesion

# Chapter 1

## 1. WORK OUTLINE

### 1.1. Background and Project Presentation

Antibiotics have proven to be powerful drugs for the control of bacterial growth. However, their extensive and unrestricted use has imposed, over the years, a continued selective pressure upon bacteria by different drugs, which led to the development of antimicrobial resistance and, even, multidrug resistance (Abreu *et al.* 2012; Alekshun & Levy 2007). This is, nowadays, one of the major challenges for the industrial, environmental and biomedical sectors, where bacterial growth (especially in the sessile form of biofilms) causes several economic and public health inconveniences (Budzyńska *et al.* 2011; Gilbert *et al.* 2002). Biofilms constitute an extra source of bacterial resistance, which due to their nature, are much more resistant to the action of current antimicrobial agents than planktonic cells and, thus, harder to eliminate (Borges *et al.* 2014a; Gilbert *et al.* 2002).

For this reason, a demand for the screening and development of potential new active products and new improved alternative strategies for biofilm control began, especially regarding new classes of antimicrobials that may not be as vulnerable as current drugs to bacterial resistance mechanisms (Borges *et al.* 2013; Trentin *et al.* 2011). In this context, there is a new interest in antibacterial products that restrict the ability of bacteria to adhere to surfaces, communicate and, consequently, inhibit biofilm formation (Borges *et al.* 2014a; 2014b). Plant secondary metabolites (phytochemicals) have thus been implicated as potentially active and new alternative antimicrobial agents (Saavedra *et al.* 2010), and because they are derived from natural sources, they are considered to present a green and safe status (Borges *et al.* 2014a; Budzyńska *et al*. 2011).

Hence, this project is based upon the action of a phytochemical (caffeic acid) and some of its alkyl ester derivatives on planktonic growth control and biofilm inhibition of two selected bacteria: a Gram-positive, *S. aureus*, and a Gram-negative, *E. coli*. To my knowledge, it is the first time caffeic acid alkyl esters are tested as antimicrobials and as agents for biofilm formation prevention and microbial adhesion control.

## 1.2. Main Objectives and Contribution

The main objectives of this Dissertation are related to the testing of caffeic acid and a homologous series of caffeic acid alkyl esters with increasingly longer alkyl ester chains on planktonic growth control (i.e. for their antimicrobial activity) and on their ability to affect the bacterial physicochemical properties and act in biofilm prevention and quorum sensing inhibition. Furthermore, it is expected that this work will contribute to a better understanding of the effects of the modification of the alkyl ester side chain length in the proposed antimicrobial and anti-biofilm activities of the compounds tested, by means of a structure-activity relationship.

In this view, the final goal and contribution of this Dissertation would be the rational development of new effective antimicrobial agents, based on molecules of natural origin, particularly from plant sources, which are considered both green and not likely to trigger further bacterial resistance responses, due to their different action mechanisms from conventional antimicrobials.

## 1.3. Thesis Organization

This Master's Dissertation is divided into five different chapters. In this first chapter (*Work Outline*), the scientific challenges that triggered the elaboration of this project are analyzed, along with the main objectives to be achieved and the general framework of the document.

A second chapter, regarding the *Literature Review*, focus on the relevant theoretical topics that allow a more insightful perspective into the issues being studied, as well as presenting an overall view on the main scientific breakthroughs and conclusions that have been described so far in the literature concerning the subject studied or related areas of studies.

In their turn, chapters three and four consist of the actual practical work developed in this project, including a theoretical understanding of the matters at hand, methodologies used and presentation and discussion of the results obtained and of their relevance for the topics. Particularly, in chapter 3, the antimicrobial activity and the mode of action of a phytochemical (caffeic acid) and a series of derivatives (alkyl esters) against two different bacteria are investigated. On the other hand, in chapter 4, the action of the abovementioned compounds is

analyzed regarding their proposed ability to prevent biofilm formation by reducing the extent of the first cell adhesion steps and by hypothetically inhibiting quorum-sensing.

Lastly, a fifth chapter is presented as a compilation of the leading conclusions withdrawn from this project and the subsequent future perspectives for the topic studied.

# Chapter 2

## 2. LITERATURE REVIEW

### 2.1. Multidrug resistant bacteria and biofilms

Control of microbial growth is required in many microbiologically sensitive environments, especially when the conditions for microbial proliferation are favorable (Ferreira *et al.* 2011). In a bacterial growth control context, the aim is to maximize bacterial inactivation or killing during the period in which active levels of chemicals are present and to minimize negative effects (such as re-growth, induction of resistance, cytotoxicity, interaction with non-target microorganisms or adverse effects on the environment), when in the presence of sub-inhibitory concentrations (Simões *et al.* 2009; Saavedra *et al.* 2010). For this purpose, antibacterial agents, routinely divided into biocides and antibiotics, are employed. These have been traditionally regarded as distinct groups of antibacterial agents by the extent of their pharmacological specificity and their degree of mammalian toxicity, being that the ideal antibiotic has a single biochemical target (i.e., a selective toxicity), whereas biocides generally possess several distinct targets, with diverse susceptibilities (i.e., a broad spectrum of usage) (Ferreira *et al.* 2011; Gilbert *et al.* 2002).

To survive in a specific environment, bacteria must respond to several stresses that lead to ill-fated growth conditions, one of which is the exposure to antimicrobial products, such as antibiotics (Simões *et al.* 2009). Antibiotics have proven to be powerful drugs for the control of infectious diseases and remain one of the most significant discoveries in modern medicine (Abreu *et al.* 2012). However, these have also been widely recognized as being used in an indiscriminate and often inappropriate fashion, by constantly being subjected to overuse, underuse and general misuse over the years, which can act as a selective pressure for the development of resistance to these compounds (Abreu *et al.* 2012; Bisht *et al.* 2009; Gilbert *et al.* 2002; Simões *et al.* 2009). The emergence of antibiotic resistance in pathogenic bacteria is a problematic and persistent concern in the medical field, with more than 70% of the bacteria that cause infections in hospitals being resistant to at least one of the most commonly used antibiotic agents (Bisht *et al.* 2009; Elmasri *et al.* 2014). Furthermore, resistant microorganisms are responsible for a decrease in the efficiency of disinfection procedures and, as a result, of

severe contamination in industrial (especially in the food industry), environmental and biomedical settings (Abreu *et al.* 2014; Borges *et al.* 2014a).

The resistance of pathogenic microorganisms to individual antibiotics is, by itself, a serious problem (Abreu *et al.* 2012). Nevertheless, over the years, continued selective pressure by different drugs has resulted in organisms bearing additional kinds of resistance mechanisms that led to multidrug resistance (Alekshun & Levy, 2007). We are now faced with a long list of microorganisms that have found ways to evade different structural classes of drugs and are no longer susceptible to most therapeutic treatments currently used (Alekshun & Levy 2007). The most problematic multidrug resistant strains include human pathogens like vancomycin-resistant enterococci, bacteria producing extended-spectrum β-lactamases (such as *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Acinetobacter baumannii*, *Escherichia coli* and *Klebsiella pneumonia*), vancomycin-resistant and/or methicillin-resistant *Staphylococcus aureus* and extensively drug-resistant *Mycobacterium tuberculosis* (Abreu *et al.* 2012; Alekshun & Levy, 2007).

Antibiotic resistance occurs when bacteria change in any way that might reduce or even eliminate the effectiveness of antimicrobial agents (Bisht *et al.* 2009). In fact, microbial susceptibility is a continuum that reflects phenotypic and genotypic variations in natural microbial populations (Abreu *et al.* 2012). Therefore, bacterial resistance may be attained through intrinsic or acquired mechanisms (i.e., bacteria may acquire resistance by *de novo* mutation or *via* the acquisition of resistance genes from other microorganisms). Intrinsic resistance to antimicrobials is a natural property of the bacteria and its mechanisms are those specified by naturally occurring genes found on the host's chromosome (even if pre-existing but previously unexpressed). These are frequently associated with cellular impermeability conveyed by the outer layers and thus, limiting the uptake of antimicrobial products. On the other hand, acquisition of new genetic material by antimicrobial susceptible bacteria from resistant ones may occur through gene transfer, either by conjugation (via plasmids and transposons), by transformation (via bacteriophages) or by transduction. The acquired resistance genes become a relatively stable part of the bacterial genome and additional resistance elements may join those already prevailing, extending the multidrug resistance phenotype. The acquired genes may enable the bacteria to produce enzymes that inactivate the antibacterial product, to modify the target site, to produce alternative metabolic pathways that bypass the antimicrobial action or to express efflux mechanisms that prevent the antimicrobial from reaching its intercellular target (Abreu *et al.* 2012; Alekshun & Levy, 2007; Simões *et al.* 2009).

### 2.1.1. Biofilms as a source of additional resistance

Microorganisms tendentiously grow and survive in a sessile form, i.e, as multicellular surface-attached communities called biofilms (Gilbert *et al.* 2002; Plyuta *et al.* 2013), being this the prevalent mode of microbial life in natural habitats, industrial processes and even in many infections (Borges *et al*. 2014a). Hence, biofilms are structured and functional consortiums of microbial cells embedded in a complex slimy matrix of extracellular polymeric substances (EPS), irreversibly attached to a surface (either biotic or abiotic) (Borges *et al*. 2012; 2014a; Gilbert *et al.* 2002; Neyret *et al.* 2014).

Biofilm formation is a dynamic and sequential process that, overall, includes the transport of microorganisms to surfaces, initial reversible/irreversible adhesion, cell-cell communication, microcolony formation, EPS production and biofilm maturation (Madigan *et al.* 2009; Simões *et al.*2010a).

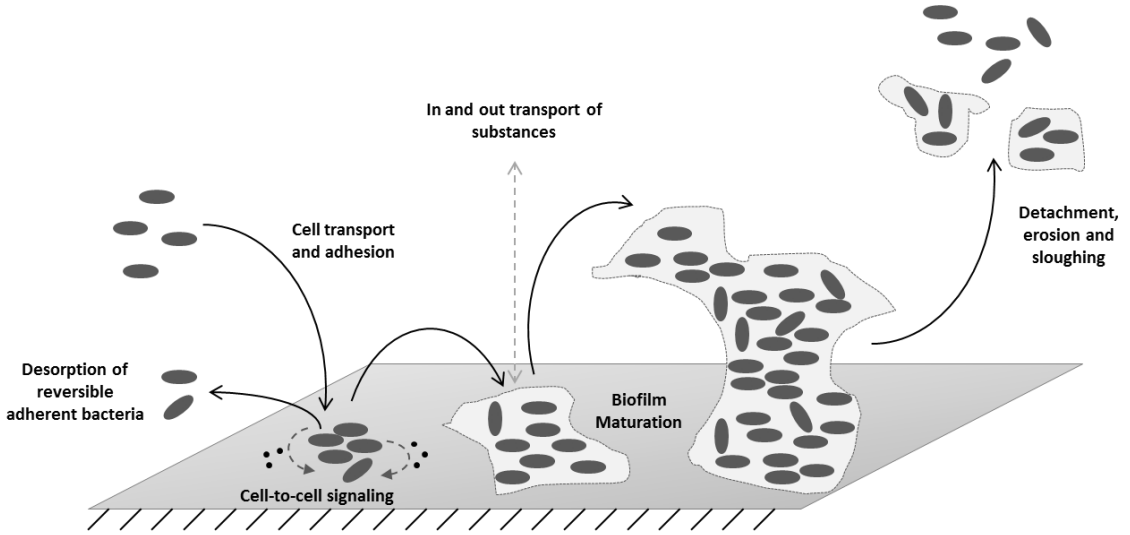



**Fig. 1 - Representation of the process leading to biofilm formation.**

The process leading to the formation of biofilms (Fig. 1) is believed to start with a pre-conditioning of the adhesion surface, either by macromolecules present in the bulk environment or intentionally coated onto the surface. Then, planktonic bacteria from the surrounding medium are transported to the surface and adhere by either a nonspecific or a specific binding reaction, meaning that, if the bond is weak (reversible), bacteria may desorb from the surface into the liquid, a phenomenon that takes place simultaneously with irreversible adsorption of bacterial cells to the surface. Once bacteria are firmly attached to the surface, they start cell-to-cell signal communications, by producing specific signaling

molecules that take a part in growth control, replication, plasmid conjugation and secretion of various virulence factors and exopolymers. At this stage, convective and diffusive transport of substrates to and within the biofilm occurs, alongside with substrate metabolism, excretion of metabolic products, cell growth, replication and extensive EPS production (Breyers & Ratner 2004), comprising a maturation stage of the biofilm. EPS are responsible for biofilm cohesion (i.e., binding cells and other particulate materials together) and adhesion to the surface. The EPS matrix is generally composed of polysaccharides, proteins, nucleic acids, lipids and phospholipids, even though proteins and polysaccharides by themselves account for 75-89% of the biofilms EPS composition (Simões *et al.*2010b). Ultimately, the biofilm may experience removal of sections by detachment, erosion or sloughing (Breyers & Ratner 2004).

Biofilms are the leading example of physiological adaptation, being one of the most important sources of bacterial resistance to antimicrobial products (Borges *et al.* 2014a; Madigan *et al.* 2009). In fact, our ability to eradicate biofilms is substantially lower than that for equivalent populations of planktonic (dispersed) bacteria, which typically present susceptibilities 10 to 1000 times higher than biofilm embedded cells (Gilbert *et al.* 2002; Neyret *et al.* 2014).

However, the conventional mechanisms of antibiotic resistance found in planktonic cells (efflux pumps, modifying enzymes and target mutations) do not seem to be responsible for the protection of bacteria in a biofilm and might rather be credited to several mechanisms that can act together (Borges *et al.* 2012). One possible example is the poor penetration or inactivation of antimicrobials in the EPS matrix, which acts as a physical barrier in which diffusive transport prevails over convective transport, thus limiting antimicrobial penetration and preventing them from reaching their target microorganisms within the biofilm. To this purpose, a number of features are implicated, such as the binding capacity of the polymeric matrix towards the antimicrobial agent, the distribution of biomass and local hydrodynamics, the rate of turnover of the microcolony relative to the molecule diffusion rate and the production and retention of extracellular products. Other examples of biofilm resistance mechanisms comprise altered (dormant) bacterial metabolic state, presence of persister cells, resistance induced by the antimicrobial itself following the use of sub-lethal concentrations and the up-regulation of efflux pumps or potential of damaged bacterial cells to undergo apoptosis or programmed cell death (feeding the community and allowing the remaining cells to survive and proliferate in the post-treatment phase) (Borges *et al.* 2012; 2014a; Gilbert *et al.* 2002).

### 2.1.2. Need for new antimicrobial approaches

The increased bacterial resistance against the classical antimicrobial treatments leads to pathogen biofilm elimination being a major challenge with serious economic and health repercussions. Biofilms have been implicated in medicine, as the cause of many chronic and biomaterial-associated infections, and in the industrial and environmental sceneries, as the cause for biofouling, biocorrosion and biodeterioration, especially in food processing and water distribution systems (Budzyńska *et al.* 2011; Gilbert *et al.* 2002; Madigan *et al.* 2009). The current concerns over bacterial multi-resistance, along with biofilm resistance to, not only the conventional treatments, but also the newest generation of drugs, and the toxicity of some of the current employed antimicrobials, has led to a demand for the screening and development of potential new active products and new improved alternative strategies for biofilm control, especially regarding new classes of antimicrobials that may not be as vulnerable as current drugs to bacterial resistance mechanisms (Borges *et al.* 2013; Trentin *et al.* 2011).

The development of the so-called resistance-modifying agents (RMAs) represents an attractive strategy to mitigate the spread of bacterial drug resistance, since it could facilitate the recycling of well-established antibiotics that are often cheaper and less toxic than new candidate antimicrobials (Borges *et al.* 2013). The proposed RMAs are capable of partly or completely suppressing bacterial resistance mechanisms (Budzyńska *et al.* 2011) by combining new or established antimicrobials with the currently used antibiotics, extending the latest useful life due to a synergism that may cause improved solubility or resorption rate, enhanced bioavailability and ability to modify or even inhibit bacterial resistance mechanisms (Abreu *et al.* 2012).

The best option so far is to discover and develop new anti-biofilm drugs, i.e., biofilm inhibitors, whose aim, unlike antibiotics, is not to inhibit cell growth, which may thus reduce the risk of drug resistance (Lee *et al.* 2014). In this context, there is a new interest in antibacterial products that restrict the ability of bacteria to adhere, communicate and, consequently, form complex biofilms or, in other words, prevent the development of biofilms. In fact, to inhibit the growth of an already established biofilm (i.e., biofilm control) is far more difficult to achieve than to impair or inhibit the initial stages of biofilm formation, namely bacterial adhesion (i.e., biofilm prevention) (Borges *et al.* 2014a; 2014b). This new approach maintains the cells in a planktonic state, switching off the virulence expression typical of biofilms and making the microorganisms more susceptible to the action of other antimicrobials

(Trentin *et al.* 2011). In this view, understanding the relationship between adhesion and biofilm formation is crucial.

Bacterial adhesion is a complex process that is affected by many factors such as the biological properties of the bacteria (presence of fimbriae or flagella, production of EPS, etc.), the physicochemical characteristics of the bacteria (hydrophobicity, surface charge, etc.), the material's surface properties (chemical composition, surface charge, hydrophobicity, roughness or texture) and environmental factors (temperature, pH, time of exposure, bacterial concentration, presence of chemical or antimicrobial treatment and flow conditions) (Simões *et al.*2010a). During biofilm formation, adhesion occurs in two different phases: in a first stage planktonic bacteria move or are moved to a surface by physical forces, such as Brownian motion, van der Waals attraction forces, gravitational forces, surface electrostatic charge or hydrophobic interactions, depending on the distance between them; on a second phase, molecular relations between bacterial surface polymeric structures and substratum surfaces become increasingly more significant (Simões *et al.*2010a). As for now, considerable resources have been directed towards technologies designed to inhibit microbial attachment. Prospects have included surface material coatings that prevent adhesion, responsive surfaces that phase change upon command or controlled orientation of surface-tethered adhesion molecules (Breyers & Ratner 2004). However, nowadays, the most popular approaches to prevent bacterial adhesion are through quorum sensing (QS) and motility inhibition, which are both important steps of biofilm formation and development (Simões *et al.*2010b).

