Faculty of Engineering of University of Porto

Integrated Master in Bioengineering

Antimicrobial activity of phenyl isothiocyanate on Escherichia coli and Staphylococcus aureus cells in planktonic and biofilm states

Dissertation for Master degree in Biological Engineering

Ana Cristina Ralha de Abreu

Supervisor: Prof. Manuel Simões

July, 2011

“There are two ways to live: you can live as if nothing is a miracle;
you can live as if everything is a miracle.”

Albert Einstein

Acknowledgments

Para a realização desta dissertação foram vários os intervenientes que colaboraram directa e indirectamente, os quais merecem o meu reconhecimento e gratidão.

Ao meu orientador, Professor Doutor Manuel Simões, por toda a dedicação, empenho e disponibilidade com que direccionou e acompanhou esta dissertação.

Quero agradecer também a todos do Laboratório E-303, Madalena, Margarida, Renato, Carla, Joanas, Dina, Paula e um especial obrigado à Anabela que me ajudou inúmeras vezes ao longo da realização do trabalho.

A todos os meus amigos e, principalmente, à Joana, Rita, Diogo e Luciana que nestes últimos meses me apoiaram e me proporcionaram óptimos momentos.

Finalmente, de modo especial, quero agradecer à minha família, pais, irmãs e ao Filipe, pelo apoio incondicional, incentivo e compreensão imprescindíveis para a realização deste trabalho.

Abstract

The inappropriate use and prescription of antibiotics to treat several and common bacterial infections contribute to the propagation of resistant variants. This situation has resulted in the constant research and development of new antimicrobials in order to maintain several effective drugs on the market. Plants are considered the best source to obtain these products since they synthesize a diverse array of secondary metabolites (phytochemicals) that protect the plant against microbial pathogens.

The purpose of the present study was to assess the *in vitro* antibacterial effects of phenyl isothiocyanate (PITC), a glucosinolate hydrolysis product (GHP) existing in several plants, against strains of *Escherichia coli* and *Staphylococcus aureus*. This activity was evaluated by disc diffusion assay. Dual combinations of several antibiotics with PITC were also performed. PITC (at 1000 µg/mL) had significant antimicrobial activities on both strains and it can act synergistically with erythromycin and ciprofloxacin in *S. aureus* strains. Physico-chemical characterization of bacterial surface by zeta potential and contact angle measurements was performed and indicated that, when incubated with PITC, the bacterial surface is less negative and more hydrophilic. The Live/Dead *Bac*Light™ kit was applied and demonstrated that, when cells were incubated with PITC for 1 hour, about 70% of the population had membrane damages.

To evaluate the antimicrobial activity of PITC in biofilm, single and multi-species biofilms of *E. coli* and *S. aureus* were developed in 96-well polystyrene microtiter plates. Biofilm mass was quantified by crystal violet staining and activity by an assay with resazurin. These bacteria were considered weak biofilm producers. The biofilm formation assays with PITC demonstrated that this compound can penetrate the biofilms efficiently and that cells (especially *S. aureus*) can adapt to this compound when a sub-lethal concentration is used. Moreover, although most biofilm were not viable at the end of the experiments, it is necessary to emphasize that bacteria could develop biofilm in the presence of PITC. The highest removal percentages of biofilms were obtained with the addition of PITC at 5000 µg/mL to cells previously exposed to this compound: 85 and 89% for *E. coli* and *S. aureus*, respectively. At this concentration, cells could not resist to PITC after a long period of exposure. The relationship between physicochemical surface parameters and adhesion of bacterial cells to polystyrene (PS) was studied. Results indicate that adhesion was underestimated when based on thermodynamic approaches and that, with PITC, bacteria have even less ability to adhere to PS. So, hydrophobic differences of cells caused by PITC have influence in the adhesion probably due to the disruption of membrane-membrane adherence. Finally, swimming, swarming and twitching motilities of the strains were evaluated. Swim was the main type of motility found for

both strains in this study. Even though *E. coli* had a greater motility, *S. aureus* is a greater biofilm producer and has more capacity to adhere to PS. So, motility of these strains does not regulate adhesion and biofilm formation. With PITC at 5000 µg/mL, motility was insignificant. PITC had always more effects for *E. coli* than for *S. aureus,* probably because *S. aureus* could develop more mechanisms of resistant to PITC.

In conclusion, PITC seems to be a promising product for antimicrobial therapy against the bacteria tested, since this compound was efficient in killing bacteria, even in biofilm. The action of PITC may involve a direct effect on adhesion, in addition to enzyme inactivation, since PITC can disrupt membrane-membrane adherence. More date is necessary about the toxicity of PITC to be used as therapeutic agent in human.

Resumo

O consumo excessivo e inadequado de antibióticos no tratamento de várias infecções tem contribuído para a propagação de estirpes bacterianas resistentes. Perante esta situação, têm sido realizados inúmeros trabalhos e pesquisas nos últimos anos com o objectivo de encontrar e aperfeiçoar novos agentes antimicrobianos de forma a manter vários medicamentos eficazes no mercado. As plantas são consideradas a melhor fonte para obtenção destes produtos uma vez que sintetizam um conjunto diversificado de metabolitos secundários (fitoquímicos) que protegem a planta contra agentes microbianos patogénicos.

O objectivo deste estudo é avaliar o efeito antimicrobiano *in vitro* do fenil isotiocianato (PITC) em estirpes de *Escherichia coli* e *Staphylococcus aureus*. Este composto é um produto da hidrólise de glucosinolatos (GHP) e existe em diversas famílias de plantas. A actividade antimicrobiana foi quantitativamente avaliada a partir do método de difusão em disco. De igual forma, a combinação do PITC com vários antibióticos foi testada de modo a averiguar a capacidade de potenciação do composto. Os resultados demonstram que o PITC apresenta uma actividade antimicrobiana significativa em ambas as bactérias e um efeito aditivo quando combinado com eritromicina e ciprofloxacina em *S. aureus*. A caracterização físico-química da superfície bacteriana (através de medições do potencial zeta e ângulos de contacto) indica que as células, na presença de PITC, ficam menos negativamente carregadas e mais hidrofílicas. Para averiguar a acção do PITC na integridade da membrana, aplicou-se o kit Live/Dead *Bac*Light™. Com este método, concluiu-se que, quando as células são incubadas com o PITC por uma hora, o número de células mortas é de cerca 70% da população total, o que indica que o PITC pode realmente causar danos da membrana citoplasmática.

Para avaliar a actividade antimicrobiana do PITC em biofilme, foram desenvolvidos biofilmes formados por espécies únicas e múltiplas das mesmas bactérias em microplacas de poliestireno com 96 poços. O biofilme formado foi quantificado através da coloração com violeta de cristal e a viabilidade das células foi avaliada pela resazurina. Ambas as bactérias foram consideradas fracas produtoras de biofilme nas condições experimentais testadas. Os ensaios de formação de biofilme com PITC demonstraram que este composto consegue penetrar os biofilmes de forma eficiente e que as células (especialmente *S. aureus*) podem adaptar-se a este composto perante uma concentração sub-letal do mesmo. Para além disso, as bactérias conseguiram desenvolver biofilme na presença de PITC, mesmo que no final da experiência este já não se encontre viável. As percentagens mais elevadas de remoção de biofilmes foram obtidas com a adição de PITC a 5000 µg/mL em células previamente expostas a este composto: 85 e 89% para *E. coli* e *S. aureus*, respectivamente. A esta concentração, as células não conseguiram resistir e adaptar-se ao PITC. A relação entre os parâmetros físico-químicos da

superfície celular e a adesão das células bacterianas ao poliestireno (PS) foi estudada. Os resultados indicam que a adesão foi subestimada quando baseada apenas em abordagens termodinâmicas e que, na presença de PITC, as bactérias têm ainda menos capacidade de aderir ao PS. Deste modo, diferenças hidrofóbicas das células causadas pelo PITC influenciam negativamente a adesão. Finalmente, as mobilidades *swimming*, *swarming e twitching* das estirpes foram avaliadas. *Swimming* foi o principal tipo de mobilidade encontrada para ambas as bactérias neste estudo. Apesar de a *E. coli* apresentar uma maior mobilidade, *S. aureus* é maior produtora de biofilme e tem uma capacidade superior de aderir ao PS. Assim, a mobilidade das bactérias não regula a adesão e formação de biofilme. Com PITC a 5000 µg/mL, as células não se adaptaram ao composto após um longo período de exposição e a mobilidade apresentada foi insignificante. PITC teve normalmente um efeito superior em células de *E. coli* comparando com *S. aureus*, provavelmente porque *S. aureus* consegue desenvolver mais facilmente mecanismos de resistência ao PITC.

O trabalho realizado permite concluir que o PITC parece ser promissor para a terapia antimicrobiana das bactérias testadas uma vez que os resultados indicam que este composto foi eficiente em eliminar bactérias, mesmo em biofilme. A acção do PITC pode envolver um efeito directo na adesão das células para além da inactivação enzimática, uma vez que o este composto induz alterações fisiológicas na hidrofobicidade e carga superficial das células o que sugere a possível desintegração da membrana. Para que este possa ser usado como agente terapêutico em humanos, muitos mais estudos são necessários, bem como mais dados sobre a toxicidade deste composto *in vivo*.

Content List

Acknowledgments I
Abstract IX
Resumo XI
Content List XIII
Figures List XV
Tables List XVI
Glossary XVII
1. Work outline 17
1.1. Background and Project Presentation 17
1.2. Main Objectives 18
1.3. Thesis Organization 19
2. LITERATURE REVIEW 20
2.1. Plant compounds with antimicrobial activity 20
2.2. Mode of action of isothiocyanates 21
2.3. Citotoxicity and benefices of isothiocyanates 22
2.4. Antibiotic resistance in cells 24
2.5. Biofilm: the main cause of bacterial infections 26
2.5.1. Development of a biofilm 27
2.5.2. Microbial adhesion to a surface 28
2.5.3. The influence of motility in biofilm formation 30
2.5.4. Mechanisms of Antibiotic Resistance in biofilms 31
3. EFFECT OF PHENYL ISOTHIOCYANATE ON PLANKTONIC BACTERIA 33
3.1. Introduction 33
3.2. Material and Methods 35
3.2.1. Bacterial Strains 35
3.2.2. Phenyl isothiocyanate and Antibiotics 35
3.2.3. Determination of minimum inhibitory concentrations (MICs) 35
3.2.4. Antibacterial Activity Assessment 36
3.2.5. Antibiotic-Phytochemical Dual Combinations Assay 36
3.2.6. Antibacterial Activity Classification 36
3.2.7. Determination of Zeta Potential 37
3.2.8. Physico-chemical characterization of bacterial surface 37

3.2.9. Assessment of membrane integrity due to propidium iodide uptake *38*
3.2.10. Statistical Analysis *39*
3.3. Results and Discussion 40
3.3.1. Antimicrobial activity of PITC alone and in combination with Antibiotics *40*
3.3.2. Surface characterization of cells in presence of PITC *42*
3.4. Conclusions 46
4. THE EFFECTS OF PHENYL ISOTHIOCYANATE ON SINGLE AND MULTI-SPECIES BIOFILM CONTROL 50
4.1. Introduction 50
4.2. Material and Methods 52
4.2.1. Bacterial Strains and Phenyl isothiocyanate *52*
4.2.2. Biofilm formation assay *52*
4.2.3. Classification of biofilm producer bacteria *53*
4.2.4. free energy of adhesion *54*
4.2.5. Motility *54*
4.2.6. Statistical Analysis *55*
4.3. Results and Discussion 56
4.3.1. Inhibition of biofilm formation by PITC in single species *56*
4.3.2. Multi-Species biofilm control with PITC *61*
4.3.3. Analysis of bacteria adhesion *63*
4.3.4. Motility assays *65*
4.4. Conclusions 68
5. CONCLUDING REMARKS AND PERSPECTIVES FOR FURTHER RESEARCH 69
5.1 General conclusions 69
5.2 Perspectives for further research 71
BIBLIOGRAPHY 73
APPENDIX 66
A. Security Data 66
B. Antibiotic Characterization 67
C. Other experimental data 69

Figures List

Fig. 1. Chemical structure of Phenyl isothiocyanate (PITC)..... 21

Fig. 2. Mechanisms of antibiotic resistance to antimicrobials. 25

Fig. 3. Mechanisms of HGT: transduction, transformation and conjugation. 26

Fig. 4. Model of biofilm development 28

Fig. 5. Four hypothesized biofilm resistance mechanisms: 1 – The antibiotic (black points) penetrates slowly or incompletely; 2 – a concentration gradient of a metabolic substrate or product leads to zones of slow non-growing bacteria (shaded cells); 3 – an adaptive stress response is expressed by some of the cells (marked cells); 4 – a small fraction of the cells differentiate into a highly protected persister state (dark cells) 32