Bacterial motility and, in particular, swimming and swarming are dependent on flagella and contribute to cell adhesion to biotic and abiotic surfaces. Swarming has long been recognized to be important for both the initial interactions with surfaces and for the movement along these and, therefore, for the early steps of biofilm formation (Lee *et al.* 2014; Wojnicz *et al.* 2012). Biofilm formation is invariably preceded by attachment mediated by the abovementioned flagellar motilities, while later stages are due to twitching motility (implicated in cell recruitment from adjacent monolayers and cell aggregate formation), which is related to type IV pili (Borges *et al.* 2012; Kumar *et al.* 2013). All considered, the importance of flagella in biofilm formation makes it an attractive target for the development of alternative biofilm control strategies (Vikram *et al.* 2013).

On the other hand, QS can regulate several bacterial activities, such as bioluminescence, virulence factor expression, swarming motility, sporulation and biofilm production. As a matter of fact, QS is an intercellular signaling system that allows bacteria to monitor their population density and, accordingly, control a variety of physiological processes by releasing and receiving small signaling molecules named autoinducers (Nazzaro *et al.* 2013;

Zhang *et al.* 2014). This cell-to-cell communication mechanism influences both initiation and maturation of bacterial biofilms and has been showed to be an important regulatory mechanism in biofilm formation/differentiation, rendering the interference with QS systems as a highly attractive and promising target to tackle biofilm control. The discovery that several products with antibiofilm activity were QS inhibitors is proof of the importance of this signaling system in biofilm prevention and began a quest for new agents that might act as nontoxic inhibitors of QS, able to control bacterial adhesion without encouraging the appearance of resistant bacterial strains (Borges *et al.* 2014b; Nazzaro *et al.* 2013; Zhang *et al.* 2014).

## 2.2. Phytochemicals

Bacteria and fungi are the leading sources of the currently available antibiotics (Cowan 1999). However, due to the already mentioned issues of toxicity and resistance, and in the quest for active products for biofilm control, an interest in products from other sources arose, particularly phytochemicals, plant secondary metabolites (Abreu *et al.* 2012). In fact, plants produce an enormous array of secondary metabolites, a number of which are commonly believed to be involved in chemical strategies to protect themselves against pathogen microbial attacks from an extensive range of microorganisms including fungi, yeasts and bacteria (Saavedra *et al.* 2010; Tegos *et al.* 2002). In order to be classified as antimicrobials, phytochemical products must normally present a minimum inhibitory concentration (MIC) in the range 100-1000 µg/mL in *in vitro* susceptibility tests (Tegos *et al.* 2002).

Phytochemicals are commonly classified either as phytoanticipins (molecules that are present constitutively in an inactive form, as is the case of glucosides, cyanogenic glucosides, and saponin glycosides, and are a part of the plant passive resistance mechanisms) or as phytoalexins (molecules that are produced *de novo* in response to tissue disruption and pathogen attack) (Abreu *et al.* 2012; Gibbons 2004; Vermerris & Nicholson 2008). However, at a chemical level, there is no boundary between phytoalexins and phytoanticipins, and in one plant species a certain chemical can function as a phytoalexin, whereas it has the function of a phytoanticipin in another species (Cowan 1999). Nevertheless, phytoalexins are typically low weight molecules that may include chemical classes such as polyphenols, alkaloids, glycosteroids, flavonoids, isoflavonoids, various sulfur products, di- and tri-terpenes, polyketides, lactones and naphthoquinones, all of them being common classes of phytochemicals (Tegos *et al.* 2002). Apart from their potential function against pathogen

invaders, it is believed that phytochemicals play other functions in plant physiology and functionality, as attraction pigments for pollination purposes or as protection mechanisms against UV damage or oxidative stress (Simões *et al.* 2009).

One of the major advantages of using phytochemicals as antimicrobial agents is the fact that, in general, their mechanisms of action differ significantly from the antibiotic ones (Saavedra *et al.* 2010), allowing for the discovery of new alternative and effective antimicrobial compounds. These, not only have broad-spectrum microbicidal and antibiofilm activities (posing a low risk for the development of resistance), but are also derived from natural sources, present a green and safe status (Borges *et al.* 2014a; Budzyńska *et al*. 2011).

Yet, pharmaceutical companies still prefer to pursue microbial derived products, of which there are many first class drug examples which can be readily fermented with few re-supply issues (Gibbons 2004). The chemical complexity of plant extracts, often undocumented toxicity, poor water solubility and the lack of standardization may be responsible for the apparent lack of industrial interest in phytochemicals. Other additional limitations are concerned with the access and supply, the inherent slowness of working with natural products and the costs of collection, extraction and isolation (Abreu *et al.* 2012).

Polyphenols are the most important and abundant group of phytochemicals (Saavedra *et al*. 2010). These compounds can be found in diverse dietary products like vegetables, fruits, chocolates and beverages (as coffee, tea or wine) and are involved in various functions in plants, such as growth and development regulation, interaction between plants, pathogenic (defense) and symbiotic microorganisms or ultraviolet radiation defense (Borges *et al.* 2014b; Manach *et al.* 2004; Plyuta *et al.* 2013). They exhibit a wide variety of biological effects including antimicrobial, antioxidant, anti-inflammatory, antiallergic, hepato- and cardioprotective, antithrombotic, antiviral, anticarcinogenic and vasodilatory actions (Merkl *et al.* 2010; Saavedra *et al.* 2010). The possible mechanisms believed to be responsible for their antimicrobial activity might include destabilization and permeabilization of cytoplasmatic membranes, efflux pump inhibition, bacterial type II fatty acid synthesis inhibition, enzyme inhibition by the oxidized products (possibly through reaction with sulfhydryl groups or through more non-specific interactions with proteins, causing a disruption of energy production) and nucleic acids synthesis inhibition of both Gram-negative and Gram-positive bacteria (Borges *et al.* 2013; Saavedra *et al.* 2010). On the other hand, regarding their antioxidant effects, polyphenols are known to delay or prevent oxidative damage by reactive oxygen species (ROS), being their abilities strongly associated with hydrogen donating and/or electron donating ability to ROS (Lee *et al.* 2014).

The polyphenol class includes simple phenolics and their derivatives: phenolic acids, such as cinnamic acids and derivatives, coumarins and derivatives, flavonoids, stilbenes, lignans and tannins. The common denominator between them is the existence of a polyphenol structure, i.e., hydroxyl groups on aromatic (benzene) rings, being classified into the different groups as a function of the number of phenol rings that they contain and of the structural elements that bind these rings to one another. Additionally, polyphenols may be associated with various carbohydrates and organic acids and with one another (Cowan 1999; Manach *et al.* 2004; Vermerris & Nicholson 2008). Moreover, phenolic compounds are usually found in plants as esters or glycosides rather than as free compounds (Vermerris & Nicholson 2008).

In particular, in the phenolic acid sub-categories, one can distinguish between two types of molecules: hydroxybenzoic acids and hydroxycinnamic acids (Manach *et al.* 2004). The numbers and positions of the hydroxyl groups on the aromatic ring causes significant changes in the properties of these molecules. In fact, the site(s) and number of hydroxyl groups on the aromatic ring are allegedly related to their relative toxicity to microorganisms, with increasing hydroxylation resulting in increased toxicity (Borges *et al.* 2012). Hydroxycinnamic acids have a C6-C3 skeleton (Vermerris & Nicholson 2008; Lee *et al.* 2014). Depending on the group substitution of the aromatic ring, different types of hydroxycinnamic acids can be found, being the most common the cinnamic, the *p*-coumaric, the ferulic, the caffeic and the sinaptic acids. However, as previously stated, these acids are rarely found in their free form (Vermerris & Nicholson 2008; Manach et al. 2004).

In plants, hydroxycinnamic acids are intermediates of the general phenylpropanoid pathway, the shikimate pathway and the lignin specific pathway (Barber *et al.* 2000; Sharma 2011). In the past, the phenylpropanoid pathway included the biosynthesis of the hydroxycinnamic acids: ferulic acid, caffeic acid and sinaptic acid from *p*-coumaric acid. However, nowadays, it is known that hydroxylation and methoxylation reactions do not occur at the level of the acid, but instead at more reduced forms. So, it appears that the hydroxycinnamic acids are, at least in part, synthesized through the oxidation of aldehydes, rather than via ring substitutions of the free acids. Nevertheless, this is not the exclusive route towards the substitution pattern of the phenyl ring or at least not in all plant species (Vermerris & Nicholson 2008).

More specifically, caffeic acid (3,4-dihydroxycinnamic acid), both free and esterified (Fig. 2), is generally the most abundant phenolic acid (representing between 75% and 100% of the total hydroxycinnamic acid content of most fruit) (Manach *et al*. 2004; Yu *et al.* 2013; Sharma 2011).

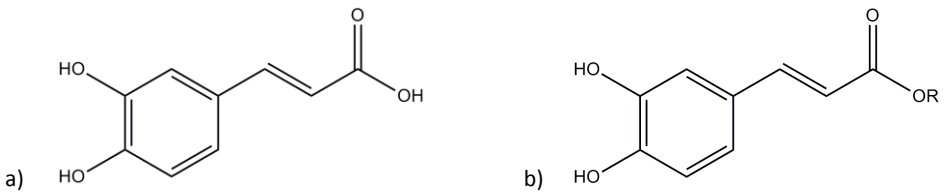



**Fig. 2 – Chemical structure of a) caffeic acid and b) caffeic acid ester (R = alkyl chain).**

Caffeic acid presents an excellent antibacterial and antioxidant activity (Manach *et al*. 2004; Yu *et al.* 2013; Sharma 2011). Furthermore, caffeic acid esters are natural components of propolis and their molecular structure contains a catechol group with an α,β-unsaturated carboxylic acid chain, which can efficiently interact with reactive oxygen species, justifying some of its activity (Uwai *et al.* 2008; Yu *et al.* 2013).

### 2.2.1. Phytochemicals in the control of planktonic and sessile bacteria

Phytochemicals have recently been receiving a lot of attention concerning their antimicrobial activity, as proven by the increasing amount of studies on this topic in the last few years. Furthermore, not only are phytochemicals tested for their potential antimicrobial activity and mode of action against several kinds of bacteria in the planktonic state, but also for their potential ability to act as biofilm formation inhibitors or even biofilm controllers.

Susceptibilities of different kinds of bacteria to phytochemicals, especially phenolics, were studied by several authors (Table 1) and, generally, results demonstrated that all compounds had antimicrobial activities against the microorganisms tested. It is interesting to notice that several studies state different susceptibilities to the phytochemicals tested for Gram-positive and Gram-negative bacteria. However, there seems to be some controversy amongst authors relating to this topic (Saavedra *et al.* 2010). Some stated that Gram-positive bacteria are more susceptible to the action of the compounds than Gram-negative ones, while others stated the exact opposite. Merkl *et al.* (2010) observed that the sensitivity of Gram-positive bacteria to the hydroxycinnamic acid esters tested was higher than in Gram-negative bacteria. Other studies also proposed that phenolics were inhibitory to Gram-positive bacteria but not to Gram-negative bacteria (Himejima & Kubo 19991; Saavedra *et al.* 2010). Borges *et al.* (2012; 2013) stated that Gram-positive bacteria were less susceptible to the phenolic acids tested than Gram-negative bacteria. These results might propose that antimicrobial action of phytochemicals is not Gram-specific.

**Table 1 - Examples of phytochemicals with proven antimicrobial activity.**

| Phytochemical compound | Bacteria | Reference |
|---|---|---|
| Alkyl gallates | *Xanthomonas citri* subsp. *citri* | Silva *et al*. (2013) |
| Alkyl gallates | *Salmonella choleraesius* | Kubo *et al.* (2002) |
| (2E)-alkenals | *S. choleraesius* | Kubo *et al.* (2004a) |
| Alkyl gallates | *Bacillus subtilis*; *Staphylococcus aureus* | Kubo *et al.* (2004b) |
| Plant extract (containing anacardic acids, cardols and cardanols) | *B. subtilis; S. aureus; Escherichia coli; Pseudomonas aeruginosa* | Himejima & Kubo (1991) |
| Ferulic acid; gallic acid | *E. coli; S. aureus; P. aeruginosa; Listeria monocytogenes* | Borges *et al.* (2013) |
| Tyrosol; gallic acid; caffeic acid; ferulic acid; chlorogenic acid;oleuropein glucoside; epicatechin; phloridzin; allylisothiocyanate; benzylisothiocyanate | *E. coli; S. aureus; P. aeruginosa; L. monocytogenes* | Saavedra *et al.* (2010) |
| 3,4-hydroxybenzoic acid alkyl esters | *E. coli; Bacillus cereus; L. monocytogenes* | Merkl *et al.* (2010) |
| *p*-coumaric acid; caffeic acid; ferulic acid; coniferaldehyde; *p*-coumaraldehyde; sinapaldehyde | *B. subtilis; E. coli; Pseudomonas syringe* | Barber *et al.* (2000) |
| Taxodione; 7-(2-oxohexyl)-taxodione | *S. aureus* | Kuźma *et al.* (2012) |

It was also established that the antimicrobial effect of hydroxycinnamic acid derivatives increases with the increasing length of the ester alkyl chain (Merkl *et al.* 2010; Uwai *et al.* 2008; Nihei *et al.* 2004). For instance, Merkl *et al.* (2010) proved that the toxicity of hydroxycinnamic acid esters increases with the increasing size of the alkyl chain, as shown by the decrease in MIC values of caffeic and ferulic acids and their correspondent alkyl esters against *Escherichia coli* and *Bacillus cereus*. However, other authors, such as Silva *et al.* (2013) and Kubo *et al.* (2002; 2004a; 2004b) demonstrated that the antimicrobial activity of alkyl esters of phenolic acids was a parabolic function of their lipophilicity, i.e., was related to the length of the hydrophobic alkyl side chain, with maximum antimicrobial activity being held by C8 to C12 alkyl esters. This means that the biological behavior of these alkyl esters could be correlated with the cutoff phenomenon, which has frequently been attributed to their

amphiphilic properties, which are structurally associated with the presence of two groups: phenolic groups (hydrophilic moiety) and an alkyl side chain (lipophilic tail). Therefore, the length of the carbon side chain must determine the release of the bioactive portion of these compounds (the phenolic acid) inside the cells (Silva *et al.* 2013).

Regarding the control of biofilms, phytochemicals have been employed and studied for a variety of purposes, mainly for inhibition of bacterial adhesion through interference of these compounds in QS systems, bacterial motility or bacterial surface properties (i.e. membrane destabilization) (Table 2).

Apart from the phytochemicals described in Table 2, several others, such as flavanones, flavonoids, flavonols, furanones, hydroxycinnamic acids, rutin, epicatechin, gamma aminobutyric acid (GABA), pyrogallol, curcumin, cunnamaldehyde, furocoumarins, ursolic acid, rosmarinic acid, salycilic acid, epigallocatechin gallate, ellagic acid, tannic acid, urolithin A/B, chlorogenic acid, vanillic acid or proanthocyanidins have also been described as phytochemicals with proven anti-quorum sensing activity, for either or both Gram-negative and Gram-positive bacteria (Nazzaro *et al.* 2013).

Special emphasis must be given to phenolic compounds, which have been definitely receiving some attention concerning their ability to prevent biofilm formation, either by altering motility or surface properties of bacteria (hydrophobicity) or by inhibiting QS. Interestingly, some authors describe no quorum sensing inhibition (QSI) activity for phenolic acids (Borges *et al.* 2014b).

Overall, the results presented in the literature regarding *in vitro* studies of the use of phytochemicals as antimicrobials and biofilm inhibitors are very enthusiastic, especially taking into account the green and natural character of these molecules (many of which are found in human dietary products, thus likely rendering a safe cytotoxic status) and their diverse and broad spectrum modes of action, which would not further inflate the bacterial multi-resistance problematic (Borges *et al.* 2013; Manach *et al.* 2004). The therapeutic potential of phytochemical products as alternatives or even potentiators of classic antibiotics is consequently established (Abreu *et al.* 2012). An interesting consideration to be made is whether or not antimicrobial phytochemical compounds are naturally present in plants in enough concentration to cease potential microbial pathogen attacks. Unluckily, there is a lack of reliable and complete information regarding their levels in plants (Barber *et al.* 2000).

Nevertheless, besides the potential practical utility of phytochemicals as antimicrobials and RMAs, the bioprospecting results and the vast knowledge of phytochemicals diversity and functionality provides new concepts with potential application for a joint action between combinatorial chemistry and computational design for the swift discovery and synthesis of

new and more effective antibacterial products. In fact, some of these compounds may provide important structural scaffolds for the rational and systematic development of new drugs, based on modifications of a known antimicrobial compound (Borges *et al*. 2013; Kubo *et al.* 2002; Madigan *et al.* 2009; Saavedra *et al.* 2010; Simões *et al.* 2008).

In this context, structure-activity relationship studies (i.e. studies in which the relationship between the molecular structure of compounds and their biological activity) may be of use, by providing a more insightful perspective into the antimicrobial modes of action of some compounds, allowing for the screening of new activity-influencing features, and thus for the designing of drugs with the greatest potency and the least side effects.

**Table 2 - Examples of phytochemical effects in biofilm formation.**

| Phytochemical compound | Bacteria | Biofilm effect | Reference |
|---|---|---|---|
| Phenyl isothiocyanate (synthetic isothiocyanate - glucosinolate) | *S. aureus; E.coli* | Prevention of biofilm formation by alteration of surface properties and motility; biofilm removal was also achieved | Abreu *et al*. (2014) |
| Resveratrol (polyphenol) | *Vibrio cholerae* | Prevention of biofilm formation by alteration of surface properties (altered binding receptors) | Augustine *et al.* (2014) |
| Allylisothiocyanate; 2-phenylethylisothiocyanate (isothiocyanates - glucosinolates) | *E.coli; P. aeruginosa; S. aureus; L. monocytogenes* | Prevention of biofilm formation by alteration of viability, motility and surface properties; biofilm removal was not observed | Borges *et al* (2014a) |
| Allylisothiocyanate; benzylisothiocyanate; 2-phenylethylisothiocyanate (isothiocyanates - glucosinolates); gallic acid; ferulic acid; caffeic acid; phloridzin; epicathechin; oleuropein glucoside (polyphenols) | *Chromobacterium violaceum* | Prevention of biofilm formation by QSI → QSI was only detected for the isothiocyanates; no QSI was observed for the phenolics | Borges *et al* (2014b) |
| Ferulic acid; gallic acid (polyphenols) | *E. coli; P. aeruginosa; S. aureus; L. monocytogenes* | Prevention of biofilm formation by alteration of motility → Gram-negative bacteria biofilm formation was more affected by phenolic acids than Gram-positive | Borges *et al.* (2012) |
| Linalool; linalyl acetate; alpha-terpineol; terpinen-4-ol (from essential oils) | *S. aureus; E.coli* | Biofilm removal → 90% reduction in biomass metabolic activity was achieved with higher susceptibilities for the Gram-negative bacteria biofilms | Buzyńska *et al.* (2011) |
| Sesquiterpenes | *S. aureus* | Prevention of biofilm formation | Elmasri *et al.* (2014) |

| | | | |
|---|---|---|---|
| Zingerone (polyphenol) | *P. aeruginosa* | Prevention of biofilm formation by alteration of motility; biofilm removal was ineffective, unless when used in synergism with an antibiotic | Kumar *et al.* (2013) |
| 7-(2-oxohexyl)-taxodione (terpenes group) | *S. aureus* | Prevention of biofilm formation; biofilm partial removal was also achieved | Kuźma *et al*. (2012) |
| Ginkgolic acids C15:1 and C17:1 | *S. aureus; E. coli* | Prevention of biofilm formation by alteration of motility → Gram-positive bacteria biofilms were less susceptible than Gram-negative ones | Lee *et al.* (2014) |
| Thymol; carvacrol; eugenol (phenolics) | multimicrobial biofilm | Biofilm removal → 4-5 log reductions were observed, especially when phenolics were used in synergism between them | Neyret *et al.* (2014) |
| 4-hydroxybenzoic acid, vanillin, gallic acid; ferulic acid, sinaptic acid, cinnamic acid, epicathechin; chlorogenic acid (phenolics) | *P. aeruginosa* | Prevention of biofilm formation by alteration of motility and QSI → no motility alterations were verified and QS was promoted at sub-MIC, which lead to an increase in biofilm formation (at higher concentrations biofilm formation was, however, prevented) | Plyuta *et al.* (2013) |
| β-sitosterol glucoside | *E. coli* | Prevention of biofilm formation by alteration of motility and QSI | Vikram *et al.* (2013) |
| Paeonidin-3-O-galactoside, paeonidin-3-O-arabinoside, cyanidin-3-O-galactoside and cyanidin-3-O-glucoside (anthocyanins) | *E. coli* | Prevention of biofilm formation by alteration of motility and surface properties | Wojnicz *et al*. (2012) |
| Phenolic extract containing: gallic acid, catechin, epicatechin, epigallocatechin gallate, benzoic acid, quercetin, tannins and kaempferol | *C. violaceum; E. coli; P. aeruginosa* | Prevention of biofilm formation by QSI | Zhang *et al.* (2014) |

# Chapter 3

## 3. ANTIMICROBIAL ACTION OF CAFFEIC ACID ALKYL ESTERS IN *ESCHERICHIA COLI* AND *STAPHYLOCOCCUS AUREUS*

### 3.1. Introduction

Apart from the widespread bacterial resistance, susceptibility to antimicrobials intrinsically depends on the type of bacteria, with different cellular permeabilities imparted by bacterial outer layers in Gram-negative and Gram-positive bacteria being frequently associated with different susceptibility to antimicrobial products. In fact, the Gram-negative cell wall is a complex multilayered structure whereas Gram-positive cell walls are typically much thicker and consist almost entirely of a single type of molecule (Madigan *et al.* 2009). Gram-positive and Gram-negative bacteria do share the inclusion of peptidoglycan in their cell walls. Peptidoglycan is a polymer composed of N-acetyl glucosamine, N-acetyl muramic acid and amino acids. In Gram-positive bacteria, peptidoglycan accounts for nearly half of the entire cell and most of the cell wall (about 90%), whilst In Gram-negative bacteria, peptidoglycan only accounts for about 10% of the cell wall (Madigan *et al.* 2009; Maisuria 2009).

Gram-negative bacteria, such as *E. coli*, surround themselves in a double membrane, where the inner or cytoplasmatic membrane is mainly composed of phospholipids and the outer membrane is a second lip bilayer containing lipopolysaccharides (LPS). The surface of the cell wall of Gram-negative bacteria is considered hydrophilic because of the LPS, which together with the outer bilayer membrane protect Gram-negative bacteria against membrane destabilizers that weaken the inner cytoplasmic membrane. The outer membrane is nevertheless a permeable barrier to hydrophilic low-molecular-weight substances due to the existence of narrow porin channels. In that view, the outer membrane acts as an efficient permeability barrier against macromolecules and hydrophobic (or even amphipathic) substances. Moreover, the low fluidity of the LPS leaflet slows down the inward diffusion of lipophilic products (Kubo et al. 2004a; Madigan *et al.* 2009; Maisuria 2009; Simões *et al.* 2009).

By contrast, Gram-positive bacteria, such as *S. aureus*, present a single membrane surrounding the cell, mainly composed of peptidoglycan, being two-fold to eight-fold larger than the cell wall of Gram-negative bacteria and not containing LPS (Madigan *et al.* 2009;

Maisuria 2009). As a result, Gram-negative bacteria are generally less susceptible to antimicrobial action, as their cell walls present a more significant barrier to surpass (Kubo *et al.* 2002; Simões *et al.* 2009; Tegos *et al.* 2002).

In the sense that cell walls differ in their hydrophilicity/lipophilicity, surface hydrophobicity is an important factor to be taken into account when studying the antimicrobial action of a molecule upon bacterial cells. In fact, alterations in the bacterial cells surface physicochemical properties upon antimicrobial treatment may be a useful indicator of its mode of action and potential as an inhibitor of biofilm formation, since hydrophobicity has been considered the most important short-range interaction in bacterial adhesion (Simões *et al.* 2007).

Other surface properties, such as surface charge, may also contribute to analyze the interaction between an antimicrobial and the bacterial surface. The surface charge of cells is frequently determined based on their zeta potential, which is calculated from the electrophoretic motility of cells in the presence of an electrical field, under defined pH and salt concentrations (Borges *et al.* 2013). When applying an electric field across a bacterial suspension, bacteria with non-zero zeta potential migrate towards the electrode of the opposite charge, with a velocity proportional to the magnitude of their zeta potential (Ferreira *et al.* 2011). Bacterial cells normally present a negative surface charge, due to the presence of anionic groups in their membranes, such as carboxyl and phosphate groups (Borges *et al.* 2013).

In particular, for the series of alkyl caffeates used in this study, it is relevant to note that these molecules possess a head-and-tail structure, similar to an amphiphile. Amphiphiles are molecules with two parts: they have a water-loving part (hydrophilic) and a water-hating part (hydrophobic), usually a long-chain alkyl group (Maisuria 2009). In this case, the amphiphile of caffeic acid esters is associated with the presence of the two groups: the phenolic groups (hydrophilic moiety) and the alkyl side chain (lipophilic tail) (Silva *et al*. 2013). This is actually the case for several phenolic acid esters, which, as already mentioned, display a parabolic antimicrobial activity as function of their lipophilicity. This biological behavior could thereby be correlated with the cutoff phenomenon, very distinctive of amphiphilic substances: antimicrobial properties of amphiphiles tend to increase with increasing alkyl chain length; however, increasing their length decreases the solubility of amphiphiles in aqueous media, lowering their biological activity levels. This phenomenon is known as the cutoff effect due to insolubility (Maisuria 2009) and is believed to explain, in part, the parabolic antimicrobial activity of such compounds.

All of the caffeic acid alkyl esters in the homologous series of compounds studied present the same hydrophilic portion, but increasingly long alkyl side chains, thus distinguishing the role of the hydrophobic alkyl portion in their antimicrobial action and allowing to perform a SAR study (Kubo *et al.* 2002).

Therefore, the main objectives of this work were to assess the antimicrobial activity and the mode of action of caffeic acid alkyl esters against Gram-positive and Gram-negative bacteria.

## 3.2. Materials and Methods

### 3.2.1. Test microorganisms

The Gram-negative bacterium *Escherichia coli* CECT 434 and the Gram-positive bacterium *Staphylococcus aureus* CECT 976 were used in this study. These microorganisms have previously been employed as model microorganisms for antimicrobial tests with phytochemical products (Saavedra *et al.* 2010; Borges *et al.* 2012). The bacteria were cryopreserved at -80$^{o}$C, in a mixture of Mueller-Hinton broth (MHB, Merck) and 30% (v/v) glycerol, and subcultured in Plate Count Agar (PCA, Merck) at 30$^{o}$C for 24 h, before testing.

### 3.2.2. Caffeic acid and alkyl ester derivatives

The compounds tested included caffeic acid and some of its ester derivatives. Caffeic acid (CAF) was obtained from Sigma-Aldrich, while its esters were kindly synthetized and provided by Prof. Fernanda Borges and her team, from Faculdade de Ciências da Universidade do Porto. The collection includes C2, C4, C6, C8 and C10 alkyl esters of caffeic acid (CAFC2, CAFC4, CAFC6, CAFC8 and CAFC10, respectively) (Appendix A).

Stock solutions of all tested compounds were prepared in dimethyl sulfoxide (DMSO, Fisher), under sterile conditions, and kept in the dark, at room temperature, for a maximum of two weeks. Serial dilutions of the stock solutions were prepared in DMSO, whenever needed.

### 3.2.3. Minimum inhibitory concentration (MIC) determination

The antimicrobial activity of the tested compounds was measured by means of their minimum inhibitory concentrations.

For each bacteria, overnight cultures were prepared in 250 mL sterile flasks containing around 50 mL of previously autoclaved (at 121ºC for 15 min) MHB, and incubated at 30ºC, under agitation at 120 rpm (in an incubation-shaking cabinet Sartorius Certomat® BS-1), after inoculation. The optical density at 600 nm ($OD_{600\ nm}$) of the overnight cultures was set to 0.1.

MIC values were determined in sterile 96-well flat-bottomed polystyrene tissue culture microtiter plates (Orange Scientific). In each well, a volume of 20 µL of compound's solution was added to 180 µL of cell culture. All test compounds were tested in a range of different concentrations (Appendix B), in duplicates. Positive and negative controls were established as follows: 200 µL of sterile distilled water; 180 µL of sterile MHB + 20 µL of the highest concentration solution tested; 180 µL of cell culture + 20 µL of DMSO; 200 µL of cell culture.

The $OD_{600}$ was measured at t=0 h in a microtiter plate absorbance reader (Biotek Synergy HT) and at t=24 h, after incubation at 30ºC and 120 rpm (in an incubation-shaking cabinet Sartorius Certomat® BS-1). The MIC was defined as the lowest concentration of test compound which would inhibit the visible growth of microorganisms after the 24 h incubation (Merkl *et al.* 2010; Nihei *et al.* 2004).

### 3.2.4. Minimum bactericidal concentration (MBC) determination

After MIC determination, 3 x 10 µL from each MIC experiment were plated out on PCA. Plates were incubated at 30ºC for 24 h and growth was visually inspected. The MBC was determined as the lowest concentration of compound in which total inhibition of growth was observed and, consequently, no CFU were detected on the solid medium (Ferreira *et al.* 2011).

### 3.2.5. Surface hydrophobicity and its components

The influence of treatment with the caffeic acid derivatites on the physicochemical surface properties of both bacteria tested was assessed by contact angle measurement.

For each bacterium, overnight cultures were prepared in 1000 mL sterile flasks containing around 250 mL of previously autoclaved MHB, and incubated at 30ºC, under agitation at 120 rpm, after inoculation. The cells were centrifuged twice at 3202 *g* for 10 min (at 25ºC) and washed with sterile saline solution (0.85% (w/v) NaCl, BDH Prolabo). The optical density at 640 nm ($OD_{640\ nm}$) of the cell suspensions was set to 0.44. A volume of 45 mL of this culture was added to 5 mL of test compound (to a final concentration of 0.1 mM), in 250 mL sterile shake flasks, and incubated for 1 h at 30ºC and 120 rpm. A negative control was prepared with DMSO. Bacterial lawns (i.e. homogeneous layers of cells) were then prepared

according to an adapted procedure described by Busscher *et al.* (1984). The treated cell cultures were filtered into nitrocellulose sterile filters (47 mm of diameter and pore size of 0.45 µm; Advantec) and kept in the fridge inside a Petri-dish to maintain the moister content, until use. Strips of around 0.5 cm were cut from the filters containing the bacterial lawns and carefully fixed onto microscope slides with double-sided adhesive tape.

Contact angle measurements were carried out according to Simões *et al.* (2010a), by using the sessile drop contact angle method to determine the surface tension of the bacterial surfaces (at least 20 determinations for each liquid and for each microorganism). The measurements were performed at room temperature using three different liquids of well-known surface tension components (two polar - ultrapure water and formamide - and one apolar - α-bromonaphtalene; Sigma). Contact angles were automatically determined using an OCA 15 Plus (Dataphysics) video-based optical measurement instrument, which allowed image acquisition and data analysis. The values of the liquids surface tension components were taken from the literature (Janczuk *et al.* 1993).

Hydrophobicity was assessed after contact angle measurements using the approach of van Oss *et al.* (1988), where 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): $\Delta G_{sws}$. If the interaction between two entities is stronger than the interaction of each one of the entities with water, then $\Delta G_{sws}>0$ mJ $m^{-2}$ and the material is considered hydrophobic. Contrariwise, if $\Delta G_{sws}<0$ mJ $m^{-2}$ the material is hydrophilic. $\Delta G_{sws}$ can be calculated from the surface tension components of the interacting entities:

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

where $\gamma^{LW}$ is the Lifshitz-van der Waals component of the surface free energy and $\gamma^+$ and $\gamma^-$ are the electron acceptor and electron donor parameters, respectively, of the Lewis acid-base component (given by $\gamma^{AB} = 2 \times \sqrt{\gamma^+\gamma^-}$). The surface tension components can be determined by simultaneous resolution of three equations (accounting for the three different liquids used for measuring the contact angles) of the form:

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

where $\theta$ is the contact angle and $\gamma^{TOT}=\gamma^{LW}\gamma^{AB}$.

### 3.2.6. Zeta potential measurement

Overnight cultures of *E. coli* and *S. aureus* were prepared in 250 mL sterile shake flasks with c.a. 50 mL of previously autoclaved MHB and incubated at 30$^{o}$C, under agitation at 120 rpm, after inoculation. The cells were centrifuged twice at 3202 *g* for 10 min (at 25 °C) and washed with sterile distilled water. The cell suspensions were adjusted to $OD_{640\ nm}$=0.22. In a sterile falcon tube, 1.8 mL of this culture were added to 200 µL of test compound (to a final concentration of 0.1 mM) and incubated for 1 h at 30$^{o}$C and 120 rpm. A negative control was prepared with DMSO. The zeta potential of the bacterial suspensions was determined using a Nano Zetasizer (Malvern Instruments) equipment, in carefully filled zeta potential cells (DTS1060, Malvern), at room temperature.

### 3.2.7. Statistical analysis

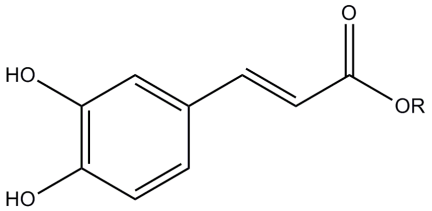
All experiments were carried out in duplicates with at least three repeats (except for the zeta potential measurements, which were only repeated twice). The data was analyzed using the *GraphPad Prism 5* software. The mean and standard deviation within samples was calculated for all cases, when relevant. To assess the statistical significance between groups, an unpaired Student's t-test was used (confidence level ≥ 95%); $p<0.05$ was considered statistically significant.

## 3.3. Results and Discussion

*S. aureus* and *E. coli* were chosen as model systems for the study of the antibacterial activity of a homologous series of caffeic acid esters (with increasing alkyl chains lengths), in the planktonic state.

As previously described, in the homologous series of caffeic acid alkyl esters, compounds differ between one another in the length of their alkyl side chain. In this view, since the head portion of all the compounds tested is the same, the data obtained can be interpreted according to changes in the hydrophobic portion of these hydroxycinnamic esters, i.e., according to the length of the alkyl side chain.

**Table 3 - Minimum inhibitory concentration and minimum bactericidal concentration of caffeic acid and its alkyl ester derivatives against *S. aureus* and *E. coli*.**

|  | | R | *S. aureus* | | *E. coli* | |
|---|---|---|---|---|---|---|
| | | | **MIC (mM)** | **MBC (mM)** | **MIC (mM)** | **MBC (mM)** |
| **Caffeic Acid** | CAF | H | 19.5 | 32.5 | 39 | 39 |
| **Caffeic Acid Esters** | CAFC2 | $C_2H_5$ | 0.4 | 6.5 | 0.4 | 13 |
| | CAFC4 | $C_4H_9$ | 1.6 | 3.25 | 0.4 | 3.25 |
| | CAFC6 | $C_6H_{13}$ | 0.4 | 0.6 | 0.15 | > 13 |
| | CAFC8 | $C_8H_{17}$ | 0.1 | 0.1 | 0.1 | > 19.5 |
| | CAFC10 | $C_{10}H_{21}$ | 0.1 | 0.15 | 0.1 | > 19.5 |

### 3.3.1. Antibacterial activity

In order to determine the bacterial susceptibility to caffeic acid and its alkyl ester derivatives, MIC and MBC were determined for *S. aureus* and *E. coli*, Gram-positive and Gram-negative bacteria, respectively. The results obtained are present in Table 3.

All compounds tested presented antibacterial activity, even though caffeic acid exhibited a MIC of 19.5 mM for *S. aureus* and 39 mM for *E. coli*, which would translate into a mass concentration of 3513 µg/mL and 7026 µg/mL, values far greater than those referred in the literature as acceptable for a phytochemical to be considered antimicrobial (100 to 1000 µg/mL; Tegos *et al.* 2002). However, this was not the case for the alkyl esters, whose MIC and MBC values ranged within (or even below) the interval for antimicrobial classification.

It is also important to note that all of the caffeic acid alkyl esters tested presented very poor solubility in water, causing severe precipitation when mixed in an aqueous environment, with subtle dissolution over time. In this view, especially for the higher concentrations tested and the higher length alkyl esters, absorbance readings for MIC determination were sometimes inconclusive, since t=0 h $OD_{600}$ values were greater than those at t=24 h. That said, it is possible that effective growth or growth inhibition were sometimes concealed over these phenomena. A common remark upon $OD_{600}$ values for MIC determination with these compounds is the fact that, for concentrations above the value of the apparent MIC (where $OD_{600}$ values did not vary over time), absorbance readings were always either greater for t=0 h samples (denoting some kind of precipitation/dissolution, as previously mentioned) or seemed to show bacterial growth. This effect denoted that the initial precipitation, upon mixing with the cell culture, prevented the compound from actually acting against the bacteria, and thus, inhibiting bacterial growth – cutoff effect.