Fig. 6. Live/Dead images (objective 100×): (A) *E. coli* in saline solution; (B) *E. coli* incubated with PITC at 1000 µg/mL for 1 hour; (C) *S. aureus* in saline solution; (D) *S. aureus* incubated with PITC at 1000 µg/mL for 1 hour. 45

Fig. 7. $OD_{570\ nm}$ as a measure of biofilm mass (a) and fluorescence (λ_{ex}: 560 nm and λ_{em}: 590 nm) results as a measure of biofilm viability (b) for *E. coli* and *S. aureus* with the effect of PITC at 1000 µg/mL in the four sequences of treatment. 57

Fig. 8. $OD_{570\ nm}$ as a measure of biofilm mass (a) and fluorescence (λ_{ex}: 560 nm and λ_{em}: 590 nm) results as a measure of biofilm viability (b) for *E. coli* and *S. aureus* with the effect of PITC at 5000 µg/mL in the four sequences of treatment. 60

Fig. 9. $OD_{570\ nm}$ as a measure of biofilm mass (a) and fluorescence (λ_{ex}: 560 nm and λ_{em}: 590 nm) results as a measure of biofilm viability (b) for multiple strains of *E. coli* and *S. aureus* with the effect of PITC at 1000 µg/mL in the four sequences of treatment. 62

Fig. 10. Pictograms of PITC: (A) Flammable liquid, (B) Skin corrosion/irritation; (C) Acute oral toxicity, Skin sensitization, Specific target organ toxicity (single exposure), Narcotic effects; (D) Aspiration hazard; (E) Hazardous to the aquatic environment (long-term hazard) 66

Tables List

Table 1. Factors affecting bacterial adhesion. 29

Table 2. MIC for *E. coli* and *S. aureus* 40

Table 3. Antibacterial activity of antibiotics and PITC on bacteria (average values ± standard deviation for at least 3 replicates) 41

Table 4. Classification of the antimicrobial potential of antibiotic-phytochemicals dual combinations. Antagonism (-); indifference (+); additive (++); synergy (+++) 42

Table 5. Zeta potential (mV) results of suspensions of *E. coli* and *S. aureus* in contact with PITC 42

Table 6. Hydrophobicity (ΔG_{bwb}^{TOT}), and apolar (γ^{LW}) and polar (γ^{AB}) components of the surface tension of untreated and PITC treated cells. The means ± SDs are illustrated 43

Table 7. Description of treatments effectuated in this work 56

Table 8. Biofilm removal/prevention by PITC in *E. coli* and *S. aureus* strains. 58

Table 9. Biofilm removal/prevention by PITC in *E. coli* and *S. aureus* strains with PITC at 5000 µg/mL. 60

Table 10. Biofilm removal/prevention by PITC for *E. coli* and *S. aureus* multi-species biofilms. The sequences were described previously 62

Table 11. Free energy of adhesion (ΔG_{bws}^{TOT}) of untreated and PITC treated cells to PS 64

Table 12. Motility results for bacteria with and without PITC 66

Table 13. Hazard and precautionary statements of PITC 66

Table 14. Characterization of antibiotics used in this study 67

Table 15. Antimicrobial activity of dual combinations (PITC – antibiotics) on bacteria observed by disc diffusion assay. The means (mm) ± SD for at least three replicates are illustrated 69

Table 16. Physico-chemical characterization of polystyrene (PS) 69

Glossary

ΔG	Free energy of interaction	mJ/m^2

Greek letters

γ	Surface free energy
θ	Contact angle

Indexes

S	Substratum
W	Water
B	Bacteria
sws	between two entities of a given surface, when immersed in water (w)
bws	between one bacteria (b) and a substratum (s) that are immersed or dissolved in water (w)
bwb	between two bacterial surfaces, when immersed in water (w)
bw	between bacteria and water
bs	between bacteria and substratum
sw	between substratum and water
LW	Lifshitz-van der Waals component
AB	Lewis acid-base component
TOT	Total
+	Electron acceptor parameter of the Lewis acid-base component (γ^{AB})
-	Electron donor parameter of the Lewis acid-base component (γ^{AB})
F	Formamide
W	Water
B	α-bromonaphtalene

Abbreviations

CIP	Ciprofloxacin
DLVO	Derjaguin-Landau-Verwey-Overbeek Theory
DMSO	Dimethyl sulfoxide

EPS	Extracellular polymeric substances
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
EUCAST	European Committee for Antimicrobial Susceptibility Testing
GHP	Glucosinolate hydrolysis products
GSH	Glutathione
HGT	Horizontal gene transfer
ITC	Isothiocyanate
LB	Luria-Bertani
L/D	Live/Dead® *Bac*Light Bacterial viability kit
LPS	Lipopolysaccharides
MIC	Minimum Inhibitory Concentration
OD	Optical Density
P	Statistical significance level
PCA	Plate count agar
PI	Propidium iodide
PITC	Phenyl isothiocyanate
PS	Polystirene
SPT	Spectinomycin
STR	Streptomycin
TET	Tetracycline
XDLVO	DLVO theory extension
WHO	World Health Organization

Chapter 1

Work outline

1.1. Background and Project Presentation

Antibiotics are one of the most successful therapeutic agents in history due to the extremely efficiency in early recognition and treatment of diseases and infections, which were previously untreatable and normally fatal (Alanis, 2005; Aminov, 2009). Consequently, the use of antibiotics resulted in a surprising reduction in the morbidity and mortality associated with these illnesses and also contributed for the dramatic rise of average life expectancy in the 20th century (WHO, 2002; Alanis, 2005; Aminov, 2009; Yim, 2009).

However, as the access to antibiotics had become eased, they are being used to treat even the most common infection diseases (Alanis, 2005). Moreover, these treatments are being applied not only in people, but also in animals and agriculture, sometimes in inadequate doses and over any time period (Levy, 2001). This situation force microbes to either adapt or die in a phenomenon known as "selective pressure" and, consequently, leads to the development of resistant microorganisms (Levy, 1997; Coutinho *et al.*, 2009). The increasing of bacterial resistance to antibiotics and antimicrobials is now a global problem posing enormous health concerns, including the increased frequency of treatment failures and severity of infections (Nascimento *et al.*, 2000; Brehm-Stecher and Johnson, 2003; Angulo *et al.*, 2004; Sibanda and Okoh, 2007). This situation continue to appeal to the moderation of antibiotic use in the

community (Gould, 1999; Nascimento *et al.*, 2000) and both the prescriber and the consumer must be educated in this reality (Levy, 2001).

To counter the increasing emergence of resistant microorganisms, it is necessary to develop more research to better understand the genetic mechanisms of resistance (Nascimento *et al.*, 2000). Moreover, substantial resources have been invested in the research of new antimicrobial compounds, mainly of microbial and plant origin (Cowan, 1999; Simões *et al.*, 2008; Zahin *et al.*, 2010).

1.2. Main Objectives

In the present study, an isothiocyanate – phenyl isothiocyanate (PITC) - was tested against *Escherichia coli* and *Staphylococcus aureus* growth. Isothiocyanates (ITCs) are compounds released by some vegetables in defense against tissue disruption and pathogen attack. Many studies confirm that ITCs have antimicrobial activity and the benefices of these compounds are many. The strains chosen are considered two of the most clinical significant bacteria involved in drug-resistant infections due to their ability to develop resistance to multiple and conventional antibiotics (Simões *et al*, 2008).

The purpose of this study was to investigate the mechanisms of antimicrobial action of PITC. This compound was also tested in order to evaluate their ability to act synergistically with some antibiotics to control bacterial growth. The antimicrobial activity of single compounds and dual combinations (antibiotic - PITC) was quantitatively assessed by measuring the inhibitory zones. To understand the mode of action of PITC, the physico-chemical characteristics of bacterial surface (zeta potential and hydrophobicity) with and without PITC were analyzed.

PITC was also tested against bacterial growth in biofilms. Surface charge, hydrophobicity, surface energy and the characteristics of polystyrene (from 96-well polystyrene microtiter plates) were analyzed to understand the factors that control bacterial adhesion. Motility was also evaluated in order to understand the role of PITC on biofilm prevention and control. So, the objective of this part of the work is to analyze the antimicrobial activity of PITC against biofilms and to evaluate the potential for adhesion of bacteria to polystyrene, comparing predicted adhesion based on thermodynamic approaches with adhesion assays.

1.3. Thesis Organization

Chapter 1 describes the main objectives, context and motivations for the development of this work and serves as a guide line to the overall work presented in the further chapters. In addition to this chapter, this work comprises four chapters.

In chapter 2 a brief review of the literature is provided. The importance of research for plant extracts with antimicrobial activity is enhanced and some relevant aspects about isothiocyanates as promising substances for therapeutic use against microbial infections are described. The toxicity studies and benefices about PITC are also reviewed, taking into account the existent literature. Due to the relevance of biofilms in most infections, the process of biofilm formation is studied, including the initial attachment of cells to surfaces. The global antibiotic resistance problem in planktonic cells and in biofilms is also focused, as well as the known mechanisms of resistance in both cases.

Chapter 3 focus the study of PITC activity against planktonic cells of *Escherichia coli* and *Staphylococcus aureus*. The antimicrobial activity of PITC against both strains is tested and the combination of PITC with several antibiotics is performed to evaluate the potentiating capacity of this compound. The physico-chemical characterization of bacterial surface after being in contact with PITC is also evaluated to understand the mechanism of action of this compound.

In chapter 4, PITC is tested against bacterial growth in biofilms of *E. coli* and of *S. aureus*. The objective is to investigate if PITC inhibits biofilm formation or if it penetrates and eliminates the biofilm already formed. Moreover, the capacity of adaption of strains to this compound is analyzed. Biofilm will be developed by single and dual species in 96-well polystyrene microtiter plates. Biofilm formation is very dependent on initial bacterial attachment, which can be influenced by the surface physicochemical properties of both bacteria and substratum. So, the adhesion of bacteria to surfaces will be analyze in this work in terms of surface charge, surface energy and the characteristics of polystyrene on bacteria to understand the factors that control bacterial attachment. Predicted adhesion based on thermodynamic approaches will be compared with adhesion assays. Motility is known to be involved in biofilm formation; therefore, the motility of *E. coli* and *S. aureus* is also evaluated in this chapter and its correlation with the biofilm formation and with the theoretical capacity to adhesion to PS is analyzed.

Finally, chapter 5 gives an overview of the work presented, describes the main conclusions and proposes future research.

Chapter 2

Literature Review

2.1. Plant compounds with antimicrobial activity

According to World Health Organization (WHO, 2002), plants would be the best source to obtain a variety of products with antimicrobial properties (Nascimento *et al.*, 2000). Plants have formed the basis for traditional medicine systems for thousands of years in many countries as China and India (Newman *et al*., 2000; Sakharkar *et al*., 2009; Javale and Sabnis, 2010; Zahin *et al*, 2010). The curative potentials of these medicinal plants are well documented and considerable amount of work have been published (Osbourn, 1996; Nascimento *et al.*, 2000; Newman *et al.*, 2000; Simões *et al.*, 2008; Javale and Sabnis, 2010; Jayaraman *et al.*, 2010; Zahin *et al.*, 2010).

A large number of plants have been reported to have compounds (phytochemicals) with antimicrobial activity. Examples of these plants are vegetables, fruits, leaves, oilseeds, cereal crops, barks and roots, spices and herbs (Kähkönen *et al.*, 1999). Some phytochemicals are known to play roles in defense and signaling on the cellular levels (Lila and Raskin, 2005). These compounds (as cyanogenic glycosides and glucosinolates) are activated in response to tissue damage or pathogen attack by microorganisms, insects, and herbivores (Osbourn, 1996;

Lewis and Ausubel, 2006; Saavedra *et al.*, 2010). Other compounds are constitutive: terpenoids, for example, are responsible for plant odor and flavor; quinones and tannins are responsible for plant pigment (Cowan, 1999). Other well-known phytochemicals include phenols and phenolic glycosides, unsaturated lactones, sulphur compounds and saponins (Osbourn, 1996).

The antibacterial activity of glucosinolates has been recognized for many decades (Gomes de Saravia and Gaylarde, 1998; Fahey *et al.*, 2001). Most described glucosinolates (approximately 120 types) are sulphur-containing glucosides (Aires *et al.*, 2009a). They have been reported almost exclusively from the order Capparales, in families as Brassicaceae (also called Cruciferae), Capparaceae and Caricaceae (Halkier and Gershenzon, 2006). They can be found in common vegetables such as cabbage, broccoli, cauliflower, brussels sprouts, kohlrabi and kale (Aires *et al.*, 2009b).