Regarding MIC values, for each bacterium, there is a clear tendency for the decrease of MIC with increasing length of the alkyl ester chain, as previously stated by Merkl *et al.* (2010) and Uwai *et al.* (2008) for similar compounds. It is interesting to note that the bacterial susceptibility increased in three orders of magnitude from caffeic acid to CAFC2, i.e., with the addition of an ethyl side chain.

For *S. aureus*, CAFC4 presents a MIC value of 1.6 mM, which is 4-times greater than the concentration needed to inhibit bacterial growth with CAFC2. Nevertheless, when regarded in the general context, this punctual decrease in bacterial susceptibility is not relevant as it is still in the same order of magnitude of the other alkyl esters. On the other hand, both octyl and decyl esters of caffeic acid (CAFC8 and CAFC10, respectively) presented the lowest MIC values, with 0.1 mM of the compound (29.2 and 32.0 µg/mL, respectively) being enough to inhibit the

growth of *S. aureus*. These two compounds exhibited the same MIC value, contrasting with the decrease of MIC tendency for increasingly bigger alkyl chains, which denotes that these alkyl lengths probably represent the maximum threshold of the parabolic curve of antimicrobial activity *vs.* lipophilicity described by Kubo *et al.* (2004a; 2004b) for phenolic acid esters. In fact, these authors described that antibacterial activity was usually maximized between C8 and C12, which is in accordance with the results obtained. This behavior is also notorious for MBC values, where the maximum of antibacterial activity is clearly located at CAFC8. Moreover, MBC values were always greater or equal to MIC values, for each compound.

For *E. coli*, the antibacterial activity of the compounds tested followed the same behavior as in *S. aureus*, even though the decrease of MIC values with the increasing alkyl length of the compounds was clearer than in the previous case. The maximum threshold of antimicrobial activity was also observed for CAFC8 and CAFC10. However, the MBC values for *E. coli* differed in several orders of magnitude from the MIC values and showed a more prominent parabolic behavior than for *S. aureus*, with maximum antibacterial activity for CAFC4. This means that whichever phenomenon causes the bactericidal activity of these compounds, it is more affected by the length of the alkyl side chain in the case of Gram-negative bacteria.

Lastly, when comparing MIC and MBC values between *S. aureus* and *E. coli*, it is possible to observe that the Gram-negative bacteria is much less susceptible than the Gram-positive one to the action of caffeate alkyl esters. The minimum inhibitory concentration for caffeic acid was double for *E. coli*, whereas it was relatively similar for the series of alkyl esters. Yet, MBC values visibly demonstrated the higher susceptibility of *S. aureus*, which is in accordance with the observations made by several authors for similar compounds (Kubo *et al.* 2002; Simões *et al.* 2009; Tegos *et al.* 2002). This behavior is most likely explained by the fact that Gram-negative bacteria possess an outer membrane with a hydrophilic coating of LPS (Maisuria 2009), posing a greater barrier to amphiphilic compounds, such as the ones tested, to overcome when attempting to penetrate the cell.

### 3.3.2. Bacterial surface charge, surface hydrophobicity and its components

The alterations in the physicochemical surface properties (especially, regarding surface charge, hydrophobicity and surface tension parameters) of *S. aureus* and *E. coli* bacterial cells, after treatment with the compounds tested was evaluated. In order to perform a SAR, all compounds were tested at the same concentration, regardless of their variant MIC and MBC. A low treatment concentration of 0.1 mM was chosen for all compounds due to solubility issues

of the compounds with higher alkyl ester chains. This procedure ensured that, for all cases, the activity of the compounds was not subjected to precipitation setbacks.

The result obtained from the zeta potential measurements allow for a better understanding of how the negatively charged caffeic acid and alkyl ester derivatives interacted with bacterial surfaces.

**Table 4 - Effects of caffeic acid alkyl esters in the surface charge of *S. aureus* and *E. coli* cells. The means ± SD of the surface charge are shown, for both bacteria, after treatment with the compounds.**

| | **Zeta Potential (mV)** | |
|---|---|---|
| | ***S. aureus*** | ***E. coli*** |
| **Control (DMSO)** | -30.9 ±3.3 | -22.6±6.2 |
| **CAF** | -10.2±4.5 | -11.8±3.0 |
| **CAFC2** | -16.4±2.8 | -28.1±4.7 |
| **CAFC4** | -24.7±3.7 | -17.4±3.2 |
| **CAFC6** | -28.3±1.3 | -18.4±0.9 |
| **CAFC8** | -25.5±4.5 | -20.5±0.8 |
| **CAFC10** | -27.0±5.6 | -19.6±2.1 |

As expected, all bacteria tested had a negative surface charge, which was less negative for *E. coli* than for *S. aureus*, a result which is in accordance with the results obtained in other studies (Borges *et al.* 2013). In general, caffeic acid (CAF) was the compound that interfered the most with the surface charge of both bacteria ($p<0.05$). Exposure to all the selected compounds increased the bacterial surface charge to less negative values. This is partly in accordance with the literature, where *E. coli* cells are said to be affected in such a manner by a phenolic acid, namely, ferulic acid, which at this pH, is ionized (in the carboxylated form) and, thus, negatively charged. However, *S. aureus* surface charge is said to be unaffected by that same compound (Borges *et al.* 2013). On the other hand, the alkyl esters of caffeic acid did not significantly altered the bacterial surface charge ($p>0.05$), except for CAFC2 treatment in *S. aureus*.

In addition, the hydrophobicity and other surface tension parameters were studied, in order to determine the influence of the alkyl caffeates in the physicochemical characteristics of both bacteria.

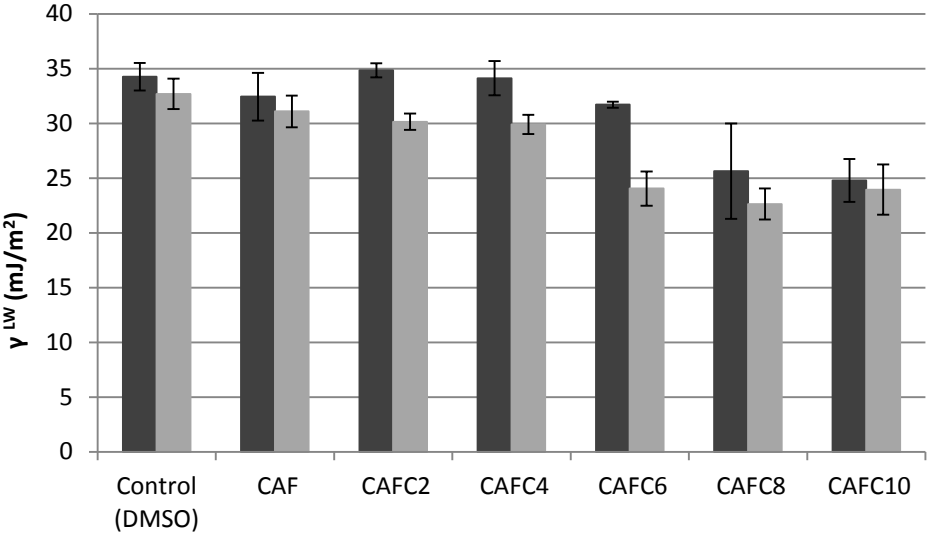



**Fig. 3 - Apolar component of the surface tension for *S. aureus* (■) and *E. coli* (■) cells after treatment with the selected compounds at 0.1 mM, for 1 h. The means ± SD (for at least three replicates) are shown.**

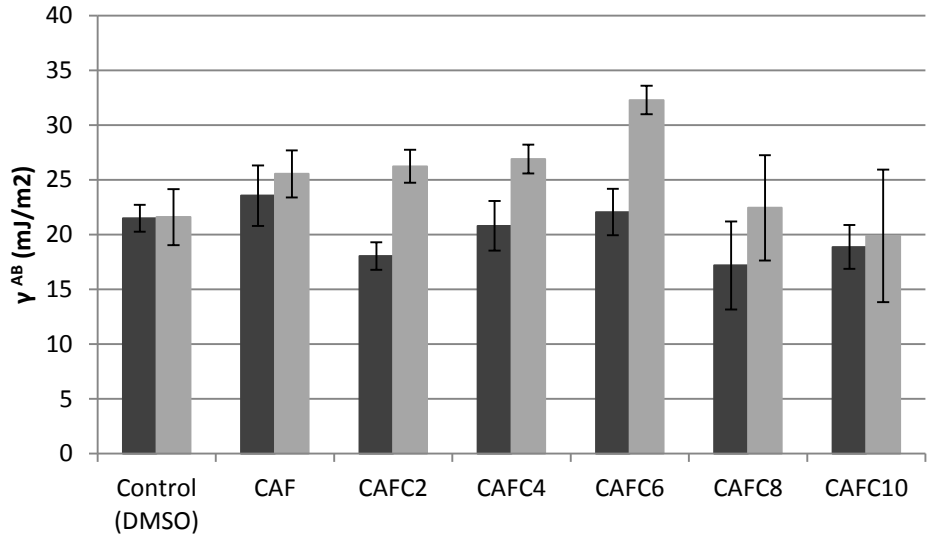



**Fig. 4 - Polar component of the surface tension for *S. aureus* (■) and *E. coli* (■) cells after treatment with the selected compounds at 0.1 mM, for 1 h. The means ± SD (for at least three replicates) are shown.**

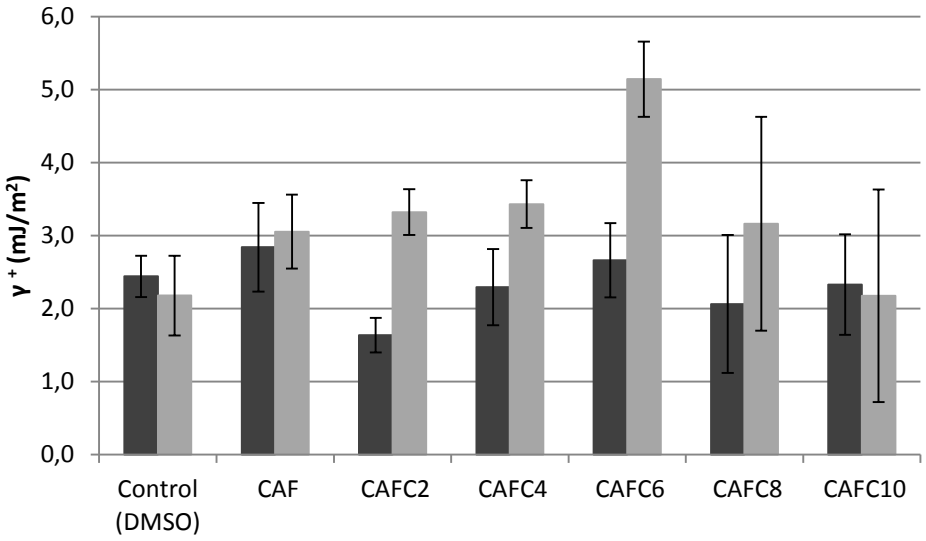



**Fig. 5 – Electron acceptor parameter for *S. aureus* (■) and *E. coli* (■) cells after treatment with the selected compounds at 0.1 mM, for 1 h. The means ± SD (for at least three replicates) are shown.**

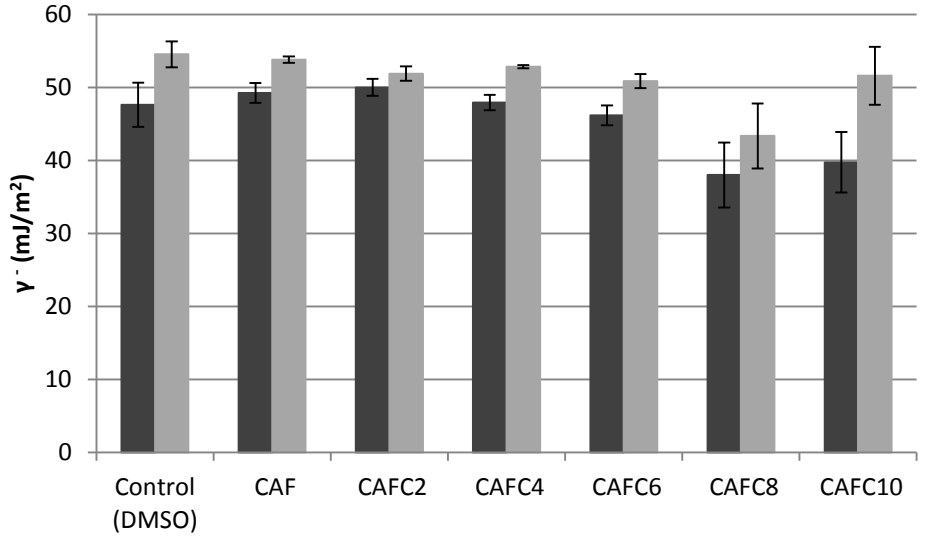



**Fig. 6 - Electron donor parameter for *S. aureus* (■) and *E. coli* (■) cells after treatment with the selected compounds at 0.1 mM, for 1 h. The means ± SD (for at least three replicates) are shown.**

As can be seen in Fig. 3, a decreasing tendency for the apolar component of the surface tension with the increase of the alkyl ester side chain was observed for both the Gram-positive and Gram-negative bacteria. This decrease is only statistically significant ($p$<0.05) for *S. aureus* from the octyl ester of caffeic acid (CAFC8) onwards. For *E. coli*, the apolar component is statistically different from the hexyl ester of caffeic acid (CAFC6) onwards, being that, for both cases, this component reaches a threshold and keeps constant. Moreover, it seems that untreated *E. coli* and *S. aureus* presented similar surface polarities ($p$>0.05), whilst

treated *E. coli* cells were less apolar than treated *S. aureus* cells when exposed to CAFC2, CAFC4 and CAFC6.

The surface tension polar component (Fig. 4), presented a distinct behavior from the apolar. For *S. aureus*, it kept similar for the untreated and treated cells, for all the compounds tested ($p$>0.05). However, for *E. coli*, $\gamma^{AB}$ presented a parabolic behavior as function of the increasing lipophilicity of the compounds tested, which reached a maximum for cells treated with CAFC6. This result proposed that the Gram-positive bacteria were unaffected by the exposure to the caffeic acid alkyl esters, while the Gram-negative ones acquired a polar character, as reflected by the increase of $\gamma^{AB}$, which was maximized for CAFC6 exposure. It is also noteworthy that the previous observations regarding similar polarities for untreated *E. coli* and *S. aureus* cells and the latest being less polar than *E. coli* cells after being exposed to the selected compounds (particularly, in the cases of CAFC2, CAFC4 and CAFC6; $p$<0.05) is also applicable for the $\gamma^{AB}$ results.

The electron acceptor component ($\gamma^+$) (Fig. 5) of *E. coli* cells described a very similar profile. *S. aureus* showed no major alteration (apart from a significant decrease - $p$<0.05 - for CAFC2 treated cells), while *E. coli* displayed a parabolic behavior, with an increase in $\gamma^+$ up to cells treated with the hexyl ester of caffeic acid. In this case, the $\gamma^+$ value is more than 1 mJ/m$^2$ higher than for the cells treated with smaller alkyl chain esters and untreated cells. The $\gamma^+$ value decreased for the cells treated with bigger alkyl chain esters, such as CAFC10 ($p$<0.05). As for the electron acceptor component ($\gamma^-$) (Fig. 6), it remained constant for untreated and treated cells of both bacteria up to CAFC6, inclusive. Both bacteria presented a decrease in the electron acceptor capability of their surface after treatment with CAFC8.

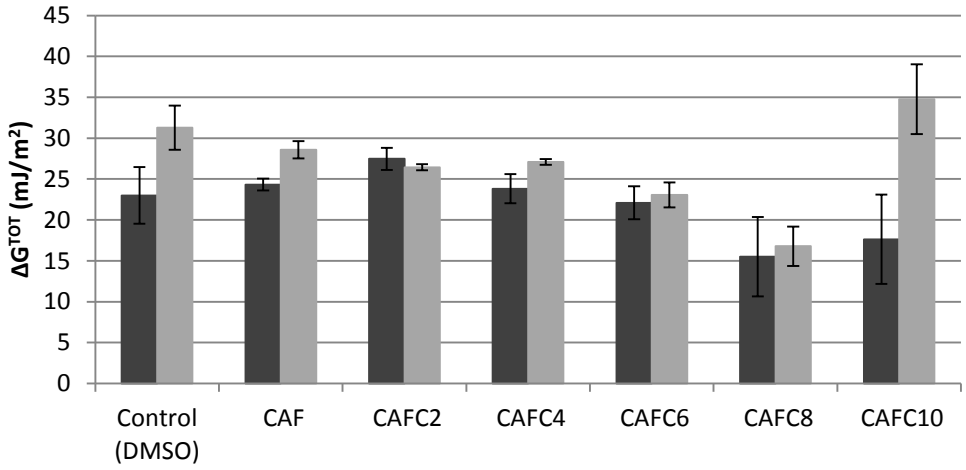



**Fig. 7 – Hydrophobicity for *S. aureus* (■) and *E. coli* (■) cells after treatment with the selected compounds at 0.1 mM, for 1 h.**

Regarding the surface hydrophobicity (Fig. 7), both bacteria had hydrophilic properties ($\Delta G^{TOT}$>0 mJ/m$^2$), when untreated or treated with the selected compounds. The application of the caffeic acid alkyl esters caused, however, some changes in the hydrophilic character of the cells. *E. coli* cell surface became less hydrophilic with the increase of the alkyl ester chain length in the test compounds, with minimum hydrophilicity being achieved for cells treated with CAFC8 ($p$<0.05). Conversely, CAFC10 did not show any effect upon the cell surface hydrophobicity, when comparing to the untreated cells ($p$>0.05). On the other hand, *S. aureus* cells were affected by the action of the alkyl caffeates in an opposite manner, increasing their hydrophilicity with the increase of the alkyl chain length, presenting maximum hydrophilicity for cells exposed to CAFC2 ($p$<0.05). For the remaining esters (with bigger length alkyl side chains), the cells showed similar hydrophilicity values to the untreated cells ($p$>0.05). In this view, it is possible to observe opposite parabolic behaviors between the Gram-positive and the Gram-negative bacteria.