Glucosinolates possess limited biological activity but numerous studies have demonstrated the broad biocidal activity of glucosinolate hydrolysis products (GHP) (Morra and Kirkegaard, 2002). Glucosinolates, which are stored in the cell vacuole, are converted to isothiocyanates (ITCs), thiocyanates, nitriles or oxazolidinethiones by an endogenous myrosinase (thioglucoside glucohydrolase EC 3.2.3.1) upon wounding of the plant (Sarwar *et al.*, 1998; Bending and Lincoln, 2000; Fahey *et al.*, 2001; Jacob, 2007). More than 20 different aliphatic and aromatic isothiocyanates together with other potential allelochemicals have been identified among degradation products of glucosinolates (Smolinska *et al.*, 2003). Isothiocyanates are recognized as the most common and predominant GHP and as major inhibitors of microbial activity (Saavedra *et al.*, 2010).

2.2. Mode of action of isothiocyanates

Most ITCs are electrophiles due to the presence of a –N=C=S group (Norsworthy and Meehan, 2005; Zhang *et al.*, 2006). This group can react with various nucleophiles compounds, especially those that are S-based (thiocarbamoylation) (Borek *et al.*, 1995a; Zhang *et al.*, 2006). The chemical structure of phenyl isothiocyanate (PITC) is demonstrated in Figure 1.

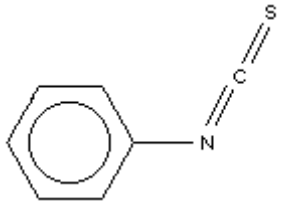


Fig. 1. Chemical structure of Phenyl isothiocyanate (PITC).

The interaction of ITCs with nucleophilic compounds can form dithiocarbamic esters with -SH groups, thiourea derivatives with -NH_2 groups, and N-monosubstituted thiocarbamic esters with -OH groups (Borek *et al.*, 1995b). So, ITCs are capable of reacting and forming a covalent bond with -SH, -NH_2, and -OH groups on membrane and/or active sites of cellular enzymes and other critical biological macromolecules important to microbial growth and survival, causing its modification and deactivation (Borek *et al.*, 1995a; Saavedra *et al.*, 2010). Soft nucleophiles such as thiol groups are easily oxidized and readily polarizable (Schultz *et al.*, 2005). Indeed, the strong reaction of ITCs with amines and cellular thiols is thought to modify various biomolecules (Zhang *et al.*, 2006; Jacob, 2007). Schultz *et al* (2005) hypothesize that all aromatic isothiocyanates have the same molecular mechanism of reactivity: Michael-type addition with an *N*-hydro-*C*-mercapto addition of cellular thiols. Typically the most main common thiol in a cell is GSH; thus, GSH is a biologically relevant thiol-containing nucleophile in eukaryotic cells (plants, fungi, animals) and in most Gram-negative bacteria (Jacob, 2007; Schultz *et al.*, 2007). This results in biochemical damage and, at sufficiently high doses, an overall effect sufficient to cause death (Borek *et al.*, 1995a). The stereoelectronic effect of the phenyl group should also be important to the antimicrobial activity (Kim *et al.*, 2004).

2.3. Citotoxicity and benefices of isothiocyanates

The use of plant extracts and phytochemicals with known antimicrobial properties can be of great significance in therapeutic treatments (Osbourn, 1996; Nascimento *et al.*, 2000).

Reactivity, as related to toxicity, is an irreversible reaction of a compound with a biological macromolecule that leads to an adverse effect. Typically, such reactivity involves covalent binding, which may lead to a series of biochemical and physiological processes. Regardless of the adverse effect, the initiating molecular event is binding to a specific molecular site and it is the nature of this site that determines the mechanism of action (Schultz *et al.*, 2005).

ITCs are regarded as the most toxic products of GHP (Morra and Kirkegaard, 2002) since they express biocidal phytotoxicity (from interaction with various proteins), growth inhibition or feed prevention (Norsworthy and Meehan, 2005). ITCs inhibit a wide variety of plant pests including mammals, birds, insects, mollusks, aquatic invertebrates, nematodes, bacteria, and fungi (Borek *et al.*, 1995a; Sarwar *et al.*, 1998; Morra and Kirkegaard, 2002; Halkier and Gershenzon, 2006; Saavedra *et al.*, 2010). Isothiocyanates are highly toxic to some fungi as proven in numerous studies with *Gaeumannomyces graminis*, *Leptosphaeria maculans*, *Rhizoctonia solani*, *Fusarium graminearum*, *Bipolaris sorokiniana*, and *Pythium irregulare*

(Smolinska *et al.*, 2003). Another notable characteristic of ITCs is their herbicidal activity. Isothiocyanates inhibit or suppress germination of certain plant species (Sarwar *et al.*, 1998; Norsworthy and Meehan, 2005). Some authors have reported the effect of isothiocyanates on germination of *Botrytis cinerea* and *Glomus mossae* spores, and sporangia of *Peronospora parasitica* (Smolinska *et al.*, 2003).

Borek *et al* (1998b) studied the contact toxicity values of 12 commercially available isothiocyanates with a broad range of molecular structures to eggs of black vine weevil, *Otiorhynchus sulcatus* (Borek *et al.*, 1998b). They found that PITC has an intermediate toxicity comparing with the others ITCs and that highest contact toxicities were obtained with the most hydrophobic or lipophilic isothiocyanates (Borek *et al.*, 1998b). This can be explained by the fact that molecular lipophilicity or hydrophobicity can affect the capability of the molecule to pass through the cell membrane, determining a different final concentration in the cell (Manici *et al*, 1997). This hypothesis could explain the high biocidal activity differences observed in the aromatic ITCs tested in plant pathogenic fungi by Manici *et al* (1997). Sarwar *et al* (1998) also showed that, when incorporated in agar, aromatic isothiocyanates were more toxic than aliphatic isothiocyanates against soil microorganisms. Aires *et al* (2009a and 2009b) and Borek *et al* (1995a) also referred that aromatic ITC were generally more effective ant toxic than aliphatic ITC. Schultz *et al* (2005) showed that, for aromatic ITCs, those with the NCS group attached directly to an aromatic ring (as PITC) are less toxic and less reactive than those attached to an aliphatic carbon for *Tetrahymena* population. These differences in potency are hypothesized to relate to difference in the ease of the Michael reaction, the proposed molecular mechanism (Schultz *et al.*, 2005). With compounds like phenyl isothiocyanate where the NCS group is bound directly to an aromatic C-atom, conjugation to the π-system increases electron density at the central C-atom, making the Michael reaction more difficult (Schultz *et al.*, 2005).

Speijers *et al* (1985) developed a subacute toxicity study in male rats with the administration of PITC by gastric intubation at several dose levels. In the highest dose group (40 mg/kg body weight/day for 4 weeks), slightly decreased growth and a significant increase in mean corpuscular hemoglobin were noted. Relative heart, liver, kidney and adrenal weights also showed a statistically significant increase in this group. Microscopic examinations revealed no abnormalities (Speijers *et al.*, 1985).

The activity of ITCs was also proven to be the responsible for the medicinal properties ascribed to some cruciferous vegetables, which have been used as wound poultices and antitumor agents for centuries (Fahey *et al.*, 2001). Evidence has been obtained about the benefices of GHP on the reduction of the incidence risk for many types of cancer (in as much as a 50% of reduction) (Fahey *et al.*, 2001; Holst and Williamson, 2004). Data showing that a high Brassica consumption is correlated with a decreased risk of cancer were most consistent for

lung, stomach, colon, and rectal, and least consistent for prostatic, endometrial, and ovarian cancer (Holst and Williamson, 2004). This situation is thought to be caused by ITCs since they can inhibit carcinogen-activating enzymes and induce carcinogen-detoxifying enzymes. Moreover, ITCs also may inhibit the proliferation of tumor cells by inducing cell apoptosis and arresting cell cycle progression (Xiao *et al.*, 2003; Zhang *et al.*, 2006). As previously explained, as most isothiocyanates are good electrophiles, they modify cysteine residues in various biomolecules, including GSH and nuclear activation factor NF-kB, which has profound biochemical implications. By transforming thiols, the function of thiol containing biomolecules is often impaired, and in the case of NF-kB, an anti-inflammatory response might occur. This has also been used to explain the chemopreventive effects of ITCs (Jacob, 2007).

So, it is of great interest to continue the examination of some glucosinolate-containing plants for their potential pharmacological value and, in particular, for cancer chemoprotection (Zhang, 2001).

2.4. Antibiotic resistance in cells

Antimicrobial resistance had been triggered by inappropriate use of antimicrobial agents. Widespread industrial and agricultural use of antibiotics played an important role as well as the reluctance of the medical community to control the prescribing and dosing of antibiotics (Wood and Moellering, 2003).

Generally, failure of microorganisms to succumb to antimicrobial treatments arises through: (1) an inherent insusceptibility to the agents employed; (2) the acquisition of resistance, by previously susceptible strains, either by mutation or by transfer of genetic material from another species or genus; or (3) the emergence of pre-existing, but unexpressed, resistance phenotypes (Gilbert *et al.*, 2002).

Several mechanisms have evolved in bacteria which confer them antibiotic resistance. The major mechanisms of bacterial resistance to antimicrobials include active drug efflux systems, mutations resulting in altered cell permeability, enzymatic degradation of antimicrobials and target gene-product modifications (Brehm-Stecher and Johnson, 2003; Dantas *et al.*, 2008; Yim, 2009). Figure 2 demonstrated these mechanisms of cell resistance to antimicrobials.

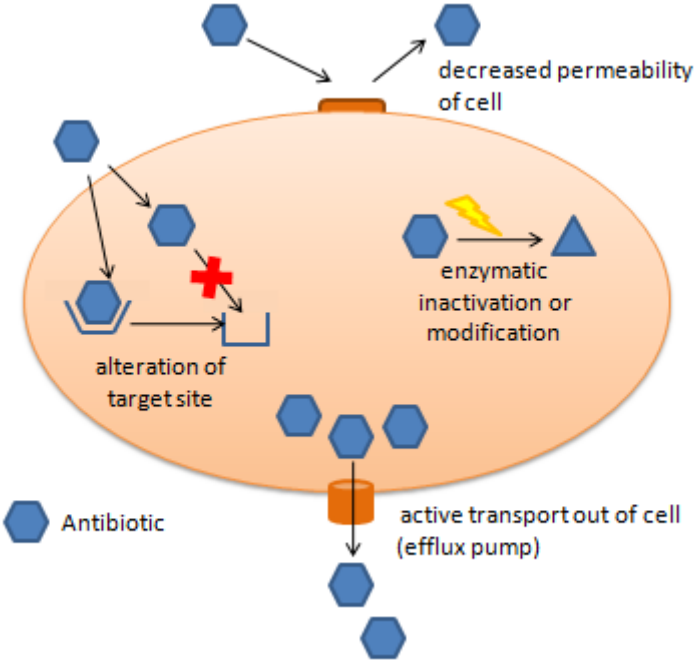


Fig. 2. Mechanisms of cells resistance to antimicrobials (adapted from Encyclopedia, 2011).

Bacteria are particularly efficient in enhancing the effects of resistance because they can rapidly transfer their resistance genes through a bacterial population (Dantas *et al.*, 2008). These resistance mechanisms are spread by vertical gene transfer at replication since daughter cells contain copies of the plasmid (Hoffman, 2001) or by lateral or horizontal gene transfer (HGT), which is a process whereby genetic material can be transferred between individual bacteria (Dantas *et al.*, 2008; Yim, 2009).

There are three possible mechanisms of HGT: transduction, transformation or conjugation (Figure 3) (Yim, 2009). Transduction occurs when bacteria-specific viruses or bacteriophages transfer DNA between two closely related bacteria. Transformation is a process where parts of DNA are taken up by the bacteria from the external environment. This DNA is normally present in the external environment due to the death of another bacterium (Yim, 2009). Conjugation is thought to be the main and most efficient mechanism of antibiotic resistant gene transfer between bacterial strains of the same species or across species within a family of bacteria (Levy, 1997; Hoffman, 2001; Yim, 2009). In this case, a copy of a plasmid or transposon with genetic information is transferred from one organism to another (WHO, 2002).

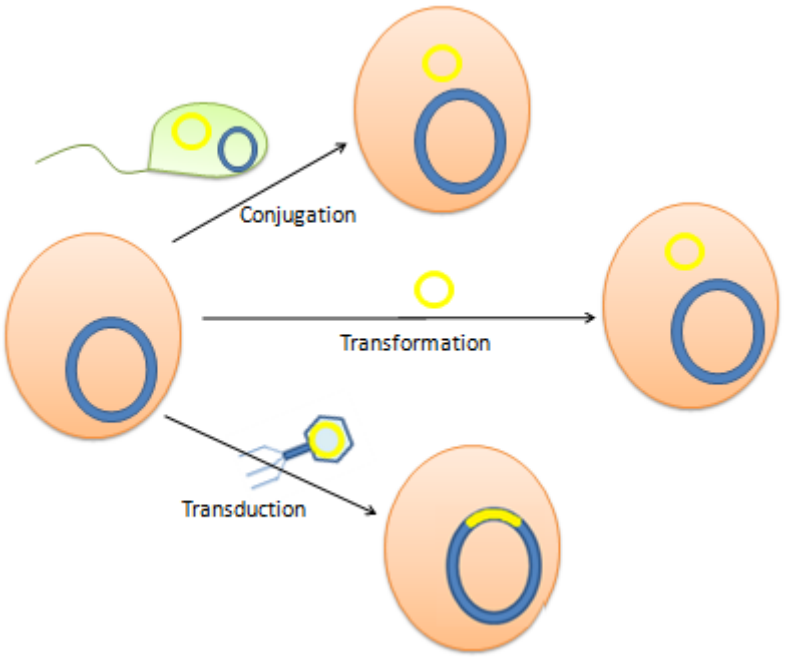


Fig. 3. Mechanisms of HGT: transduction, transformation and conjugation (adapted from Andersson, 2010).