In a general perspective, the exposure to caffeic acid alkyl esters caused changes in the physicochemical properties of both bacteria. To sum up, treatment with compounds with increasingly long alkyl ester chains led, in *E. coli*, to:

- a decrease in MIC values and parabolic MBC values variations (with minimum MBC values for CAFC4);
- inverted parabolic zeta potential variations (with maximum surface charge for CAF);
- parabolic $\Delta G^{TOT}$ variations (with minimum hydrophilicity for CAFC8);
- a decrease in $\gamma^{LW}$, i.e., lower surface apolarities;
- inverted parabolic $\gamma^{AB}$ variation (with maximum surface polarity for CAFC6);
- inverted parabolic $\gamma^{+}$ variations (with maximum electron acceptance for CAFC6);
- a decrease in $\gamma^{-}$, i.e., in the electron donor capability, for CAFC8 and CAFC10;

and, in *S. aureus*, to:

- a decrease in MIC values and parabolic MBC values variations (with minimum MBC values for CAFC8);
- inverted parabolic zeta potential variations (with maximum surface charge for CAF);
- inverted parabolic $\Delta G^{TOT}$ variations (with maximum hydrophilicity for CAFC2);
- a decrease in $\gamma^{LW}$, i.e., lower surface apolarities;
- no significant alterations of $\gamma^{AB}$;
- parabolic $\gamma^{+}$ variations (with minimum electron acceptance for CAFC2);
- a decrease in $\gamma^{-}$, i.e., in the electron donor capability, for CAFC8 and CAFC10;

First of all, this compilation of results highly suggests that caffeic acid and its alkyl ester derivatives most likely act as membrane permeabilizers, i.e., antimicrobial agents that weaken

the outer membrane of the cells, inducing changes in its permeability and, consequently, in the bacterial most visible physicochemical characteristics, such as polarity, hydrophobicity and surface charge. Due to their known amphiphilic character, these compounds may cross the membrane by passive diffusion, disturbing the membrane structure and possibly acidifying the cytoplasm due to their acid nature and causing protein denaturation. This acidification phenomenon requires, however, further investigation, once the caffeic acid alkyl esters, unlike caffeic acid, are not prone to grant an acidic character (their pKa is around 8.7). Protein denaturation might also cause interference with redox reactions by, for e.g., inhibiting the electron transport chain (Borges *et al.* 2013; Campos *et al.* 2009). Furthermore, this hyperacidification phenomenon may cause further permeabilization changes, such as local rupture and pore formation in the membranes, which, in the end, might lead to leakage of cytoplasmatic constituents (including proteins, nucleic acids, and inorganic ions such as potassium or phosphate) and, finally, to cell death (Borges *et al.* 2013; Campos *et al.* 2009). Actually, some authors have also concluded that this is, in fact, the most likely *modus operandi* of phenolic acids as antimicrobials (Campos *et al.* 2009; Lacombe *et al.* 2010; Kubo *et al.* 2002). These conclusions might be inferred from the alterations of zeta potential, polarity, electron donor/acceptor behaviors and hydrophilicity observed for both bacteria tested.

Secondly, these observations lead to one more conclusion regarding the elongation of the alkyl ester chains: it is possible that the antimicrobial activity of this series of compounds is associated with a fine balance between affinity for the lipid bilayers of cell membranes and the ability to cause disruption of the membrane, which visibly differs from Gram-positive to Gram-negative bacteria, due to their different cell wall structure and composition. However, no clear relationship between bacterial susceptibility and the effect of each compound in the bacterial surface physicochemical properties can be withdrawn from the results obtained. As an example, one might say that CAFC8 was the compound that caused the lowest hydrophilic properties for both *E. coli* and *S. aureus*, i.e. that caused the cells to become more hydrophilic. This might lead to a conclusion that this would be the compound that caused higher membrane interference and thus, the most effective as an antimicrobial agent. However, this is only true for the Gram-positive bacterium *S. aureus*, for which the lowest MBC value found in the compound series tested was for CAFC8. In the case of *E. coli*, the lowest MBC value was found for CAFC4. A possible hypothesis is that this smaller alkyl ester might be more effective in crossing the LPS layer outside the outer membrane (typical of Gram-negative bacteria), once LPS render a more hydrophilic environment to endure until the lipidic bilayer of the outer membrane is reached (Maisuria 2009). It is also important to note that lower susceptibilities observed for Gram-negative bacteria (i.e., the need for higher concentrations of antimicrobials

to achieve cell death), might also be credited to the fact that, as already mentioned, the outer membrane is an effective barrier (Kubo et al. 2004a; Madigan *et al.* 2009). It is thus reasonable to assume that most of the compound molecules would be incorporated in the outer membrane (due to their partial lipophilic character), and hence not able to reach the inner membrane and the cytoplasm in order to trigger all destabilization steps that might lead to cell death.

## 3.4. Conclusions

Bacterial susceptibility of *S. aureus* and *E. coli* was assessed by MIC and MBC determination and it was concluded that all of the compounds tested (caffeic acid and caffeic acid alkyl ester homologous series) presented antimicrobial activity, even though caffeic acid led to MIC values that exceeded, in both bacteria, the acceptable range for a phytochemical to be considered an antimicrobial. It was also concluded that there was a very clear tendency for increasing antimicrobial activity (i.e., for decreasing MIC values) with increasing length of the alkyl ester chain in the compounds. Indeed, bacterial susceptibility increased in 3 orders of magnitude from caffeic acid to its ethyl ester CAFC2, for both bacteria.

In *S. aureus*, the lowest MIC values determined correspond to CAFC8 and CAFC10. Susceptibility to these compounds was similar for the Gram-positive bacterium, which means that a length of C8 to C10 alkyl ester chain is the maximum threshold in the lipophilicity *vs*. antimicrobial activity parabolic curve described in the literature. In fact, the MBC values determined show maximum bactericidal activity for CAFC8. In *E. coli* the MIC values were minimum for CAFC8 and CAFC10, while MBC values were minimized for CAFC4. Overall, the Gram-negative bacterium was less susceptible to the action of the compounds tested, with MBC values several orders of magnitude higher than for *S. aureus*. This means that *E. coli* cells are more resistant to antimicrobial action of these phenolic acid derivatives than *S. aureus*, as was expected, due to their different cell wall compositions.

The compounds also showed an ability to interfere with the physicochemical properties of both bacteria. The surface charge of both bacteria was, as expected, negative. Caffeic acid was able to significantly alter the surface charge of both *E. coli* and *S. aureus* to less negative values. However, the alkyl esters did not significantly affect the surface charge of the bacteria. Also, polarity and hydrophobicity of both bacteria were affected by exposure to the tested compounds, seldom showing a parabolic behavior dependent on the alkyl ester

length. The apolar character of bacteria was reduced with the increase of lipophilicity of the compounds, for both bacteria. The polar character was maximized for CAFC6 in *E. coli* and showed no significant alterations for *S. aureus*. The electron acceptor properties were also maximized for CAFC6 in *E. coli* and were minimized for CAFC2 in *S. aureus*, while CAFC8 and CAFC10 caused a reduction of the electron donor properties for both bacteria. Lastly, hydrophobicity was minimized for CAFC8 for *E. coli* and maximized for CAFC2 in *S. aureus*.

It was thus concluded that, due to their amphiphilic character, caffeic acid and its alkyl ester derivatives mode of action includes membrane disturbance and permeabilization, which induces changes in the physicochemical characteristics of bacteria. Hence, it is proposed that the compounds cross the membrane by passive diffusion, disturbing the cell membrane structure and possibly acidifying the cytoplasm due to their acid nature and protein denaturation, causing permeabilization changes in the outer membranes, such as local rupture and pore formation in the membranes, which, in the end, might lead to leakage of cytoplasmatic constituents and, finally, to cell death.

Furthermore, the antimicrobial activity of these compounds is associated with a fine balance between affinity for the lipid bilayers of cell membranes and the ability to cause disruption of the membrane structure, which visibly differs from Gram-positive to Gram-negative bacteria and depends on the length of the alkyl ester chain of the compounds. It is proposed that, in Gram-negative bacteria, due to the existence of the hydrophilic LPS layer and of an outer membrane, smaller length alkyl ester chain compounds have a better lipophilicity balance that allows both the crossing of the LPS layer as well as of the hydrophobic outer membrane.

# Chapter 4

## 4. THE EFFECTS OF CAFFEIC ACID ALKYL ESTERS IN THE EARLY STEPS OF BIOFILM FORMATION

### 4.1. Introduction

In order to predict the microbial behavior in the early steps of biofilm formation, several theoretical approaches have been applied to describe adhesion of bacteria to surfaces, such as the classical Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, the extended DLVO (XDLVO) theory and the thermodynamic approach (based on the surface Gibbs energy) (Absolom *et al.* 1983; Simões *et al*. 2007; Van Oss *et al.* 1988). When a microorganism and a surface enter into direct contact, the existing water film between their interacting entities has to be removed. This is in accordance with the thermodynamic theory of adhesion and is expressed by the Dupré equation, which states that the Gibbs free energy of interaction can be calculated assuming that the interfaces between bacteria/liquid medium and solid/liquid medium are replaced by a bacteria/solid interface (Absolom *et al.* 1983). In this view, the interaction between a microbial cell and solid surface is only possible from a thermodynamic point of view if this interaction leads to a decrease in the surface Gibbs free energy (Busscher *et al.* 1984).

However, important biological factors, which significantly contribute to the adhesion process, have been left out in these models, such as the production of polysaccharides, lipopolysaccharides and extracellular appendages (adhesins), causing a lack of agreement, i.e., an underestimation of adhesion when comparing theoretical adhesion evaluations and *in-vitro* adhesion assays (Simões *et al.*2010a).

On the other hand, as already described, QS plays an important role in initial bacterial attachment to surfaces and, consequently, in biofilm growth and development. QS is an intercellular signaling system that allows bacteria to mediate cell-cell interactions. In QS, small signaling molecules, called autoinducers, that accumulate in the surrounding environment and allow bacteria to sense the size/density of the neighboring bacterial population and control a variety of physiological processes and auto-regulate their gene expression accordingly (Simões *et al.*2010b; Madigan *et al.* 2009; Zhang *et al*. 2014). In this sense, these signals are produced

while the bacterial population grows, until a threshold concentration of cells (quorum) is perceived by the bacteria, resulting in repression or activation of specific genes.

The autoinducers differ depending on the bacteria. Gram-negative bacteria generally produce acylated homoserines lactone (AHLs) as autoinducers. These signaling molecules are synthesized by enzymes of the LuxI family (AHL synthethases) and are constantly being produced and received at basal levels by bacterial cells, until the cell density of the population increases, causing an increase in the concentration of AHLs being diffused through the membrane and accumulated intra- and extracellularly in proportion to cell density. After quorum is reached, the AHLs induce the expression of a set of target genes. Conversely, unlike Gram-negative bacteria, Gram-positive bacteria employ secreted peptides processed from precursors that can be used as autoinducers for QS. This signal peptide is usually transported out of the cell via an ABC transporter (Madigan *et al.* 2009; Nazzaro *et al.* 2013). When the extracellular concentration of the peptide signal accumulates to the minimal stimulatory level, a histidine sensor kinase protein, belonging to a two-component signaling system, detects it, autophosphorylating a conserved histidine residue, being the phosphoryl group subsequently transferred to an equivalent response-regulator protein. The response regulator is then phosphorylated on a conserved aspartate residue, which activates the transcription of a target gene or genes (Madigan *et al.* 2009; Nazzaro *et al.* 2013). Lastly, a furanosyl boronated diester molecule (AI-2) and a non-boronated diester molecule (vAI-2) are known to be involved in inter- and intra-species communications amongst both Gram-positive and Gram-negative bacteria (Simões *et al.* 2009).

The quest for new antimicrobial agents led to the search of compounds that interrupt bacterial communication in biofilms, with several phytochemicals, such as polyphenols, being capable of affecting biofilm formation in some bacteria (Nazzaro *et al.* 2013). Inhibition of bacterial QS may take place through different mechanisms including inhibition of auntoinducer synthesis, inhibition of autoinducer transport and/or secretion, sequestration of autoinducers, antagonistic action and inhibition of targets downstream of the autoinducer receptor binding site (Nazzaro *et al.* 2013).

Usually, the QS system mechanism of *Chromobacterium violaceum* has been studied and used to evaluate the quorum and antiquorum activity of compounds (Nazzaro *et al.* 2013). The QS systems of *C. violaceum* consist of CviI/CviR, which are homologs of the LuxI/LuxR systems. This strain mediates QS by autoinducers of the type of AHLs. *C. violaceum* also produces violacein, a purple pigment, under the control of its QS system, as the binding of AHLs to the transcriptional regulator CviR triggers the expression of violacein production (Borges *et al.* 2014b). That being said, these bacteria are used as biosensors for QS studies due

to both the high similarity of QS systems that use the same type of AHL signaling molecules (making it possible to test if compounds can interfere with other QS system homologues) and the direct visualization of QSI, by lack of violacein production (Borges *et al.* 2014b; Madigan *et al.* 2009).

Hence, the objectives of this work were to assess the effects of caffeic acid alkyl esters in the early steps of biofilm formation, specifically regarding their action against bacterial adhesion to surfaces and QS, and thus, in biofilm prevention.

## 4.2. Materials and Methods

### 4.2.1. Free energy of adhesion

In order to predict the influence of caffeic acid and its alkyl esters in the ability of the microorganisms to adhere to surfaces, the free energy of interaction between the isolated microorganisms and three different materials (polystyrene, silicone and AISI316 stainless steel) was calculated (Simões *et al*. 2007), following the contact angle measurement described in section 3.2.5. The hydrophobicity and surface tension parameters of the abovementioned materials was taken from the literature, particularly from Simões *et al.* (2010a), for PS, and Simões *et al.* (2007), for SS and silicone.

When studying the interaction between substances 1 and 2 that are immersed or dissolved in water (w), the total interaction energy ($\Delta G_{1w2}^{TOT}$) can be expressed as:

$$\Delta G_{1w2}^{TOT} = \gamma_{12}^{LW} - \gamma_{1w}^{LW} - \gamma_{2w}^{LW} + 2\left[\sqrt{\gamma_w^+}\left(\sqrt{\gamma_1^-} + \sqrt{\gamma_2^-} - \sqrt{\gamma_w^-}\right) + \sqrt{\gamma_w^-}\left(\sqrt{\gamma_1^+} + \sqrt{\gamma_2^+} - \sqrt{\gamma_w^+}\right) - \sqrt{\gamma_1^+\gamma_2^-} - \sqrt{\gamma_1^-\gamma_2^+}\right] \quad (3)$$

Thermodynamically, if $\Delta G_{1w2}^{TOT} < 0$ mJ/m$^2$, adhesion is favorable, while if $\Delta G_{1w2}^{TOT} > 0$ mJ/m$^2$ , adhesion is not expected to occur.

### 4.2.2. Adhesion assay

Adhesion assays were performed using three different representative adhesion surfaces for *E. coli* and *S. aureus* cells: PS, silicone and SS. Coupons of the abovementioned materials with 1 x 0.8 cm were prepared for further use by immersing them in diluted

commercial detergent (Sonasol) for 30 min. In order to remove any detergent, the coupons were rinsed in distilled water and subsequently immersed in ethanol at 70% (v/v) for 15 min. The coupons were then stored in sterile distilled water until needed. Directly before use, they were separately placed in a Petri-dish and subjected to UV light for approximately 30 min. The coupons of each material were vertically inserted, in sterile conditions, in sterile 48-well plates (BioLite Multidish, Orange Scientific).

Overnight cultures of *E. coli* and *S. aureus* were prepared in 250 mL sterile shake flasks with c.a. 50 mL of previously autoclaved MHB and incubated at 30$^{o}$C, under agitation at 120 rpm, after inoculation. The cells were centrifuged twice at 3202 *g* for 10 min (at 25$^{o}$C) and washed with sterile saline solution (0.85% (w/v) NaCl, BDH Prolabo). The cell suspensions were set to $OD_{610\ nm}$=0.22. Adhesion to each material was allowed to occur by adding 1 mL of cell suspension to each coupon-containing well and the plates were incubated for 2 h at 30$^{o}$C and 150 rpm (Simões *et al*. 2007). The coupons were then transferred to different wells containing 1 mL of 10% (v/v) solutions of each test compound (in saline solution, to a final concentration of 0.1 mM) and incubated for 1 h at 30$^{o}$C and 150 rpm. A negative control was set using DMSO. The coupons were afterwards immersed in sterile saline solution to remove weakly/reversibly adherent bacteria and placed in a falcon tube containing 10 mL of sterile saline solution. Adherent bacteria were resuspended by agitation (for 1 min), using a vortex. Serial 10-fold dilutions of each sample were prepared (up to $10^{-3}$, in sterile saline solution), and 3 x 10 µL from each dilution were plated out in PCA, for cultivability assessment using an adaptation of the drop plate method (Herigstad *et al.* 2001), in which the drop is let to slide across the solid medium for better visual accounting. Plates were incubated at 30$^{o}$C for 24 h and, after, CFUs were counted and results are presented in terms of log CFU/cm$^2$.

### 4.2.3. Quorum sensing inhibition

A standard disk diffusion assay (Bauer *et al*. 1966) was used with biosensor strain *Chromobacterium violaceum* ATCC 12472 to detect possible QSI activity of the test compounds. Overnight cultures of *C. violaceum* were prepared in 100 mL sterile shake flasks with c.a. 25 mL of previously autoclaved Luria-Bertani broth (LB; Liofilchem) and incubated at 30$^{o}$C, under agitation at 120 rpm, after inoculation. The cell suspensions were set to $OD_{620\ nm}$=0.1. LB agar (LBA – LB broth + 1.8% (m/v) Agar-Agar, Merck) plates were inoculated with 100 µL of the cell suspension. Sterile paper disks (6 mm in diameter) were then placed over the plates and loaded with 10 µL of different concentrations of each tested compound (concentrations of 0.1 and 1 mM were tested). A negative control using DMSO was also

prepared. After incubation for 24 h at 30 °C, the inhibition halos around the disk (either by antimicrobial activity, indicated by the lack of microbial growth, or by bacterial growth presenting violacein pigment inhibition) were measured, according to the procedure described by Borges *et al.* (2014b).

### 4.2.4. Statistical analysis

All experiments were carried out in duplicates with at least three repeats. The data was analyzed using the *GraphPad Prism 5* software. The mean and standard deviation within samples was calculated for all cases. To assess the statistical significance between groups, One-way ANOVA with Bonferroni post-test was used (confidence level ≥ 95%); $p<0.05$ was considered statistically significant.

## 4.3. Results and Discussion

### 4.3.1. Bacterial adhesion

In order to assess the effects of caffeic acid and its alkyl ester derivatives on the adhesion potential of *S. aureus* and *E. coli* cells, a theoretical characterization of the free energy of adhesion to three different materials was undertaken according to the thermodynamic approach (Absolom *et al*. 1983; Busscher *et al.* 1984). In this approach, both the hydrophobicity of the cell surface (with and without treatment) and of the adhesion surface itself are taken into account because the ability of bacteria to attach to each other and to surfaces depends, in part, on the interaction of hydrophobic domains (Simões *et al.* 2010b).

In this case, hydrophobicity characterization of the both bacteria upon treatment with the test compounds had already been established (Chapter 3), showing that both presented a hydrophilic character ($\Delta G^{TOT}>0$ mJ/m$^2$) with or without exposure to the antimicrobials. In addition, all three of the materials tested (polystyrene, silicone and stainless steel) had a hydrophobic character ($\Delta G^{TOT}<0$ mJ/m$^2$), being silicone the most hydrophobic ($\Delta G^{TOT}$=-85.6 mJ/m$^2$), followed by SS ($\Delta G^{TOT}$=-55.1 mJ/m$^2$) and, lastly, PS ($\Delta G^{TOT}$=-44.0 mJ/m$^2$) (Simões *et al.* 2007; 2010a).

Based on the thermodynamic approach, the theoretical thermodynamic ability of both bacteria to adhere to the test materials was calculated and the results are compiled in Table 5.

From a thermodynamic point of view, adhesion is only possible if this interaction leads to a decrease in the surface Gibbs free energy (Busscher *et al.* 1984), i.e., if $\Delta G^{TOT}<0$ mJ/m$^2$.

**Table 5 - Free energy of adhesion between *S. aureus* and *E. coli* (untreated and treated bacteria) and the different material surfaces (when immersed in water).**

| | | $\Delta G^{TOT}$ (mJ/m$^2$) | | |
|---|---|---|---|---|
| | | **PS** | **Silicone** | **SS** |
| ***S. aureus*** | **Control** | 1.7 | -7.2 | -1.7 |
| | **CAF** | 3.8 | -5.4 | 0.4 |
| | **CAFC2** | 2.2 | -7.7 | -1.5 |
| | **CAFC4** | 1.8 | -7.4 | -1.7 |
| | **CAFC6** | 1.6 | -8.2 | -2.0 |
| | **CAFC8** | -3.7 | -17.5 | -8.6 |
| | **CAFC10** | -1.7 | -15.5 | -6.6 |
| ***E. coli*** | **Control** | 6.7 | -3.3 | 2.9 |
| | **CAF** | 7.6 | -1.9 | 4.1 |
| | **CAFC2** | 6.9 | -2.8 | 3.3 |
| | **CAFC4** | 7.7 | -2.0 | 4.2 |
| | **CAFC6** | 9.7 | -1.3 | 5.8 |
| | **CAFC8** | 2.8 | -11.1 | -2.1 |
| | **CAFC10** | 7.2 | -7.3 | 2.2 |

As it can be observed from the calculated results, both the Gram-positive and the Gram-negative bacteria are thermodynamically able to adhere to silicone ($\Delta G^{TOT}<0$ mJ/m$^2$). It appears that the interaction between the Gram-negative bacterium and the silicone surface was, however, less favorable than the one between *S. aureus* and silicone, due to smaller variations upon the Gibbs free energy for these interactions, which means that, both untreated and treated cells of *E. coli* will tend to adhere less to silicone than *S. aureus*. It is also interesting to observe that CAF and compounds with smaller alkyl ester chains, such as CAFC2, CAFC4 or CAFC6, appear to cause a propensity for bacteria to adhere less to silicone surfaces, when comparing to the control cells, than higher alkyl ester chain compounds, such as CAFC8 and CAFC10.

On the other hand, PS surfaces were generally not thermodynamically favorable for bacterial adhesion ($\Delta G^{TOT}>0$ mJ/m$^2$), except for the cases of *S. aureus* cells exposed to CAFC8 and CAFC10 treatments. This fact appears to corroborate the previous observation regarding compounds with smaller length alkyl ester chains causing a decrease in the ability of bacteria

to attach. In fact, even though *E. coli* is not thermodynamically favored to adhere to PS, regardless of the compound used for antimicrobial treatment, a similar observation may be reached. On the other hand, yet again, *E. coli* cells present higher $\Delta G^{TOT}$ values, being less prone to adhere to PS than *S. aureus* cells.

Conversely, SS surfaces showed a mixed behavior. Untreated *S. aureus* cells showed theoretical thermodynamic ability to adhere to SS, with an aggravated tendency towards treatment with alkyl esters. In this view, only caffeic acid-treated cells rendered adhesion unfavorable for *S. aureus*. As for *E. coli* cells, those were thermodynamically not prone to adhere to SS ($\Delta G^{TOT}$>0 mJ/m$^2$), a condition which became increasingly true for cells treated with CAF and alkyl esters up to CAFC6. In their turn, CAFC8 and CAFC10-treated cells were thermodynamically favored to adhere to SS.

To sum up, adhesion was thermodynamically less favorable for PS surfaces, as it was the lesser hydrophobic material tested and for the Gram-negative bacterium, which was also found to be, generally, less hydrophobic than the Gram-positive bacterium. Concerning the effects of the compounds tested, CAF was the one which enabled *S. aureus* cells to be less prone to adhere to the surfaces. For *E. coli* cells, it was CAFC6, regardless of the surface material.

Nevertheless, whilst the prediction of the adhesion potential on the basis of physicochemical properties gives us useful information on the possible real-life microbial behavior, it is not often accurate, due to the existence of other cellular mechanisms that play an important role on the bacterial adhesion process, such as flagella, pili or fimbrae and EPS production (Simões *et al.* 2007). In fact, a comparison between the theoretical thermodynamic prediction of adhesion and adhesion assays usually shows an underestimation of bacterial adhesion for the first case (Simões *et al.* 2007). Thereby, adhesion assays to assess treated and untreated *E. coli* and *S. aureus* adhesion were performed using small coupons of PS, silicone and SS.

Regarding adhesion of *E. coli* to PS surfaces (Fig. 8), it is possible to observe that, unlike thermodynamically predicted, cells did adhere to the PS surfaces. Besides, no major variation on the number of adhered cells is visible with the increasing alkyl ester chain of the tested compounds. The treated cells presented similar log CFU/cm$^2$ values to the control ($p$>0.05), with exception of cells exposed to CAFC6 ($p$<0.05). As for *S. aureus*, adhesion to PS varied greatly with antimicrobial treatment. In fact, CAFC6, CAFC8 and CAF10 exposures caused a severe reduction ($p$<0.05) on the number of adhered cells when comparing to untreated cells (log reductions of 1.9, 2.2 and 1.8 for CAFC6, CAFC8 and CAFC10, respectively), which translates to a percentage reduction of over 98% of the number of cells adhered to the PS

surface, for all three cases (practically inhibiting bacterial adhesion to this material). This is an interesting result considering that adhesion of cells exposed to CAFC8 and CAFC10 was thermodynamically favorable, unlike it was for the remaining compounds. In addition, it is also possible to verify that the Gram-negative bacterium was more adherent to PS coupons than the Gram-positive ($p$<0.05), even though this bacterium was thermodynamically less expected to adhere to surfaces than the Gram-positive, regardless of being or not exposed to the action of the alkyl caffeates, but with special emphasis for CAFC6, CAFC8 and CAFC10. That being said, *E. coli* cells were less susceptible to the action of the compounds in which concerns to their adhesion to PS surfaces. This behavior is in accordance with the antimicrobial susceptibility of these bacteria to the selected compounds, as proven by MIC and MBC determination.

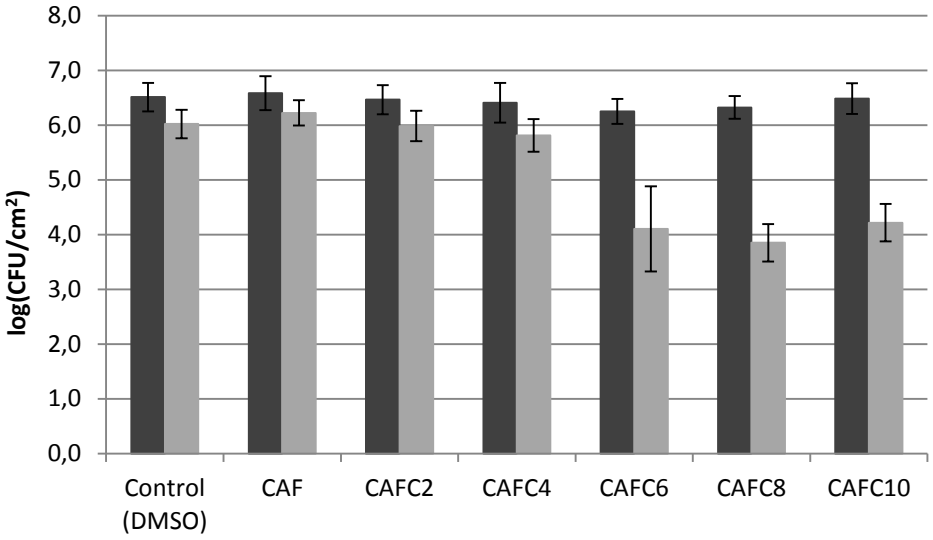



**Fig. 8 – Adhesion of *E. coli* (■) and *S. aureus* (■) cells to PS coupons, after exposure to the selected compounds at 0.1 mM, for 1 h. The means ± SD of cell logs (i.e., log[CFU/cm$^2$]) are shown.**

Regarding adhesion to silicone surfaces (Fig. 9), a similar behavior is observed, when comparing to PS. For *E. coli* cells, adhesion was also observed (this time, as was expected) and no distinction was also observed between the extent of adhesion of untreated and treated cells ($p$>0.05), regardless of the compound used. As for *S. aureus*, adhesion was also observable, as thermodynamically predicted, but only exposure to CAFC8 caused a significant variation in the adhesion of cells to silicone ($p$<0.05), with a log reduction < 1, but even so, a percentage reduction of c.a. 76%. When comparing *E. coli* and *S. aureus* adhesion to silicone, a similar conclusion to the one withdrawn from PS adhesion can be reached: *E. coli* cells were less susceptible to the action of the selected compounds, presenting higher adhesion rates

than *S. aureus*, regardless of the size of the alkyl ester chain. In reality, the percentage reduction of *S. aureus* adhesion when comparing to *E. coli* rounds the 96% for untreated conditions and, in general, over 90% for the treated ones.

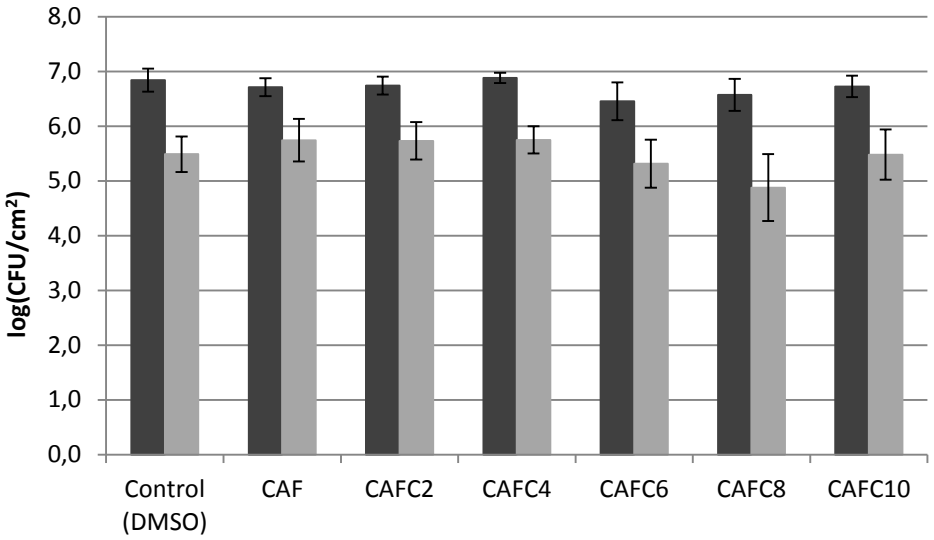



**Fig. 9 - Adhesion of *E. coli* (■) and *S. aureus* (■) cells to silicone coupons, after exposure to the selected compounds at 0.1 mM, for 1 h. The means ± SD of cell logs (i.e., log[CFU/cm$^2$]) are shown.**

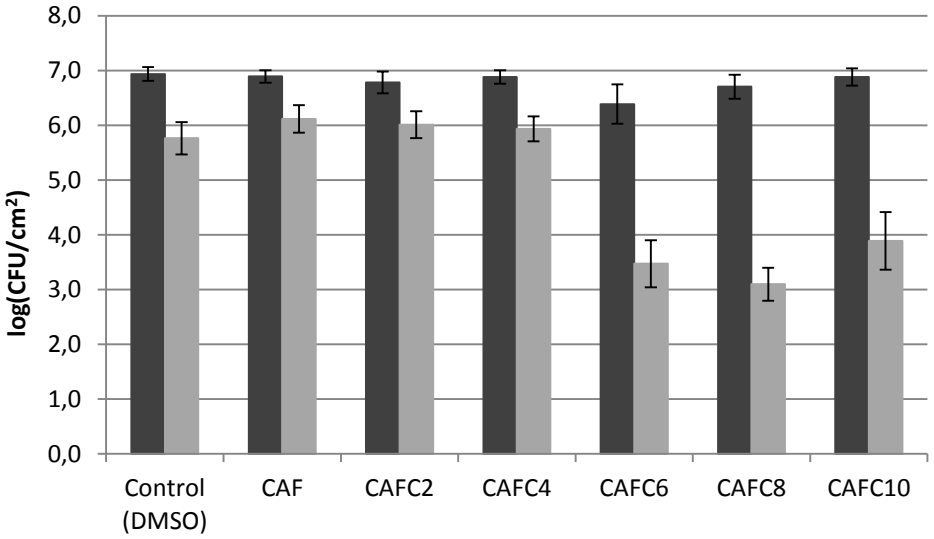



**Fig. 10 - Adhesion of *E. coli* (■) and *S. aureus* (■) cells to SS coupons, after exposure to the selected compounds at 0.1 mM, for 1 h. The means ± SD of cell logs (i.e., log[CFU/cm$^2$]) are shown.**

Additionally, taking into account the adhesion of *E. coli* cells to SS surfaces (Fig. 10), one can observe that no significant variations of adhesion levels occurred for the different compounds tested ($p$>0.05), except for CAFC6 ($p$<0.05), where a log reduction of 0.5 was shown, which translates into a decrease of 72% on the number of *E. coli* cells that adhere to

the SS coupons, when comparing to the control. Interestingly, CAFC6 was also the compound that revealed to be the most effective in adhesion prevention for *E. coli* on SS, in the thermodynamic predictions made. As for *S. aureus*, adhesion was less favored for cells exposed to CAFC6, CAFC8 and CAFC10, with cell log values that are significantly different from the untreated situation ($p$<0.05). For these cases, log reduction of 2.1, 2.7 and 1.9 were observed, respectively, which translates into percentage reductions of 99.5, 99.8 and 98.7% for the number of adhered cells on the SS surfaces. This means that these compounds practically inhibited bacterial adhesion to stainless steel. Moreover, also for this material, adhesion of the Gram-negative bacterium was, in a general perspective, higher than for the Gram-positive bacterium ($p$<0.05).

**Table 6 - Comparison between the percentage reductions of bacterial cell adhesion in *E. coli* for the different materials tested.**

| | | Adhesion percentage reduction (%) | |
|---|---|---|---|
| | | **PS *vs*. SS** | **PS *vs.* Silicone** |
| ***E. coli*** | **Control** | 62.2 | 52.9 |
| | **CAF** | 50.7 | 25.3 |
| | **CAFC2** | 52.0 | 47.2 |
| | **CAFC4** | 66.6 | 66.4 |
| | **CAFC6** | 26.7 | 37.4 |
| | **CAFC8** | 58.4 | 43.3 |
| | **CAFC10** | 59.9 | 42.4 |

Lastly, comparing amongst materials, it appears that for *E. coli* cells, PS was the material upon which less bacterial adhesion was observed. In Table 6, it is possible to verify that this was the case for all cells, regardless of the compound used for treatment. Moreover, it is possible to observe that this difference in bacterial adhesion for PS surfaces was most significant for cells exposed to CAFC4. However, CAFC6 was the compound which globally caused a worst interaction between cells and the surfaces, decreasing adhesion levels for all three materials, as proven by the lowest percentage difference between PS and the remaining materials.

Yet, for *S. aureus*, it is not possible to distinguish between materials for which was the one that rendered lower adhesion levels. Nevertheless, considering that CAFC6, CAFC8 and CAFC10 were the compounds for which bacterial adhesion of *S. aureus* was minimized for all materials, it is possible to perceive that silicone was the material that rendered smaller variations of cell log for cells treated with the abovementioned compounds. In fact, bacterial

adhesion was 93.8, 90.6 and 94.6% higher for these compounds (respectively) in silicone than PS and 98.6, 98.3 and 97.4% higher (respectively) in silicone than in SS. That said, for cells treated with these larger alkyl caffeates, silicone rendered higher adhesions and thus, made cells more resistant to antimicrobial activity and adhesion prevention. In fact, besides being thermodynamically favorable for bacterial adhesion (Simões *et al.* 2007), silicone has also been shown to allow significant amounts of non-specific protein adsorption, which might, in its turn, favor bacterial adhesion (Lin *et al.* 2011).

Nonetheless, it is worth mentioning that CFU counts often lead to an underestimation of viable cells, due to the presence of injured/starved cells and/or of potentially "viable but non-culturable" cells (Ferreira *et al.* 2010). In this view, the obtained adhesion results might be underestimated and would require a cell viability assessment to eliminate this putative error factor.

### 4.3.2. Quorum sensing inhibition

QSI has been suggested to be one of the effective mechanisms through which prevention of bacterial adhesion may be achieved (Borges *et al.* 2014b). In this manner, the caffeic acid alkyl esters were studied for their ability to inhibit QS, thus suggesting a mechanism for their activity as cell adhesion inhibitors.