2.5. Biofilm: the main cause of bacterial infections

Studies of planktonic cells living in batch culture have provided extensive information describing the mechanisms controlling bacterial growth (Pratt and Kolter, 1998). However, in order for bacteria to survive within hostile environments and unfavorable environmental conditions (including predation, antimicrobials presence and host immune responses), they have adapted by existing as adherent populations - sessile bacteria (Olson *et al.*, 2002; Verstraeten *et al.*, 2008). Over 80% of bacterial infections in humans involve the formation of biofilms (Brooun *et al.*, 2000; Simões, 2008; Busetti *et al.*, 2010). Biofilms are functional consortia of bacteria populations adherent to a surface and to each other and/or embedded within extracellular polymers matrices (glycocalyx), concentrated products of their own metabolism and ions and nutrients from the environment (Gilbert *et al.*, 1997; Pratt and Kolter, 1998; Smith, 2005; Atabek, 2006; Stepanović *et al.*, 2007; Toté *et al.*, 2008; Verstraeten *et al.*, 2008).

Reports of microbial recalcitrance towards more aggressive biocidal treatments were being related in the industrial, medical and environmental sector (Gilbert *et al.*, 2002). Some examples consist on biofouling of heat exchange systems and marine structures, microbial induced corrosion of metal surfaces, deterioration of dental surfaces, indwelling biomedical

implants and devices, contamination of household products, food preparations and pharmaceuticals (Mah and O'Toole, 2001; Simões, 2005; Toté *et al.*, 2008).

Staphylococcus aureus is one of the most frequent bacterial causes of community and hospital acquired infections due to the easy transmission from the nasal membranes and skin to wounds where bacteria produce numerous extracellular proteins and toxins (Valle *et al.*, 2003). Examples of biofilm-related animal infections caused by *S. aureus* are mastitis, endocarditis, osteomyelitis, meningitis, sepsis and wound infections (Fantin and Carbon, 1992; Teuber, 2001; Olson *et al.*, 2002; Kaito and Sekimizu, 2007; Toté *et al.*, 2009). In addition, *S. aureus* has the capacity to adhere implanted indwelling devices such as catheters, tracheotomy and peritoneal dialysis tubing, heart valves, prostheses and to contact lens (Cramton *et al.*, 1999; Stewart, 2002; Valle *et al.*, 2003; Toté *et al.*, 2009).

As the best studied bacterium, *E. coli* should serve as an excellent model for biofilm formation (O'Toole *et al.*, 2000). Biofilms of *E. coli* causes, for example, enteritis, urinary catheter infections and bacterial prostatitis (Olson *et al.*, 2002; Fux *et al.*, 2005).

2.5.1. DEVELOPMENT OF A BIOFILM

The structure of biofilms provides an ideal environment for gene transfer and cell-to-cell interactions (Atabek, 2006). Cell-to-cell signaling, termed quorum sensing, control a variety of physiological functions including motility, conjugation, competence, sporulation, virulence, cell attachment and detachment and biofilm differentiation and formation (Hammer and Bassler, 2003; Atabek, 2006).

The development of a biofilm is believed to occur in a sequential process that includes transport of microorganisms to surfaces, initial reversible/irreversible adhesion to a solid surface, cell–cell communication, formation of microcolonies on the surface, extracellular polymeric substances (EPS) production and, finally, differentiation of microcolonies into exopolysaccharide-encased, mature biofilms (Costerton *et al.*, 1999; Simões *et al.*, 2010).

EPS participate in the formation of microbial aggregates and are responsible for binding cells and other particulate materials together - cohesion - and to the surface – adhesion (Simões, 2005).

Numerous changes in gene regulation lead biofilm cells to become phenotypically and metabolically different from their planktonic counterparts (Kolari, 2003). Once a biofilm has successfully formed, specific metabolic processes begin, which may include the degradation of substratum, uptake of environmental pollutants, secretion of more protective EPS matrix and production of toxins which are transported into the bulk phase (Emerson IV, 2006).

Afterwards, cells in the biofilm can return to a planktonic lifestyle. Two possible ways can lead to cells dispersion from biofilms. To colonize new areas, a programmed set of events leads to hydrolysis of the extracellular polysaccharide matrix and conversion of a subpopulation of cells into motile planktonic cells, which can rapidly multiply and leave the biofilm sessile biofilm communities (Costerton *et al.*, 1999). Additionally, physical detachment pathway can occur, in which a fragment from the biofilm simply detaches and is carried by the bulk to a new location where initiates a new sessile population (Costerton *et al.*, 1999). One possible signal for detaching may be starvation (O'Toole *et al.*, 2000). Figure 4 presents the model described for development and posterior maturation of biofilms.

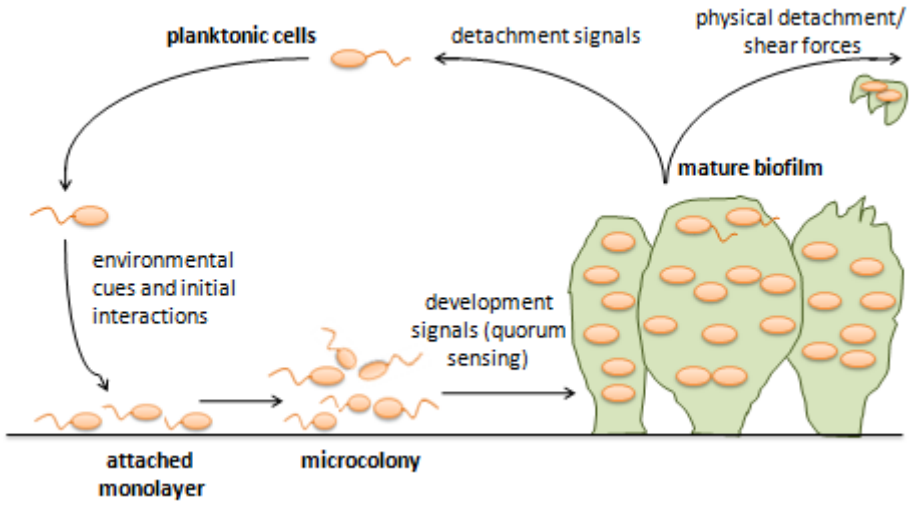


Fig. 4. Model of biofilm development. Individual planktonic cells can form cell-to-surface and cell-to-cell contacts resulting in the formation of microcolonies. Since the initial attachment is reversible, quorum sensing serves as a maturation signal leading to the formation of differentiated, thick mature biofilm structures (Costerton *et al.*, 1999). Cells in the biofilm can return to a planktonic lifestyle to complete the cycle (adapted from O'Toole *et al*, 2000).

2.5.2. MICROBIAL ADHESION TO A SURFACE

To explain the adhesion process of bacteria to surfaces, the classical Derjaguin-Landau-Verwey-Overbeek (DLVO) theory was developed, where the electrostatic and van der Waals forces have been combined. DLVO theory has been extended by the inclusion of acid-base interactions (XDLVO) which accounts for the hydrophobicity of the surfaces involved (Atabek, 2006).

Even some properties of bacteria such as hydrophobicity, DLVO calculations and surface charge have been used to explain bacterial adhesion to a surface, a molecular level understanding of the initial bacterial adhesion process was still lacking (Atabek, 2006). The theories previously explained consider only the physical-chemical and thermodynamic of accession and do not take into account the fact that the organisms are living entities and their microbiological characteristics may condition the adhesion to a surface (Simões, 2004). The biological properties of bacteria, such as outer membrane proteins, fimbriae, flagella, and the production of extracellular polymeric substances (EPS) also influence the attachment to surface (Walker *et al.*, 2004; Simões *et al.*, 2010). On all Gram-negative bacteria, besides EPS, one additional important surface molecule, the lipopolysaccharide (LPS), have also been implicated in bacterial adhesion, transport and in cell interaction with different surfaces such as epithelial cells and medical implants (Kolari, 2003; Walker *et al.*, 2004; Atabek, 2006).

So, bacterial adhesion is a complex process affected by many factors, including those described at Table 1.

Table 1. Factors affecting bacterial adhesion (Pompermayer and Gaylarde, 2000; Walker *et al.*, 2004; Simões, 2005; Stepanović *et al.*, 2007; Simões, *et al*. 2009; Extremina *et al.*, 2011).

Cell properties		**Surface properties**	**Environmental factors**
Physicochemical	**Biological**		
Hydrophobicity	Fimbriae	Chemical composition	Temperature
Surface charge	Flagella	Presence of organic material	pH
	Production of EPS	Surface charge	Time of exposure
	Outer membrane proteins	Hydrophobicity	Bacterial concentration
	Extracellular appendages	Roughness	Growth medium
	Signaling molecules	Texture	Fluid flow conditions
			Hydrodynamics
			Chemical treatment
			Washing procedures
			Presence of antimicrobials

Recently, microbial adhesion has been described as a two-phase process. Initial adhesion between bacteria and non-living surfaces is usually mediated by physical forces, such as brownian motion, van der Waals attraction forces, hydrogen bonding, acid-base, electrostatic interactions (often repulsive as most bacteria and conditioned surfaces are negatively charged), gravitational forces, hydrophobic interactions and biospecific interactions (Kolari, 2003; Atabek, 2006; Simões *et al.*, 2010). Magnitudes of these forces are affected by the distance of the bacteria from the surface (long-range and short-range forces) and by ionic strength (Kolari, 2003). In the second phase, during the biofilm growth, molecular reactions between bacterial surface structures and substratum surfaces become predominant (Simões *et al.*, 2010).

2.5.3. THE INFLUENCE OF MOTILITY IN BIOFILM FORMATION

The ability to react with active motions to internal or external stimuli is one of the most striking characteristics of living organisms (Komnick *et al.*, 1973). Motility was demonstrated as another important aspect in several stages of biofilm formation and development (Pratt and Kolter, 1998; Simões *et al.*, 2007a). The initial adsorption of cells to a surface is mediated by pili, fimbriae and flagella (Lai *et al.*, 2009). The primary function of flagella in biofilm formation is assumed to be in transport and in initial cell-to-surface interactions (Simões, 2005). Type IV pili are presumably the principal adhesins, mediating the initial cell aggregation and microcolony development on a surface (Costerton *et al.*, 1999; Lai *et al.*, 2009). Finally, motility may also enable attached, growing cells to migrate along the surface, thereby facilitating biofilm expansion (Pratt and Kolter, 1998).

The motility mediated by type IV pili is denominated twitching motility. Swimming motility is a flagella-dependent motility type and represents individual cell movement in aqueous environment (Inoue *et al.*, 2008; Verstraeten *et al.*, 2008). Swarming motility is also mediate by flagella and is a multicellular phenomenon involving the rapid and coordinated migration of cells across semisolid surfaces (Inoue *et al.*, 2008). The relationship between biofilms and swarming is more elusive but is increasingly being reported (Deziel *et al.*, 2001; Lai *et al.*, 2009). This complex multicellular behavior requires the integration of chemical and physical signals, which leads to a profound modification of cell morphology, and short planktonic cells differentiate into elongated and multiflagellated swarm cells (Verstraeten *et al.*, 2008; Lai *et al.*, 2009). In addition to flagella, swarmer cells require an increased production of certain extracellular components (wetting agents) that reduce surface friction and enable the smooth migration of a group of cells on viscous surfaces (Inoue *et al.*, 2008). Swarming has

been reported in many genera of Gram-negative (including *E. coli*) and in some Gram-positive flagellated bacteria (Inoue *et al.*, 2008; Verstraeten *et al.*, 2008).

As bacteria might select between motility, such as swarming, and biofilm formation at certain stages, the link between motility and biofilm formation tends to be complex (Verstraeten *et al.*, 2008). For example, the initiation of biofilm formation through reversible attachment often requires flagella, and motility on a surface can be crucial for biofilm architecture. However, motility is also involved in the release of bacteria from mature biofilms (Verstraeten *et al.*, 2008). The actual role of flagella in attachment is probably dependent on the specific strain of bacterium as well as growth conditions (Dickson and Koohmaraie, 1989).

2.5.4. MECHANISMS OF ANTIBIOTIC RESISTANCE IN BIOFILMS

Biofilm cells are more complex and have different characteristics compared to planktonic cells (Atabek, 2006), including the enhanced resistance to antimicrobial agents, which plays a major role in the ineffectiveness of many antibiotic therapies (Simões *et al.*, 2010). In fact, cells existing in biofilm can become 10 to up 1000 times more resistant to the effects of antimicrobial agents than their planktonic counterparts (Amorena *et al.*, 1999; Mah and O'Toole, 2001; Smith, 2005; Zahin, 2010). So, biofilms are very hard to eradicate and antibiotic therapy typically fails to kill the biofilm (Costerton *et al.*, 1999). For some antibiotics, the concentration required to kill sessile bacteria may be a thousand times greater than that required to kill planktonic bacteria of exactly the same strain (Olson *et al.*, 2002; Toté *et al.*, 2009).

Biofilm resistance is not completely understood and the persistence of infections caused by microbial biofilms, even face aggressive antibiotic therapy, continues to motivate the search for the fundamental mechanisms of biofilm reduced susceptibility (Xu *et al.*, 2000; Inoue *et al.*, 2008).

Biofilm resistance involves multiple mechanisms (Inoue *et al.*, 2008). One obvious difference between planktonic cells and biofilm is the presence of a polymeric matrix enveloping the community that retards diffusion of antimicrobials into the biofilm (Costerton *et al.*, 1999; Brooun *et al.*, 2000; Fux *et al.*, 2005). Many papers have investigated this hypothesis as an explanation of biofilm resistance (Stewart, 1996; Xu *et al.*, 2000; Mah and O'Toole, 2001; Smith, 2005). Costerton *et al* (1999) conclude that antibiotics have been shown to penetrate biofilms readily in some cases and poorly in others, depending on the particular agent and biofilm (Costerton *et al.*, 1999). However, given that, in many cases, biofilms consist of stacks of cells with aqueous channels flowing in between, only impenetrability seems unlikely (Smith,

2005). Other theories included a reduced susceptibility of biofilm microorganisms compared to their freely suspended counterparts (Stewart, 1996): reduced metabolism and growth rate and the existence of persister cells, a small population of cells with a highly protected phenotype (Costerton *et al.*, 1999; Brooun *et al.*, 2000; Lai *et al.*, 2009). Figure 5 represents some hypotheses of antibiotic resistance in biofilms.

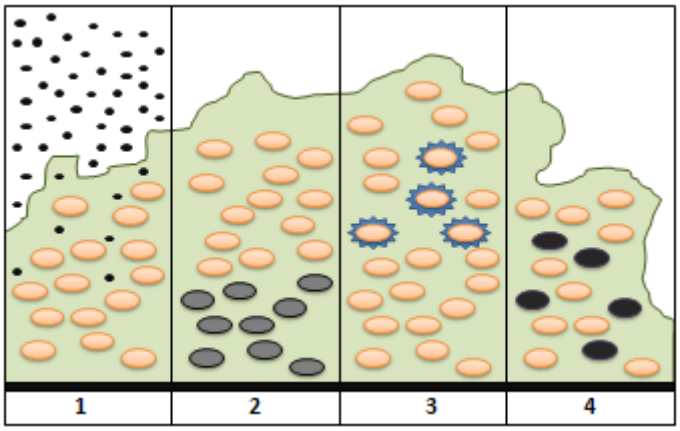


Fig. 5. Four hypothesized biofilm resistance mechanisms: 1 – The antibiotic (black points) penetrates slowly or incompletely; 2 – a concentration gradient of a metabolic substrate or product leads to zones of slow non-growing bacteria (shaded cells); 3 – an adaptive stress response is expressed by some of the cells (marked cells); 4 – a small fraction of the cells differentiate into a highly protected persister state (dark cells) (adapted from Stewart, 2002).

The microorganisms generate physiological changes when cells attach to a surface by expressing a biofilm phenotype that can confer resistance face to stress environmental conditions such as nutrient limitation, heat shock, cold shock, changes in pH and many chemical agents (Simões, 2005). Sublethal concentrations of antimicrobial agents might act as inducers/ transcriptional activators of more tolerant phenotypes (Simões, 2005). A novel hypothesis for the considerable uprising of biofilm relates to the potential of damaged bacterial cells to undergo apoptosis or programmed cell death (Gilbert *et al.*, 2002) and to survive treatment phases and proliferate in the post-treatment phase (Simões, 2005). The difference between planktonic and biofilm communities is that the frequency of persisters is much higher in the biofilm (Stewart, 2002).

Chapter 3

The effect of phenyl isothiocyanate on planktonic bacteria

3.1. Introduction

Due to the emergence of bacterial resistance, the utility of many antibiotics is decreasing over time. The assessment of the efficacy of new antimicrobial agents is very important face the current situation. In this work, the antimicrobial activity of PITC will be compared with those obtained for five antibiotics: two aminoglycosides (streptomycin - STR - and spectinomycin - SPT), one quinolone (ciprofloxacin - CIP), one macrolide (erythromycin - ERY) and one polyketide (tetracycline - TET). The security data of PITC are described in Appendix A (Fig. 10 and Table 13) and the characterization of these antibiotics are described in Appendix B (Table 14).

Plant extracts exhibiting strong antibacterial activity may interact with antibiotics and restore their efficacy (Taylor *et al.*, 2002; Zahin *et al.*, 2010). Several studies have proposed that this combination is a new strategy for developing therapies for bacterial infections since many natural plant products can potentiate the activity of antibiotics (Jayaraman *et al.*, 2010). It is interesting to note that phytochemicals that have different antibacterial modes of action can

potentiate the activity of the same antibiotic class (Simões *et al.*, 2009). Moreover, it is conceivable that phytochemicals with other mechanisms of action, such as those with membrane permeability effects, may potentiate the antibacterial potential of antibiotics that target intracellular sites (aminoglycosides, macrolides, quinolones, tetracyclines). In fact, this is an interesting chemotherapeutic strategy (Simões *et al.*, 2009). So, PITC was combined with the five antibiotics previously referred.

The interactions may be synergistic, neutral or antagonistic (Zahin *et al.*, 2010). Positive interactions that intensify the potency of a bioactive product are generally called potentiating. According to European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID):

- indifference is present when a combination of antibacterial products promotes equal effects to those obtained with the most active product;
- an additive effect is observed when a combination of antibacterial products is equal to that of the sum of the effects of the individual products;
- synergism is present when the combination of antibacterials exceeds the additive effects of the individual products,
- Antagonism interactions occur when certain components of the mixture inhibit full biological activity of pharmacologically-active compounds by reducing their stability or bioavailability or by enhancing their metabolism and, for that reason, promotes a reduced effect comparatively to that of the most effective individual product (Lila and Raskin, 2005; Simões *et al.*, 2009).

However, the fact that a combination of two antibiotics is more effective than either agent alone does not necessarily mean that the combination has synergistic activity *in vivo*, but it could reflect an additive effect. In practice, most investigators use statistical methods to evaluate the *in vivo* effectiveness of combinations, and they call *in vivo* synergism a statistically significant difference between the activity of a combination and that of the most effective agent alone (Fantin and Carbon, 1992).

The physico-chemical characterization of bacterial surface – surface charge and hydrophobicity -, after being in contact with PITC, will be also evaluated to understand the mechanism of action of this compound.

3.2. Material and Methods

3.2.1. BACTERIAL STRAINS

The bacteria used in this study were obtained from the Spanish Type Culture Collection (CECT): the Gram-negative bacteria *Escherichia coli* (CECT 434) and the Gram-positive bacteria *Staphylococcus aureus* (CECT 976). The bacteria were distributed over the surface of Plate Count Agar (PCA – Merck) and incubated for 24h at 27 ± 3 ºC.

3.2.2. PHENYL ISOTHIOCYANATE AND ANTIBIOTICS

PITC was obtained from Sigma-Aldrich (Portugal) and it was prepared in dimethyl sulfoxide (DMSO) (Sigma; Portugal). Ciprofloxacin (CIP), erythromycin (ERY), streptomycin (STR), tetracycline (TET) and spectinomycin (SPT) were obtained from Sigma (Portugal). Every antibiotic were prepared with sterile water, with exception of ciprofloxacin that was prepared in DMSO. DMSO was filtrated before utilization to avoid contamination. After preparation, antibiotics were frozen and thawed in several cycles. The concentration of antibiotics used was according to NCCLS M100-S15: Performance Standards for Antimicrobial Susceptibility Testing (Fifteen Informational Supplement): ciprofloxacin – 5 µg/disc; erythromycin – 15 µg/disc; streptomycin – 30 µg/disc; tetracycline – 30 µg/disc; and spectinomycin – 100 µg/disc.

3.2.3. DETERMINATION OF MINIMUM INHIBITORY CONCENTRATIONS (MICS)

The Minimal Inhibitory Concentration (MIC) is defined as the lowest concentration of an antimicrobial agent needed to inhibit bacterial growth under laboratory conditions (Hoffman, 2001; Jayaraman *et al.*, 2010). MIC of antibiotic was determined by the micro-dilution method. The cell suspensions obtained by overnight cultures growth in Mueller-Hinton medium (Fluka, Portugal) were adjusted to a OD_{640nm} of 0.2 ± 0.02 and were added to sterile 96-well polystyrene microtiter plates with PITC in several concentrations (100, 200, 500, 700, 1000, 1500, 2000 µg/mL) in a final volume of 200 µL. PITC was added so as not to exceed 10% of total volume. After 24 h at 37 °C, the MIC of each sample was determined by measuring the optical density in the spectrophotometer (640 nm). MIC corresponds to the concentration in which the final OD is inferior or equal to the initial OD.

3.2.4. ANTIBACTERIAL ACTIVITY ASSESSMENT

Colonies of bacteria were picked from overnight PCA cultures (log phase cultures) in solid medium (Merck, Portugal). The inoculum was prepared my making a 0.9% NaCl suspension of bacteria, which was adjusted to match to 0.5 McFarland turbidity standards. The suspension was spread with a sterile cotton swap into Petri dish (90 mm of diameter) containing 20 ml of Mueller-Hinton Agar. Sterile filter paper discs (with 6 mm in diameter), impregnated with 15 µL of PITC (at MIC), were placed on the agar plate seeded with the respective bacteria. The plates were incubated at 37 ºC for 24 hours. Discs of ciprofloxacin, erythromycin, streptomycin, tetracycline and spectinomycin were used as positive controls and discs impregnated with DMSO were used as negative controls. After incubation, the diameter in mm of the inhibitory or clear zones around the disks was recorded. All tests were performed in triplicate and the antibacterial activity was expressed as the mean of inhibition diameters (mm).

3.2.5. ANTIBIOTIC-PHYTOCHEMICAL DUAL COMBINATIONS ASSAY

To study the antimicrobial effects of PITC conjugated with antibiotics, the phytochemical was inserted in Mueller-Hinton agar medium (at a final concentration corresponded to the MIC). This insertion was performed after sterilize and cooling the medium, to avoid the deterioration of the phytochemical. Bacteria from overnight growth cultures were suspended in saline solution with a final cell concentration of approximately 0.5 McFarland standards, as previously mentioned. After pressing the discs impregnated with 15 µL of each antibiotic onto the surface of the inoculated agar plate, plates were incubated for 24 h at 37 ºC. After incubation, zones of growth inhibition were measured. All tests were performed in triplicate and the antibacterial activity was expressed as the mean of inhibition diameters (mm).

3.2.6. ANTIBACTERIAL ACTIVITY CLASSIFICATION

The antibacterial effects of the tested phytochemicals were classified according to the scheme proposed by Aires *et al* (2009a):

- non-effective (-): inhibition halo = 0;
- moderate efficacy (+): 0 < inhibition halo < antibiotic inhibition halo;
- good efficacy (++): antibiotic inhibition halo < inhibition halo < 2 × antibiotic inhibition halo;
- strong efficacy (+++): inhibition halo > 2 × antibiotic inhibition halo.

The effect of dual combinations of antibiotics and phytochemicals was classified according to Saavedra *et al* (2010):

- antagonism (-): if [inhibition halo – (antibiotic inhibition halo + phytochemical inhibition halo)/2] < 0;
- indifference (+): if 0 ≤ [inhibition halo – (antibiotic inhibition halo + phytochemical inhibition halo)/2] < antibiotic inhibition halo or phytochemical inhibition halo;
- additive (++): if antibiotic inhibition halo < [inhibition halo – (antibiotic inhibition halo + phytochemical inhibition halo)/2] < 2 × antibiotic inhibition halo or phytochemical inhibition halo;
- synergy (+++): if inhibition halo > 3 × antibiotic inhibition halo or phytochemical inhibition halo.

For the classification was selected the highest inhibition halos caused by the antibiotic or phytochemical application for each condition tested.