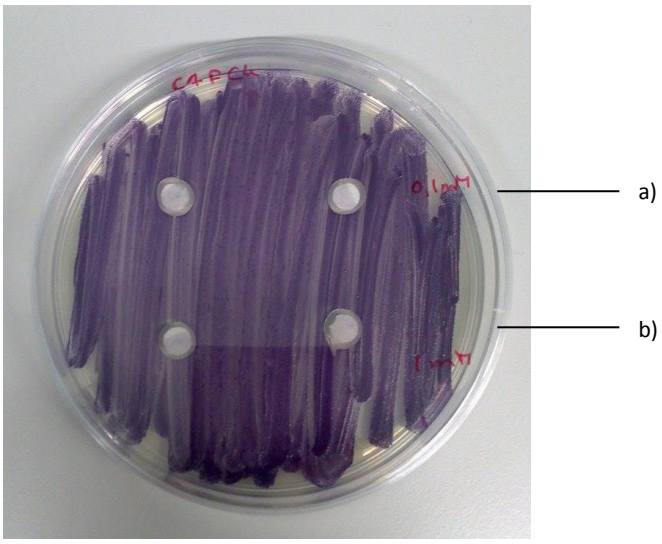



**Fig. 11 - Example of a standard disk diffusion assay for QSI determination in *C. violaceum*, with CAFC4 at concentrations of a) 0.1 mM and b) 1 mM.**

A standard disk diffusion assay (Fig. 11) was chosen as the bioassay for the detection of QSI. In this way, an inhibition of the production of violacein by *C. violaceum* would indicate

inhibition of cell-to-cell communication and thus prove caffeic acid esters as active agents against QS and, consequently, against biofilm formation.

Despite the fact that the biosensor used for this purpose was different from the microorganisms upon which this project was based, a concentration of 0.1 mM for each compound was also used, both to preserve result comparability and because bacterial susceptibility to caffeic acid in *C. violaceum* is in the same magnitude range (Borges *et al.* 2014b) as the ones found in this study for *E. coli* and *S. aureus*. That said, it was assumed that a similar behavior would be observed for the caffeic acid alkyl esters. Furthermore, a 10-times higher concentration (1 mM) was tested.

For the compounds tested, only one halo was observed: a clear halo, typical of bacterial growth inhibition, demonstrating the antimicrobial activity of the compounds. However, as no opaque halo (corresponding to bacterial growth without production of violacein) was visible, besides the one previously referred to, it can be concluded that caffeic acid and its alkyl esters did not cause QSI in *C. violaceum*. The growth inhibition halos measured for each compound are depicted in Fig. 12.

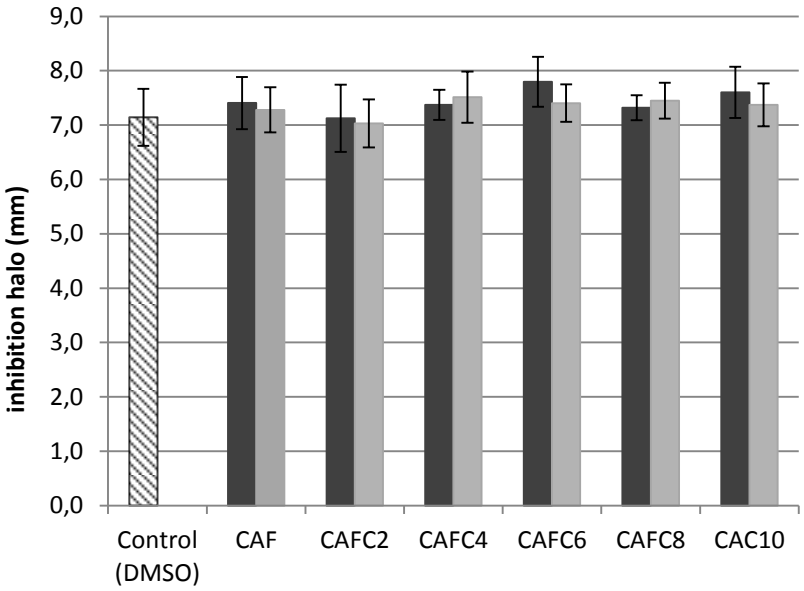



**Fig. 12 - Growth inhibition halos observed for *C. violaceum* subjected to 1 mM (■) and 0.1 mM (■) of the compounds tested.**

The growth inhibition halos did, nonetheless, appear to be very similar and no significant difference ($p$>0.05) was observed either between the control sample (with DMSO) and the compounds tested, for each concentration, or between different concentrations, for each compound tested. So, in fact, it cannot be assumed that the compounds had any

antimicrobial activity and, most likely, the inhibition halos observed were only a direct cause of the dispersion of the DMSO into the LBA plate.

However, several factors, such as different susceptibility of *C. violaceum* to the test compounds or limitations of the QSI assay used, may be implicated in the obtained results and, thus, no final conclusions regarding the QSI activity of these alkyl caffeates may be withdrawn. In fact, the application of *C. violaceum* as a biosensor is a very preliminary and speculative approach, as it only represents one specific QS system that might respond differently than the QS systems of *E. coli* and will definitely respond differently than the QS system of *S. aureus*, which, as a Gram-positive bacterium, uses peptides and not AHL-type molecules as autoinducers (Nazzaro *et al*. 2013). Moreover, the MIC of the test compounds for *C. violaceum* should have been previously determined, in order to define the real susceptibility of the bacteria to these molecules. As an example, Borges *et al.* (2013b) reported a violacein inhibition of 75% for caffeic acid at 1000 µg/mL, i.e., at 5.5 mM (a concentration which is more than 5-times greater than the higher concentration tested), even though caffeic acid did not show any positive result for QSI in the disk diffusion assays. This also indicates that other methods to asses QSI must be considered, once phenolics such as caffeic acid and caffeic acid alkyl esters interfere with membrane permeability, as previously discussed, which might, in its turn, affect the flux of AHLs and not its synthesis (Borges *et al*. 2014b).

## 4.4. Conclusions

In the quest for new antibacterial products that restrict the ability of bacteria to adhere, communicate and, consequently, prevent biofilm formation, *S. aureus* and *E. coli* adhesion to surfaces was studied under the influence of a homologous series of alkyl caffeates.

Thermodynamic predictions based on theoretical models rendered PS as the surface material that would promote lower levels of bacterial adhesion for both bacteria tested. Silicone was predicted to stimulate adhesion for both bacteria and SS to cause *E. coli* adhesion to be unfavorable and *S. aureus* adhesion to be favorable when subjected to the action of most of the selected compounds, except caffeic acid. Cells exposed to caffeic acid and alkyl esters CAFC2, CAFC4 and CAFC6 were thermodynamically less prone to adhere to surfaces, whilst cells treated with CAFC8 and CAFC10 were predicted to adhere to surfaces. In general, adhesion was thermodynamically less favorable for the Gram-negative bacterium and CAF and

CAFC6 were the compounds which, regardless of the surface material caused *S. aureus* and *E. coli*, respectively, to be less likely to adhere.

However, despite thermodynamic predictions, adhesion assays revealed different patterns than the ones predicted. Firstly, bacterial adhesion was observed for both bacteria, for all three materials tested, independently of the thermodynamic predictions. *E. coli* cells adhered to surfaces in a greater extent than *S. aureus* even though the opposite was expected. This observation is in accordance with the antimicrobial susceptibility of these bacteria to the selected compounds, as proven by MIC and MBC determination, since *E. coli* cells were less susceptible to their action. Moreover, PS was in fact, the material which rendered lower adhesion levels for *E. coli*, while silicone was the material which caused the most resistant bacterial adhesion for both bacteria, with antimicrobial treatment being pretty much ineffective in inhibiting bacterial adhesion of *E. coli* for all compounds tested, as well as for *S. aureus*, for which only CAFC8 produced significant cell log variations. Finally, for both PS and SS, CAFC6 was the only compound to cause a significant decrease in *E. coli* adhesion (45% and 72% percentage reduction, comparing to the control), whilst CAFC6, CAFC8 and CAFC10 practically inhibited *S. aureus* bacterial adhesion (percentage reductions higher than 98% for all cases), even though those compounds were the ones that, thermodynamically, favored most significantly adhesion of the Gram-positive bacterium.

In this view, it is proven that alkyl caffeates do possess an ability to inhibit biofilm formation by decreasing the extent of the first adhesion steps. These compounds were much more effective in preventing biofilm formation for the Gram-positive bacterium (which was more susceptible to the action of the compounds), especially in PS and SS surfaces, where the longer alkyl esters of the series were able to practically inhibit bacterial adhesion. *E. coli* adhesion was only affected (yet, only at reasonable levels) by CAFC6, a compound with a medium length alkyl ester chain that was implicated in increasing the bacterial polarity and electron acceptor properties of *E. coli* in the hydrophobicity studies.

Furthermore, QSI has been suggested to be one of the effective mechanisms through which prevention of bacterial adhesion may be achieved. Nevertheless, no QSI activity or significant antimicrobial activity was observed for any of the caffeic acid alkyl esters or caffeic acid itself, in *C. violaceum.* The fact that *C. violaceum* is a biosensor that only represents one specific QS system and that the method used to determine QSI is very limited leads to the conclusion that further analysis is required before discarding QSI as one of the mechanisms employed by caffeic acid alkyl esters to prevent bacterial adhesion, apart from cell membrane destabilization.

# Chapter 5

## 5. Concluding Remarks and Research Needs

### 5.1. General Conclusions

Caffeic acid alkyl esters proved to be effective antimicrobial agents for both Gram-positive and Gram-negative bacterium in both planktonic and biofilm states.

Bacterial susceptibility to these phytochemical derivatives was much superior to that observed for caffeic acid, proving that the presence of the alkyl ester side chain contributed significantly for the antimicrobial activity of these compounds. In fact, a clear tendency for increasing antimicrobial activity with increasing length of the alkyl ester side chain was verified. Bacterial growth inhibition in the planktonic state was maximized for the octyl and decyl esters of caffeic acid (CAFC8 and CAFC10) for both bacteria, whilst bactericidal activity was maximized for the butyl ester of caffeic acid (CAFC4) in *E. coli* and for CAFC8 in *S. aureus*.

The observed effects of caffeic acid alkyl esters in planktonic bacteria include alterations in the physicochemical properties of both bacteria with parabolic variations dependent on the alkyl ester length, namely hydrophobicity and polarity. In contrast, surface charge was not significantly affected by the action of these compounds. The modifications on the physicochemical properties of the bacteria led to a proposed mechanism of action for this family of compounds. Accordingly, it is proposed that these compounds act as permeabilizers and, due to their amphiphilic character, caffeic acid and its alkyl ester derivatives disturb the bacterial membrane and induce its permeabilization due to a possible acidification of the cytoplasm and subsequent local rupture and pore formation in the membranes, which might lead to leakage of cytoplasmatic constituents and cell death. The antimicrobial activity of these compounds is thus associated with a fine balance between affinity for the lipid bilayers of cell membranes and the ability to cause disruption of the membrane structure, which visibly differs from Gram-positive to Gram-negative bacterium and depends on the length of the alkyl ester chain of the compounds.

On the other hand, the effects of caffeic acid alkyl esters in the first steps of biofilm formation were also established. The compounds proved to be effective in impairing bacterial

adhesion of *E. coli* and *S. aureus* to surfaces, but QSI was not possible to correlate to these effects as no observable influence in cell-to-cell communications was verified in *C. violaceum*.

Although bacterial adhesion was always observed for both bacteria exposed to the antimicrobial agents, in all three materials tested, it was almost completely inhibited in some cases, namely for *S. aureus* cells exposed to CAFC6, CAFC8 or CAFC10, which presented adhesion inhibitions of over 98% in PS and SS surfaces, and for *E. coli* cells exposed to CAFC6, which showed adhesion inhibitions of 45% and 72% in PS and SS, respectively. Adhesion to silicone was, however, not effectively inhibited by exposure to the caffeic acid esters, once this material induced stronger and more resistant bacterial adhesions.

In summary, the alkyl caffeates were much more effective in preventing bacterial growth and biofilm formation for the Gram-positive bacterium, which were more susceptible to the action of these antimicrobials than Gram-negative bacterium, as demonstrated by MIC and MBC values and lower adhesion levels. Therefore, *E. coli* cells were more resistant to the antimicrobial action of the selected compounds, apparently due to the composition of their cell walls, which are considered to be an additional source of bacterial resistance.

Overall, the structure-activity relationship study conducted showed that caffeic acid is a potential scaffold for antimicrobial agents development, by proving the influence of the addition of an alkyl ester side chain on the antimicrobial potentiation of this phenolic acid. To sum up, caffeic acid alkyl esters were more effective than caffeic acid in bacterial control in both the planktonic and the sessile states. However, the influence of the alkyl side chain length is not yet fully understood, once no obvious pattern was observed apart from the fact that longer alkyl side chain compounds had better results in inhibiting bacterial growth and bacterial adhesion for the Gram-positive bacterium, while medium length alkyl side chain compounds were more effective for the Gram-negative bacterium. These compounds are considered as green and have the potential to circumvent the current problems of bacterial resistance.

## 5.2. Future Work

In a final analysis, several aspects still need to be clarified in order to completely understand the effects of caffeic acid alkyl esters in the control of planktonic and sessile cells.

First of all, for a better understanding of the adhesion inhibition activity of these compounds, motility of bacterial cells upon exposure to these compounds should be

investigated. As a matter of fact, impairment of motility is, like QSI, an attractive mechanism to prevent bacterial adhesion and biofilm formation. Coupled with this suggestion, comes the fact that, in this project, *C. violaceum* was used as a biosensor for QSI activity. However, *C. violaceum* only represents one specific QS system and, in order to maintain conformity in the study and completely understand the influence of these compounds in QS, other biosensors should have been used, such as genetically modified *E. coli* or *S. aureus* that allow QSI detection. In addition, other QS detection methods should also be attempted, once the disk diffusion method does not make it possible to actually know the exact amount of violacein inhibition.

Secondly, the proposed mode of action of these compounds should be further analyzed. For that purpose, a $K^+$ leakage assessment is suggested, in order to evaluate the veracity of the assumptions made regarding membrane permeabilization and cytoplasmatic outflow through pore and local rupture formation in the membrane.

Moreover, CFU counts in the adhesion assays should be replaced and/or validated with a cell viability assessment (such as epifluorescence microscopy with viability-indicator stains), so as to eliminate errors arising from underestimation of viable cells characteristic of CFU quantification due to the existence of “viable but non-culturable” cells that, despite losing the ability to grow on media typically used for culture, remain alive and capable of regaining their metabolic activity once conditions become favorable.

A final suggestion comprises the testing of caffeic acid alkyl esters in synergism with classic antibiotics in order to assess if their ability as RMAs surpasses their ability to act as sole antimicrobial agents.

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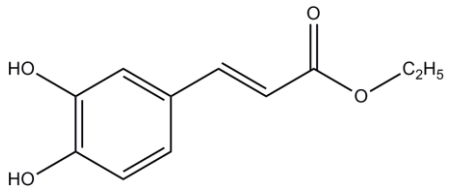
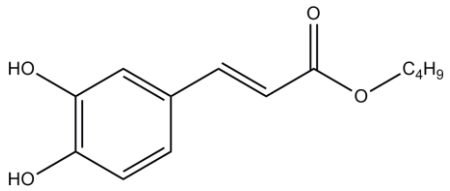
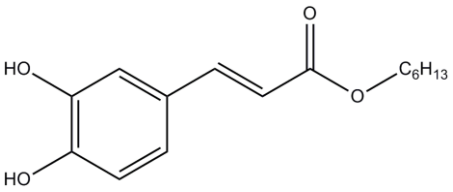
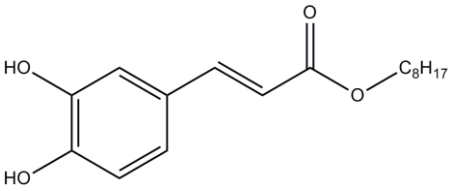
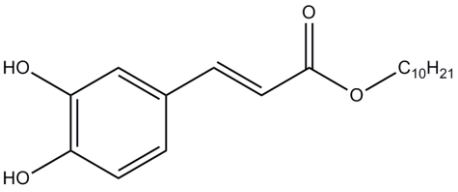
# APPENDIX

## A. Caffeic alkyl esters (general data and synthesis)

**Table A. 1 - Chemical structure and molecular weight of the caffeic acid esters tested.**

| | | Molecular Weight (g/mol) |
|---|---|---|
| CAFC2 |  | 208.2101 |
| CAFC4 |  | 236.2631 |
| CAFC6 |  | 264.3169 |
| CAFC8 |  | 292.3701 |
| CAFC10 |  | 320.4232 |

The caffeic acid esters were chemically synthetized by someone else using similar protocols to the ones described by Garrido *et al.* (2012) and Menezes *et al.* (2011), for short and long chain alkyl hydroxycinnamates, respectively. After purification, the compounds were identified by spectroscopy (NMR and MS). The following $^{1}H$ NMR spectra were obtained.