3.2.7. DETERMINATION OF *ZETA* POTENTIAL

The overnight cultures of *E. coli* and *S. aureus* were centrifuged at 4000 rpm for 10 min and washed twice with sterile water. The $OD_{640\ nm}$ of strains was adjusted to 0.2 ± 0.02 and samples were incubated with PITC for 30 minutes at 37 ºC. PITC concentration used was the MIC and also 5000 µg/mL. Cells suspensions without PITC were used as control. The zeta potential experiments were performed using a Malvern Zetasizer instrument (Nano Zetasizer, Malvern instruments, UK). All experiments were carried out in triplicate at room temperature and were repeated at least at three different occasions.

3.2.8. PHYSICO-CHEMICAL CHARACTERIZATION OF BACTERIAL SURFACE

The physico-chemical properties of the bacterial surface were determined using the sessile drop contact angle method on bacterial lawns, which were prepared as described by Busscher (Busscher *et al.*, 1984). Samples with PITC were incubated for 1 hour. PITC concentration used was the MIC and also 5000 µg/mL. Determination of contact angles was performed automatically using a model OCA 15 Plus (DATAPHYSICS, Germany) video based optical contact angle measure instrument, allowing image acquisition and data analysis. Hydrophobicity was evaluated after contact angles measurements, following the van Oss approach (van Oss *et al.*, 1987; van Oss *et al.*, 1988; van Oss *et al.*, 1989). The degree of hydrophobicity of a given surface (s) is expressed as the free energy of interaction between two

entities of that surface, when immersed in water (w) - ΔG_{sws} (mJ/m^2). When $\Delta G_{sws} < 0$, the interaction between the two entities is stronger than the interaction of each entity with water and the material is considered hydrophobic. Conversely, if $\Delta G_{sws} > 0$, the material is hydrophilic. ΔG_{sws} can be calculated through the surface tension components of the interacting entities, according to to Equation 1:

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

where γ^{LW} accounts for the Lifshitz-van der Waals component of the surface free energy, and γ^+ and γ^- are the electron acceptor and electron donor parameters of the Lewis acid-base component (γ^{AB}), respectively, being $\gamma^{AB} = \sqrt{\gamma^+\gamma^-}$.

The measurements were carried out at room temperature (23 °C ± 2) using three different liquids: water, the polar formamide and the apolar α-bromonaphtalene (Sigma, Portugal). The liquids surface tension components were obtained from literature (Janczuk *et al.*, 1993). Once the values are obtained, three equations of the type of (2) can be solved:

$$(1+\cos\theta)\gamma_1^{Tot} = 2\left(\sqrt{\gamma_s^{LW}\gamma_w^{LW}} + \sqrt{\gamma_s^+\gamma_w^-} + \sqrt{\gamma_s^-\gamma_w^+}\right) \quad (2)$$

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

Contact angle measurements were performed at least with 20 determinations for each liquid and for each microorganism at three independent experiments.

3.2.9. ASSESSMENT OF MEMBRANE INTEGRITY DUE TO PROPIDIUM IODIDE UPTAKE

The Live/Dead *Bac*Light™ kit (Invitrogen) is a fast method applied to estimate both viable and total counts of bacteria (Ferreira *et al.*, 2011). The kit consists of two stains, propidium iodide (PI) and SYTO9, which both stain nucleic acids. Green fluorescing SYTO9 is able to enter all cells and is used for assessing total cell counts, whereas red fluorescing PI enters only cells with damaged cytoplasmic membranes (Berney *et al.*, 2007). The emission properties of the stain mixture bound to DNA change due to the displacement of one stain by the other and quenching by fluorescence resonance energy transfer (Berney *et al.*, 2007). After an overnight, strains were centrifuged and washed twice with 0.9% NaCl solution. The OD_{640nm} was adjusted to 0.2 ± 0.02 and the suspensions were incubated with PITC for 30 minutes and for 1 hour.

PITC concentration used was the MIC and also 5000 μg/mL. Controls of cells without PITC were used. Afterwards, bacteria were transferred to saline solution and diluted 1:10. Three hundred microliters of each diluted suspension were filtered through a Nucleopore® (Whatman) black polycarbonate membrane (pore size 0.22 μm) and stained with 250 μL diluted SYTO 9™ and 100 μL diluted component PI. The dyes were left to react for 7 min in the dark. The membrane was then mounted on *Bac*Light mounting oil, as described in the instructions provided by the manufacturer. The microscope use for observation of stained bacteria was a LEICA DMLB2 with a mercury lamp HBO/100W/3 incorporating a CCD camera to acquire images using IM50 software (LEICA) and a 100× oil immersion fluorescence objective. The optical filter combination for optimal viewing of stained mounts consisted of a 480 to 500 nm excitation filter in combination with a 485 nm emission filter (Chroma 61000-V2 DAPI/FITC/TRITC). Three independent experiments were performed for each condition tested.

3.2.10. STATISTICAL ANALYSIS

All experiments were done in triplicate. The data was analyzed using One-Way Anova. The results were presented as the Means ± SEM (standard error of the mean). Significance level for the separation was set at $P < 0.05$. The data were analysed using the statistical program SPSS 14.0 (Statistical Package for the Social Sciences).

3.3. Results and Discussion

3.3.1. ANTIMICROBIAL ACTIVITY OF PITC ALONE AND IN COMBINATION WITH ANTIBIOTICS

The MIC of PITC against both bacteria was found to be 1000 µg/mL, as indicated in Table 2. The minimal inhibitory concentrations (MICs) of plant-derived antimicrobials are often reported to be between 100 and 1000 mg/L (Sakharkar *et al.*, 2009).

Table 2. MIC for *E. coli* and *S. aureus*

	$OD_{640\ nm}$						
Strains	**PITC concentration (µg/mL)**						
	100	**200**	**500**	**700**	**1000**	**1500**	**2000**
E. coli	7.5±0.1	0.52±0.1	1.0±0.2	0.63±0.1	0.17±0.1	0.22±0.05	0.16±0.02
S. aureus	5.5±0.3	3.6±0.4	5.0±0.6	2.1±0.3	0.19±0.07	0.25±0.1	0.16±0.05

Since the concentration of an antimicrobial for therapeutic purposes should not be more than 1000 µg/mL, this compound may still be useful for these applications. PITC could also be used in some disinfection processes, since its MIC is much lower than the concentration of biocides commonly used in these cases (approximately 50 mg/L) (Simões *et al.*, 2007; Ferreira *et al.*, 2010).

The synergistic effect from the association of antibiotics with phytochemicals against resistant bacteria leads to new choices for the treatment of infectious diseases, which enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment (Purushotham *et al.*, 2010). In the present study, the antimicrobial activity of PITC was tested alone and in combination with five antibiotics. The purpose is to maximize the positive effects of bacterial inactivation and killing. PITC demonstrated antimicrobial activity against the bacteria tested as all antibiotics used in this study. Table 3 shows the antimicrobial activity of antibiotics and PITC for both strains.

Table 3. Antibacterial activity of antibiotics and PITC on bacteria (average values ± standard deviation for at least 3 replicates)

	Diameter of inhibition zone (mm)					
	ERY	**STR**	**CIP**	**TET**	**SPT**	**PITC**
E. coli	16.0±1.0	28.3±1.5	39.3±1.2	27.7±1.2	25.7±0.6	13.7±0.6
S. aureus	23.5±1.5	21.5±2.1	30.0±2.7	43.0±1.0	12.0±1.0	9.67±0.6

The strains are considered susceptible to all antibiotics, according to the NCCLS M100-S15 (Clinical and Laboratory Standards Institute, 2005), where the organisms are reported as susceptible, intermediate or resistant to the agents that have been tested. The most effective antibiotics against *E. coli* and *S. aureus* strains were ciprofloxacin and tetracycline while the lowest effect was observed with erythromycin and spectinomycin, respectively ($P < 0.05$). The negative control performed with the solvent (DMSO) used in the preparation of phytochemical solutions had no effects on bacterial growth.

PITC had significant antimicrobial activity on *E. coli* ($P < 0.05$). This fact was not expected since Gram-negative species are generally less susceptible to phytochemicals comparatively to Gram-positive, as its outer membrane and a set of multi-drug resistance pumps constitute stronger barriers to antibiotic entry (Simões *et al*, 2008). On the contrary, Gram-positive bacteria possess a permeable cell wall that usually does not restrict the penetration of antimicrobials (Simões *et al*, 2008). Javale and Sabnis (2010) also referred many studies in which plant extracts have always shown high antimicrobial activity towards Gram-positive comparing to Gram- negative bacteria. However, Taguri *et al* (2006) suggested that Gram-staining does not correlate with antimicrobial potency and the susceptibility to phytochemicals was dependent on the bacterial species. The differences also may depend on phytochemical mode of action, as well as on chemical-physical properties (Simões *et al*, 2008). As PITC reacts with active sites of cellular enzymes and other critical biological macromolecules, probably can cause membrane disintegration and, in this case, the outer membrane of Gram-positive strains could not serve as a protective barrier.

According to the classification scheme previously explained about the antibacterial effects of PITC - non-effective (-), moderate efficacy (+), good efficacy (++); strong efficacy (+++) -, moderate antimicrobial efficacy was verified with the application of PITC on the strains comparing to the inhibitory activities of the antibiotics tested.

Dual combinations of antibiotic-PITC were performed. Table 4 shows the effect of these combinations and the classification of the action as antagonism (-), indifference (+), additive (++) and synergy (+++).

Table 4. Classification of the antimicrobial potential of antibiotic-phytochemicals dual combinations. Antagonism (-); indifference (+); additive (++); synergy (+++)

	ERY	STR	CIP	TET	SPT
E.coli	+	+	+	+	+
S.aureus	++	+	++	+	+

The inhibition zones obtained with these combinations are presented in Appendix C (Table 14). PITC showed an additive behavior when combined with erythromycin and ciprofloxacin in *S. aureus* growth. This additive effect may be due to a double attack of both agents on different target sites of the bacteria (Adwan and Mhanna, 2008). The other combinations produced insignificant antibacterial activities according to the classification scheme used. This situation shows that, despite PITC had more effects against *E. coli, S. aureus* is more susceptible to interactions of PITC-antibiotics. Zago *et al* (2009) demonstrated that *S. aureus* is more susceptible to interactions of this type when compared with some *E. coli* strains. *E. coli* demonstrated a low frequency of synergism in tests between essential oils and some drugs (Zago *et al.*, 2009).

3.3.2. SURFACE CHARACTERIZATION OF CELLS IN PRESENCE OF PITC

The charge properties of the cell surfaces have a very important role in the stability, mode of action and resistance mechanisms of microorganisms (Ferreira *et al.*, 2011). The surface charge of cells was determined by zeta potential measurements (Table 5).

Table 5. Zeta potential (mV) results of suspensions of *E. coli* and *S. aureus* in contact with PITC

Strain	PITC Concentration (µg/mL)	Zeta Potential (mV)
E. coli	0	-18.4 ± 0.4
	1000	-15.8 ± 1.6
	5000	-16.2 ± 0.8

	0	-25.4 ± 0,6
S. aureus	1000	-20.5 ± 0.5
	5000	-19.8 ± 1.1

Both strains are negatively charged. Most microorganisms have a negative surface charge under physiological conditions due to anionic groups, such as carboxyl and phosphate, in their membrane (Ferreira *et al.*, 2011). These results allow a better understanding how PITC interacts with the bacteria: the adsorption of the positively charged PITC changes the cells surface charge to less negative values *(P* < 0.05).

The results with PITC at 5000 µg/ mL were very similar to those obtained with PITC at 1000 µg/mL (*P* > 0.05), suggesting a possible cell surface saturation with PITC at the lower concentration.

Microbial hydrophobicity is defined by the energy of attraction between apolar or slightly polar cells immersed in an aqueous phase and can be assessed by several methods; although, the best method to determine bacterial hydrophobicity is by contact angles measurements (Cerca *et al.*, 2006). The surface hydrophobicity was determined using the approach of van Oss, which allows the assessment of the absolute degree of hydrophobicity of any surface in comparison with their interaction with water (van Oss, 1995). The results demonstrate that cell-PITC interaction results in an alteration of cell surface hydrophobicity, as can be observed in Table 6.

Table 6. Hydrophobicity (ΔG_{bwb}^{TOT}), and apolar (γ^{LW}) and polar (γ^{AB}) components of the surface tension of untreated and PITC treated cells. The means ± SDs are illustrated

	PITC concentration (µg/mL)	**Contact Angle (º)**			**Surface tension parameters (mJ/m^2)**				**Hydrophobicity (mJ/m^2)**
		θ_w	θ_F	θ_B	γ^{LW}	γ^{AB}	γ^+	γ^-	ΔG_{bwb}^{TOT}
E. coli	0	17±0.9	18±0.6	43±1.3	33	11	2.3	52	28
	1000	23±0.2	37±1.4	58±0.1	26	10	1.8	60	40
	5000	22±1.2	36±2.1	52±1.9	29	8.9	1.3	61	42
S. aureus	0	31±1.1	30±0.5	51±1.6	29	11	2.5	47	24
	1000	26±0.5	32±0.4	42±0.8	34	7.3	1.0	52	33
	5000	21±0.8	28±0.7	50±1.8	30	11	2.2	55	32

It is possible to estimate the hydrophobic or hydrophilic character of surfaces from surface tension components: the higher the value of LW component, more apolar is the surface and, therefore, lower would be its affinity for polar liquids; the higher the value of AB component, more hydration water has the surface, so more hydrophilic it would be (Simões, 2004). However, several studies conclude that:

- The hydrophobicity of a substance is not dependent on its polar component, but the greater the value of this component is, more hydrophobic or less hydrophilic, becomes the surface;
- The γ^- parameter has a great influence on the hydrophobic or hydrophilic character (Simões, 2004).

According to the surface tension parameters, the Lifshitz–van der Waals (γ^{LW}) component of the bacteria had similar values and all the bacteria were predominantly electron donors (γ^-). The γ^- parameter is inferior for bacteria without PITC, which means that the bacterial surface is less hydrophilic.

The hydrophobicity of surfaces can also be quantified by calculating the energy of hydrophobic attraction (ΔG_{bwb}^{TOT}) through the components of surface tension, according to the calculation methodology developed by van Oss (1995). As previously explained, when the overall free energy of interaction between the molecules of a surface immersed in water is attractive ($\Delta G^{TOT} < 0$), means that the surface molecules have less affinity for water than among themselves, and in this case is considered hydrophobic. When the overall free energy of interaction between the molecules of a surface immersed in water is sufficiently repulsive ($\Delta G^{TOT} > 0$) the surface is considered hydrophilic. The more negative the value of ΔG^{TOT} more hydrophobic is the surface and the more positive the value of ΔG^{TOT} more hydrophilic is the surface.

Therefore, *E. coli* and *S. aureus* cells have hydrophilic properties ($\Delta G^{TOT} > 0$ mJ/m^2). The application of PITC promotes the increase of their hydrophilic characteristics ($P < 0.05$), especially for *E. coli*. Results with cells incubated with PITC at 5000 µg/mL were very similar to those obtained with PITC at 1000 µg/mL ($P > 0.05$).

The Live/Dead *Bac*Light™ kit (Invitrogen) assesses membrane integrity by selective stain exclusion (Ferreira *et al.*, 2011). The Live/Dead *Bac*Light viability kit allows the distinction of viable and dead cells in a cell population: those cells fluorescing green (without membrane damage) are considered viable cells while those fluorescing red (with membrane damage) are considered dead cells. Figure 6 shows the images obtained with this method in bacteria without the PITC effect and in bacteria incubated with PITC at 1000 µg/mL for 1 hour.

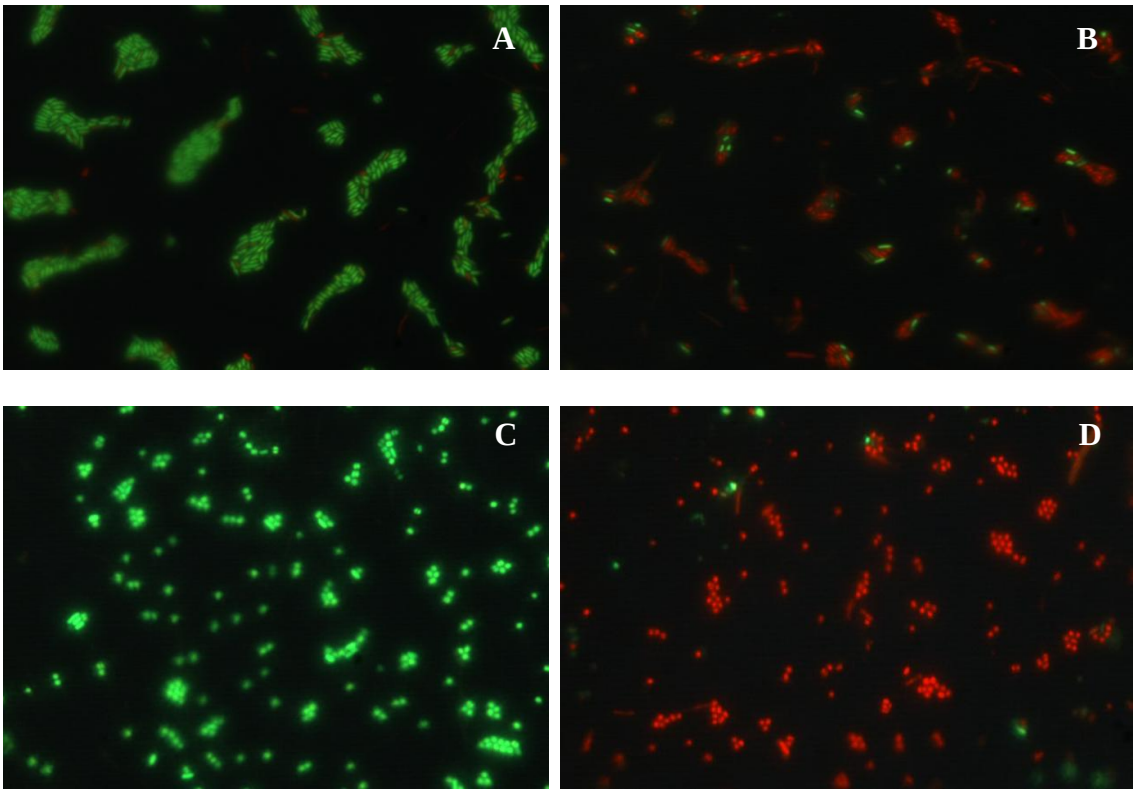


Fig. 6. Live/Dead images (objective 100×): (A) *E. coli* in saline solution; (B) *E. coli* incubated with PITC at 1000 µg/mL for 1 hour; (C) *S. aureus* in saline solution; (D) *S. aureus* incubated with PITC at 1000 µg/mL for 1 hour.

Without PITC all cells are colored green. When incubated for 30 minutes with PITC, approximately 40% of cells are red colored but when incubated with PITC for 1 hour, the number of red cells increased (about 70% of the total population was PI stained). PITC at 5000 µg/mL produced similar results ($P > 0.05$). So, PITC may cause cytoplasmic membrane damage, which can corroborate the data obtained from zeta potential and hydrophobicity assessment.

Isothiocyanates have long been known to bind to the external proteins of cell membranes and poorly penetrating the cell cytoplasm (Gomes de Saravia and Gaylarde, 1998). As previously explained, most ITCs are electrophiles because of the presence of a –N=C= S group, which can react with various nucleophiles (Zhang *et al.*, 2006). So, since PITC can act with several biological molecules, this compound is considered a multi-target antimicrobial and can react with cell at multiple points. Due to the reaction with membrane proteins, PITC may form a monolayer around the cell that changes the electrostatic potential and membrane integrity, which explain the results obtained in this study. The loss of thiol groups in peptides and proteins by PITC may trigger cell death (Zhang, 2001). The scarcity of green cells seen in Figure 6 suggests that this might be so. However, this speculation remains to be investigated.

3.4. Conclusions

Plant extracts have a great potential as antimicrobial compounds against microorganisms. ITCs are highly promising antimicrobial agents. The results showed that PITC had significant antimicrobial activities on both strains, especially for *E. coli*. This compound also had a good efficacy against *S. aureus* strains when combined with erythromycin and ciprofloxacin due to an additive effect. On *E. coli*, the combination of PITC-antibiotics produced indifferent results. This phenomenon might be related with the bacteria type.

Physico-chemical characterization of bacterial surface by zeta potential and contact angle measurements indicated that, when incubated with PITC, the bacterial surface is less negative and more hydrophilic. The reaction of PITC with thiol groups in peptides and proteins at the membrane may be the cause of the changes on physical bacteria surface observed. These actions can lead to cell death as indicated by Live/Dead staining.

Chapter 4

The effect of phenyl isothiocyanate on single and multi-species biofilm control

4.1. Introduction

Bacteria are equipped with a host of stress responses that allow them to cope with environmental fluctuations, such as abrupt temperature changes, oxidative stress, low water activity, DNA damage, starvation, and others (Stewart, 2002). Biofilm represent an important survival strategy for microorganisms since they enjoy a number of advantages over their planktonic counterparts, namely the increased resistance to antimicrobials (Busetti *et al.*, 2010; Simões *et al.*, 2010). Infections that involve a biofilm mode of growth are generally chronic and are often difficult to treat (Olson *et al.*, 2002).

Biofilms can be made up of single or multiple bacterial species. There has been an explosive increase of biofilm knowledge in the last two decades. However, most of the mechanisms regarding biofilm formation are revealed by means of studying single-species biofilms (Yang *et al.*, 2011). This is important since single-species biofilms exist in a variety of infections and on the surface of medical implants (O'Toole *et al.*, 2000). However, multi-species biofilms represent the most important lifestyles of microorganisms in nature (Yang *et al.*, 2011).

Soft-tissue infection or infections associated with indwelling medical devices are often the result of the growth of mono-species biofilms (Gilbert *et al.*, 1997). Contrarily, it has been estimated that, for example, dental biofilms contain more of 500 different bacterial taxa (Mah and O'Toole, 2001).

Biofilms associated with indwelling medical devices may be composed of Gram-positive or Gram-negative bacteria or yeasts. Bacteria commonly isolated from these devices include the Gram-positive *Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis,* and *Streptococcus viridans;* and the gram-negative *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis,* and *Pseudomonas aeruginosa* (Donlan, 2001). However, the bacterial species in multi-species biofilms vary a lot, depending on their environment (Yang *et al.*, 2011).

In this chapter, the effect of PITC will be tested on biofilms formed by single and multi-species of *E. coli* and *S. aureus*. A broad range of model systems have been described for the study of biofilm formation and development *in vitro*. In most of these model systems, the quantification of biofilm biomass was done by conventional plating, which is labor and slow (Peeters *et al.*, 2008). Over the past years, biofilm quantification in microtiter plates has been described. This quantification can be of two types: biofilm biomass assays (based on the quantification of matrix and both live and dead cells) and viability assays (based on the quantification of viable cells) (Peeters *et al.*, 2008). Cristal violet (CV) is a basic dye which binds to negatively charge surface molecules and polysaccharides in the extracellular matrix in both live and dead cells (Peeters *et al.*, 2008; Extremina *et al.*, 2011). Resazurin, the main component of Alamar Blue, is a blue redox indicator that can be reduced by viable bacteria in the biofilm to pink (Extremina *et al.*, 2011). Thus, continued growth maintains a reduced environment (pink) and the extent of conversion from blue to pink is a reflection of cell viability.

Moreover, the cells adhesion to polystyrene will be studied as well as the mechanisms responsible for this process. As described before, bacterial adhesion may be influenced by hydrophobicity, surface charge, motility, flagellation and release of extracellular substances, such as polysaccharides, proteins and metabolite molecules (van Loosdrecht *et al.*, 1987; Dickson and Koohmaraie, 1989; Li and McLandsborough, 1999; Van Bambeke *et al.*, 2003; Simões *et al.*, 2007b; Di Bonaventura *et al.*, 2008; Simões *et al.*, 2010).

4.2. Material and Methods

4.2.1. BACTERIAL STRAINS AND PHENYL ISOTHIOCYANATE

Escherichia coli and *Staphylococcus aureus* strains used are the same as those referred in Chapter 3. The bacteria were distributed over the surface of Plate Count Agar (PCA – Merck, Portugal) and incubated for 24h at 27 ± 3 ºC. PITC was obtained from Sigma-Aldrich (Portugal) and it was prepared in DMSO.

4.2.2. BIOFILM FORMATION ASSAY

The strains were obtained from overnight cultures (log phase cultures) in Mueller-Hinton medium (Fluka, Portugal). The inoculum was prepared my adjusting OD_{620nm} to 0.04±0.02 with fresh Mueller-Hinton medium and, then, 200 µl of cell suspension were added to eight wells of sterile 96-well polystyrene microtiter plates. To promote biofilm formation, the plates were incubated aerobically on a shaker at 150 rpm, at room temperature, for 24 h. Negative controls were performed with sterile water and medium. Cells suspension incubated for 24 hours and with no contact with PITC were used as positive control (to quantify the total formation of biofilm). In total, four sequences were developed to test the effect of PITC in several steps of the process. The treatments applied were:

(A) The strains were incubated overnight with PITC → biofilms were formed in microtiter plates for 24 hours → after 24 hours, biofilms were incubated again with PITC for 1 hour;

(B) The strains were incubated overnight with PITC → biofilms were formed in microtiter plates for 24 hours;

(C) The strains were incubated overnight without PITC → biofilms were formed in microtiter plates for 24 hours → after 24 hours, biofilms were incubated again with PITC for 1 hour;

(D) The strains were incubated overnight without PITC → biofilms of the strains were formed in microtiter plates for 24 hours in the presence of PITC.