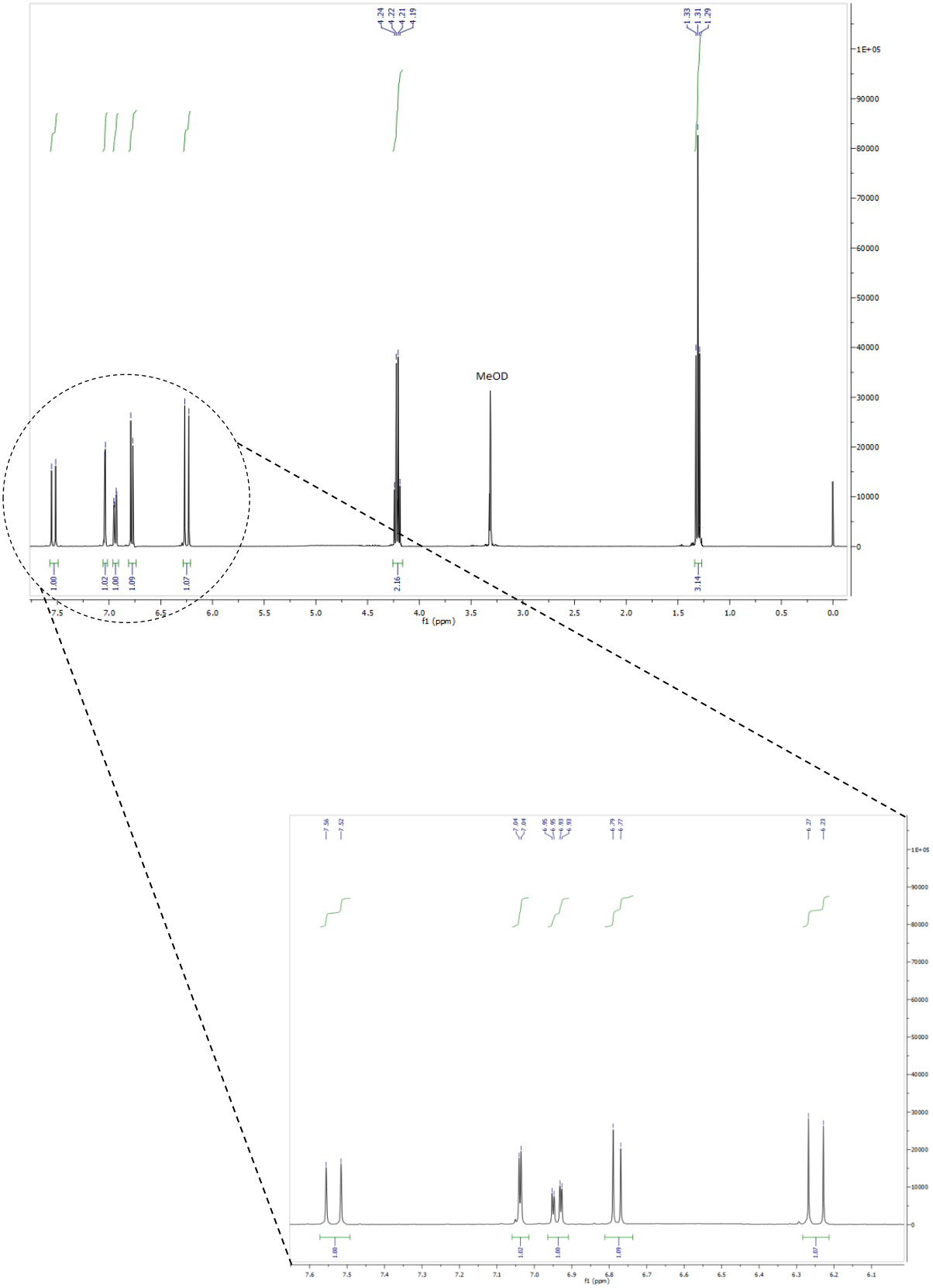



**Fig. A. 1 – $^{1}$H NMR spectra for CAFC2.**

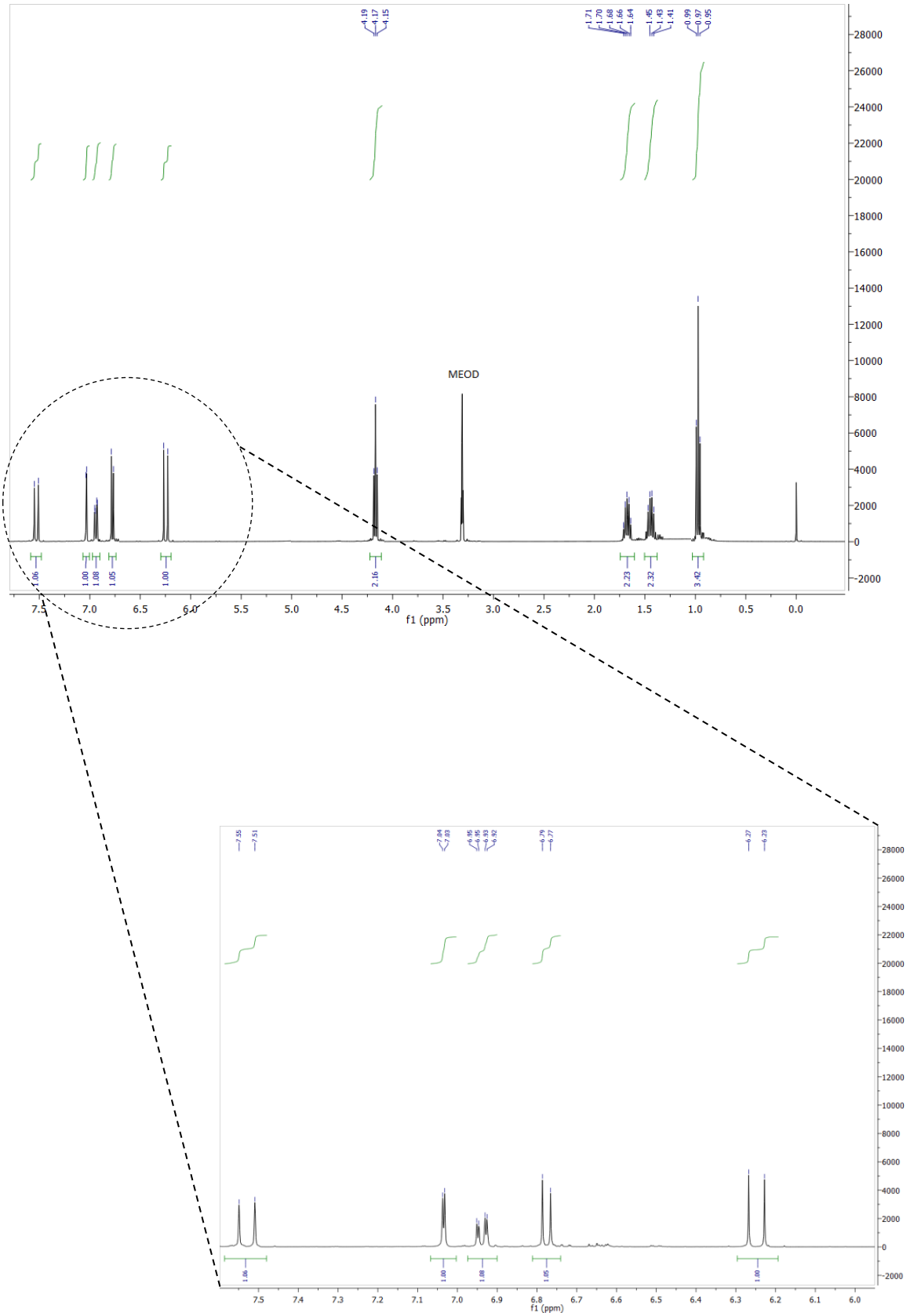



**Fig. A. 2 – $^{1}$H NMR spetra for CAFC4.**

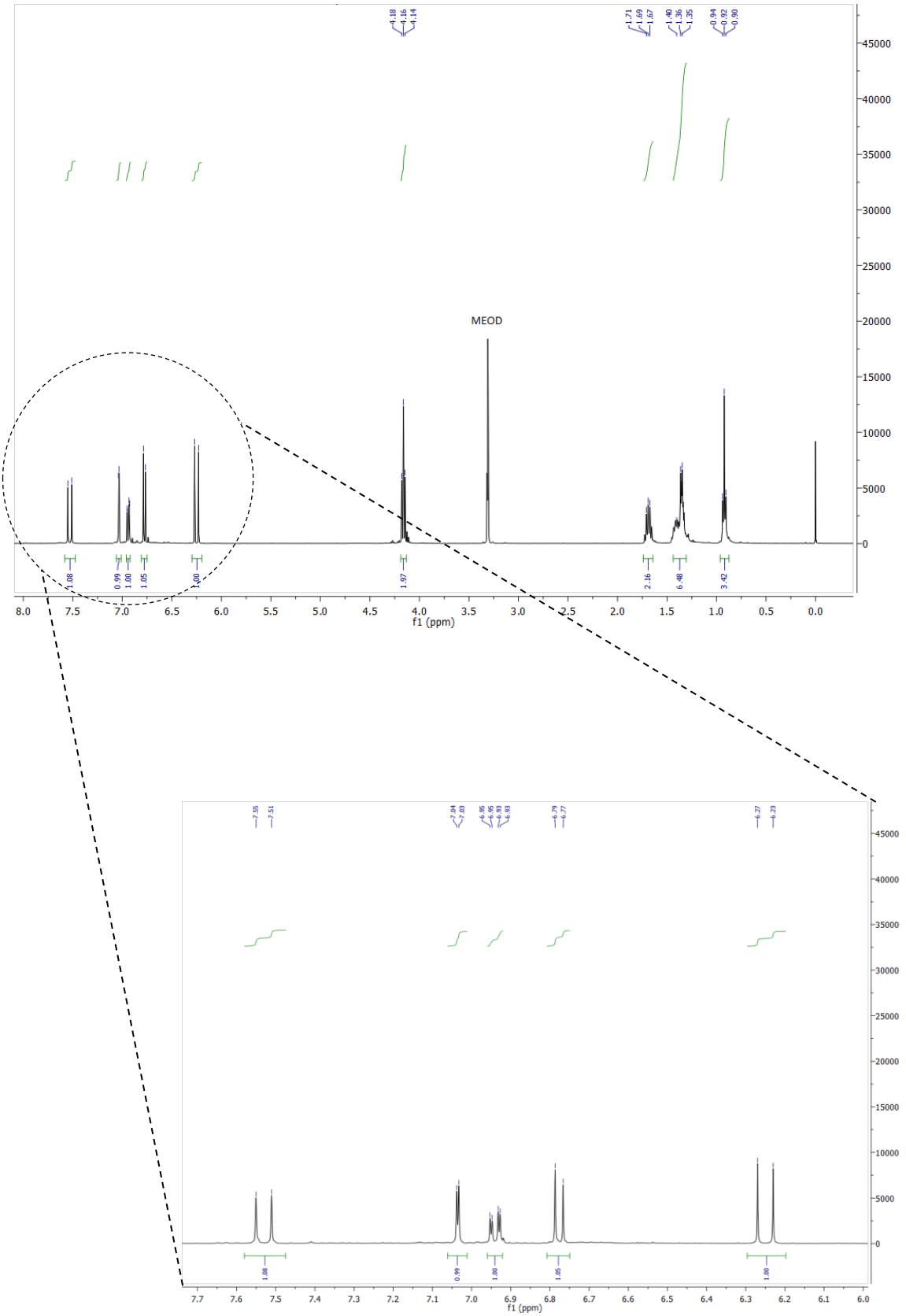



**Fig. A. 3 – $^{1}$H NMR spectra for CAFC6.**

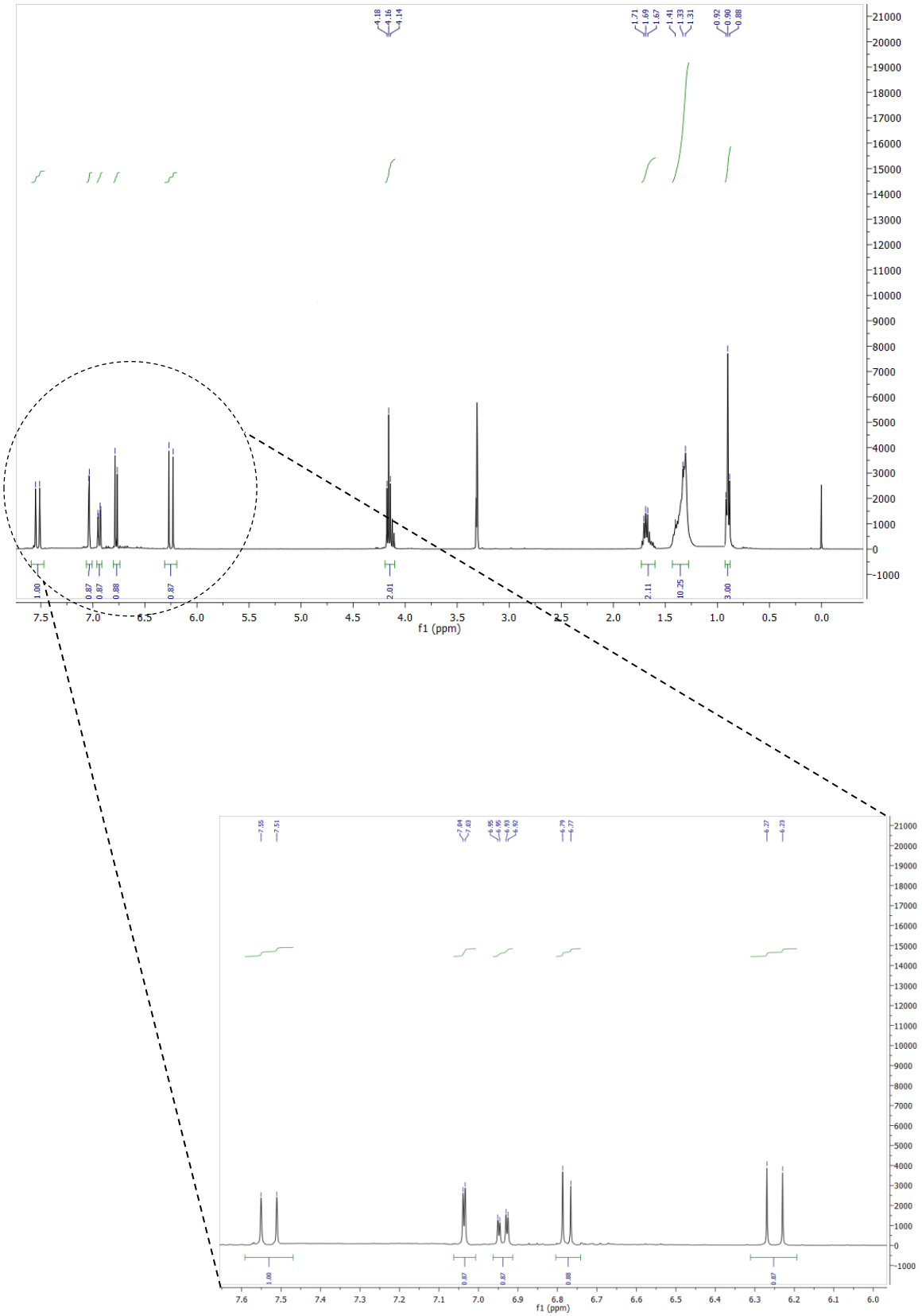



**Fig. A. 4 – $^{1}$H NMR spectra for CAFC8.**

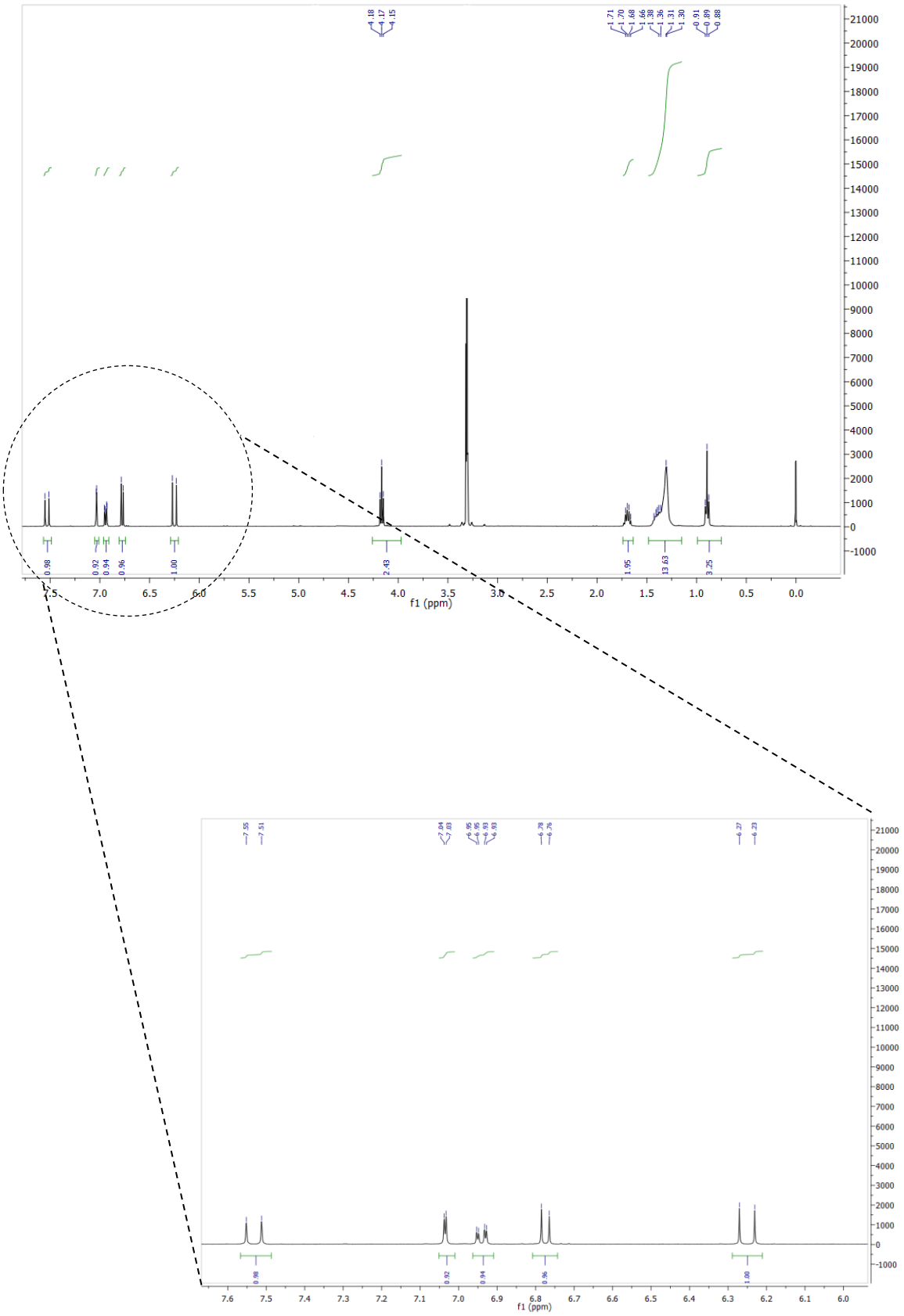



**Fig. A. 5 – $^{1}$H NMR spectra for CAFC10.**

## B. MIC and MBC determination: range of concentrations tested

**Table A. 2 – Range of concentrations tested for MIC and MBC determination in *E. coli* and *S.aureus*.**

| Compound | Concentrations tested (mM) * | |
|---|---|---|
| | *E. coli* | *S. aureus* |
| CAF | 6.5 – 52 | 6.5 – 52 |
| CAFC2 | 0.4 – 19.5 | 0.4 – 19.5 |
| CAFC4 | 0.2 – 13 | 0.2 – 13 |
| CAFC6 | 0.05 – 13 | 0.1 – 3.25 |
| CAFC8 | 0.025 – 19.5 | 0.025 – 0.8 |
| CAFC10 | 0.025 – 19.5 | 0.025 – 0.8 |

* In between the range of concentrations presented, serial two-fold or 3/4-fold dilutions were prepared.