To test the effect of PITC after biofilm development, the content of the wells was removed after incubation, and the wells were washed three times with 200 µL of NaCl (8.5 g/L) to remove reversibly adherent bacteria. Then, PITC was added to the wells. Concentrations of PITC used were the MIC and 5000 µg/mL. The plates were incubated for 1 hour (PITC was

added for three times, twenty minutes each of incubation). The remaining attached bacteria were analyzed in terms of biomass adhered on the surfaces of the microtiter plates and in terms of biofilm activity: the total biofilm biomass (both live and dead cells) was estimated by using crystal violet and viable cells/activity were quantified using resazurin.

4.2.2.1. Biomass quantification (crystal violet)

First, the inoculum in the walls was removed, the walls were washed with 200 µL of sterile water and 250 µL of ethanol were added for 15 minutes to promote biofilm fixation. After this, supernatants were removed and the plates were air-dried. Then, the fixed bacteria were stained for 5 minutes with 200 µL of crystal violet solution (Gram color-staining set for microscopy, Merck). After gently washing in water, the plates were left to dry and, finally, the walls were immersed in 200 µL ml of acetic acid 33% (v/v) (VWR) to release and dissolve the stain. The optical density of the obtained solutions was measured at 570 nm using a microtiter plates reader (SpectraMax M2E, Molecular Devices).

4.2.2.2 Resazurin Assay

In the resazurin assay, a commercially available resazurin solution (Sigma) was used. Stock solutions were stored at −20 °C. In this method, 190 µl of sterile Mueller-Hinton medium were added to all wells followed by the addition of 10 µl of resazurin solution. Fluorescence (λ_{ex}: 560 nm and λ_{em}: 590 nm) was measured after 20 min of incubation at room temperature using the microtiter plates reader.

4.2.3. CLASSIFICATION OF BIOFILM PRODUCER BACTERIA

Bacteria were classified using the scheme of Rodrigues *et al* (2010) as follows:

- nonbiofilm producer (0): OD less than or equal to $OD_{control}$;
- weak biofilm producer (+): OD greater than $OD_{control}$ and less than or equal to $2xOD_{control}$;
- moderate biofilm producer (++): OD greater than $2xOD_{control}$ and less than or equal to $4xOD_{control}$;
- strong biofilm producer (+++): OD greater than $4xOD_{control}$.

The $OD_{control}$ refers to negative control.

4.2.4. FREE ENERGY OF ADHESION

The free energy of adhesion was calculated through the surface tension components of the entities involved in the adhesion process by the thermodynamic theory expressed by Dupré equation (Simões *et al.*, 2010). When studying the interaction between one bacteria (b) and a substratum (s) that are immersed or dissolved in water (w), the total interaction energy, ΔG_{bws}^{TOT}, can be expressed by the interfacial tensions components as:

$$\Delta G_{bws}^{TOT} = \gamma_{bs} - \gamma_{bw} - \gamma_{sw} \quad (3)$$

For instance, the interfacial tension for one dysphasic system of interaction (bacteria/substratum - γ_{bs}) can be defined by the thermodynamic theory according to the following equations:

$$\gamma_{bs} = \gamma_{bs}^{LW} + \gamma_{bs}^{AB} \quad (4)$$

$$\gamma_{bs}^{LW} = \gamma_{b}^{LW} + \gamma_{s}^{LW} - 2 \times \sqrt{\gamma_{b}^{LW} \times \gamma_{s}^{LW}} \quad (5)$$

$$\gamma_{bs}^{AB} = 2 \times \left(\sqrt{\gamma_{b}^{+} \times \gamma_{b}^{-}} + \sqrt{\gamma_{s}^{+} \times \gamma_{s}^{-}} - \sqrt{\gamma_{b}^{+} \times \gamma_{s}^{-}} - \sqrt{\gamma_{b}^{-} \times \gamma_{s}^{+}} \right) \quad (6)$$

The other interfacial tension components, γ_{bw} (bacteria/water) and γ_{sw} (substratum/water), were calculated in the same way. The value of the free energy of adhesion was obtained by the application of Eqs. 4-6, which allowed the assessment of thermodynamic adhesion. Thermodynamically, if $\Delta G_{bws}^{TOT} < 0$ mJ/m^2, the adhesion of one bacteria to substratum is favorable. On the contrary, adhesion is not expected to occur (Simões *et al.*, 2010).

4.2.5. MOTILITY

The swarming (flagella-directed rapid movement onto solid surfaces), swimming (flagella-directed movement in aqueous environments), and twitching (pilus-directed movement onto solid surfaces) motilities of *E. coli* and *S. aureus* were investigated using the following media:

(1) swim plate (10 g/L tryptone, 2.5 g/L NaCl, 0.3% agar (Merck)),

(2) swarm plate (10 g/L nutrient broth, 2.5 g/L NaCl, 0.7% agar), and

(3) twitch plate (10 g/L tryptone, 2.5 g/L NaCl, 1.5% agar).

To test the effect of PITC in cell motility, the same mediums were prepared and PITC was added after autoclave and cold the mediums. Concentrations of PITC used were the MIC and 5000 µg/mL. Control was performed with mediums without PITC. The agar media were air-dried for 1 day before preparation. The cell suspensions (*E. coli* and *S. aureus*) obtained by overnight cultures grown in LB medium (Merck) were adjusted to OD_{640nm} of 0.4 ± 0.02. Then, 15 µL of the cell suspensions were added at the center of each plate. The plates were incubated and after 24, 48 and 72 hours the inhibition zones were measured.

4.2.6. STATISTICAL ANALYSIS

All experiments were done in triplicate. The data was analyzed using One-Way Anova. The results were presented as the Means ± SEM (standard error of the mean). Significance level for the separation was set at $P < 0.05$. The data were analyzed using the statistical program SPSS 14.0 (Statistical Package for the Social Sciences).

4.3. Results and Discussion

4.3.1. SINGLE-SPECIES BIOFILM CONTROL WITH PITC

After concluding that phenyl isothiocyanate had significant antimicrobial activities against *E. coli* and *S. aureus* strains and that PITC can act synergistically with erythromycin and ciprofloxacin in *S. aureus* strains (Chapter 3), in this chapter, the effect of PITC in biofilms of the same strains was evaluated. So, biofilm formation was performed in sterile 96-well polystyrene microtiter plates. For better analysis of the results, the sequences tested are explained again in Table 7.

Table 7. Description of treatments performed in this work

Test	Description
A	The strains were incubated by overnight with PITC → the biofilms were formed for 24 hours → After 24 hours, the biofilms were incubated again with PITC for 1 hour
B	The strains were incubated overnight with PITC → the biofilms were formed for 24 hours
C	The strains were incubated overnight without PITC → the biofilms were formed for 24 hours → After 24 hours, the biofilms were incubated again with PITC for 1 hour
D	The strains were incubated overnight without PITC → the biofilms were formed for 24 hours in presence of PITC

With these sequences it would be possible to conclude about the efficacy of PITC in: control (removal and inactivation) of biofilms never exposed to PITC; control of biofilms formed by cells previously exposed to PITC; prevention of biofilm formation by PITC.

Figure 7 shows the results of CV (Fig. 7a) and resazurin (Fig. 7b) staining for *E. coli* and *S. aureus* strains for the several conditions tested. The negative control was performed with sterile medium. The positive control represented the quantity of biofilm formed in the absence of PITC.

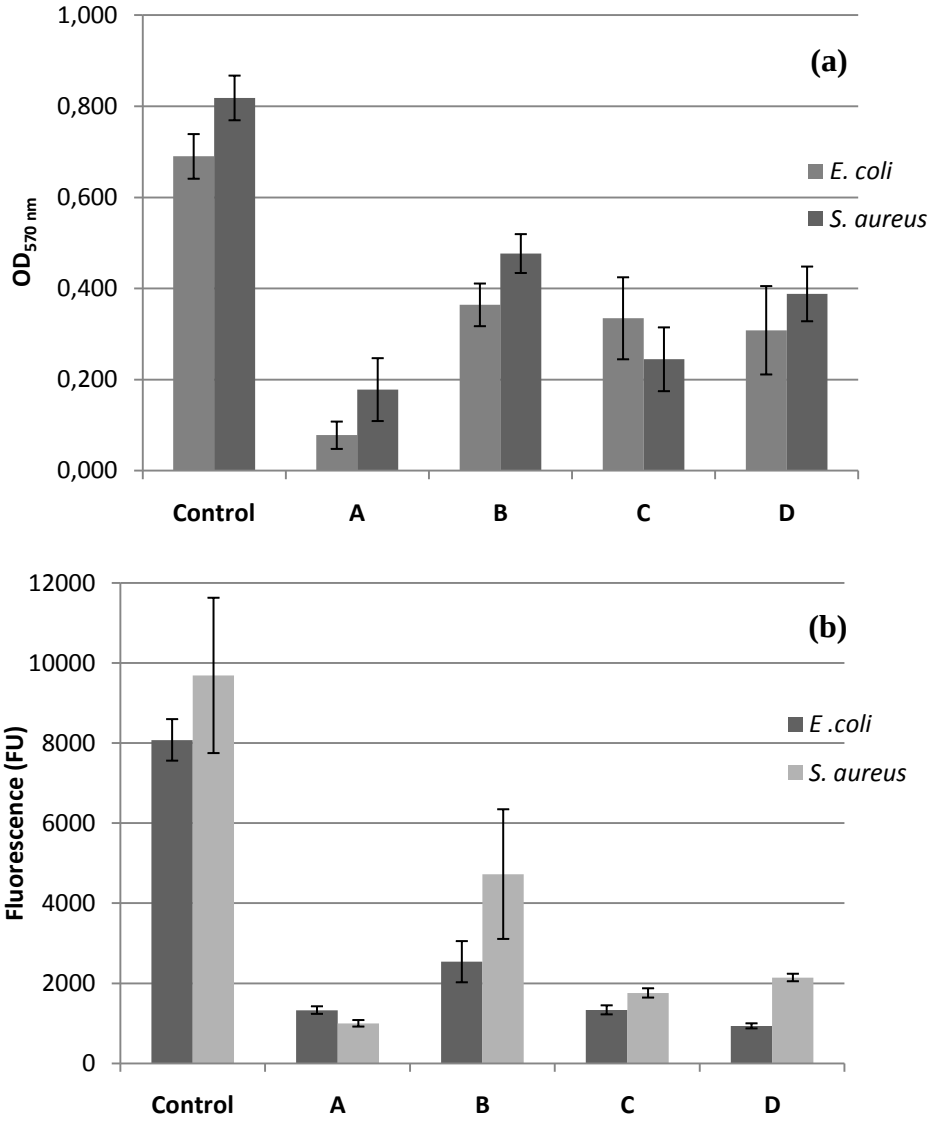


Fig. 7. $OD_{570\ nm}$ as a measure of biofilm mass (a) and fluorescence (λ_{ex}: 560 nm and λ_{em}: 590 nm) results as a measure of biofilm viability (b) for *E. coli* and *S. aureus* with the effect of PITC at 1000 µg/mL in the four sequences of treatment.

According to classification scheme proposed by Stepanović *et al* (2000), both strains are weak biofilm producers (+) in these conditions and with PS as substrate. The same result was demonstrated by Rodrigues *et al* (2010).

With the addition of PITC, the quantity of biofilm measured by CV was always lower comparing with that obtained by positive control, for both strains ($P < 0.05$) (Fig. 7a). Table 8 shows the percentage of biofilm removal (in cases A and C) or prevention (in cases B and D) with PITC for the several sequences previously described.

Table 8. Biofilm removal/prevention by PITC in *E. coli* and *S. aureus* strains

Biofilm removal/prevention by PITC (%)		
	E. coli	***S. aureus***
A	89	78
B	47	42
C	42	39
D	55	53

In sequence C, the capacity of PITC to act against biofilms with cells never exposed to this compound was evaluated. PITC was added to biofilms already developed and the removal percentages obtained were 42 and 39% for *E. coli* and *S. aureus*, respectively. The most important difference between planktonic and biofilm cells is that the last ones have greater opportunity to express protective traits as the result of retarded antimicrobial penetration or slow growth, enabling biofilm cells to respond to an antimicrobial challenge (Stewart, 2002). Gomes de Saravia and Gaylarde (1998) reported that penetration of isothiocyanates into cells is, in general, poor and it may therefore penetrate biofilms inefficiently. However, resazurin results (Fig. 7b) conclude that, for both strains, the cells were not viable (the samples were blue stained). So, although most biofilm was still attached to the surface, cells were killed by PITC. This indicated that PITC could penetrate biofilms and react with cells membrane.

The objective of sequence B is to evaluate the capacity of cells to adapt to PITC. The results show that the incubation overnight with PITC did not kill bacteria totally and the survival bacteria can form biofilm as well. Although, the biofilm mass in this case was inferior 47% and 42% for *E. coli* and *S. aureus* strains, respectively (Table 8). In chapter 3, we concluded that only after 1 hour of contact with PITC, approximately 70% of cells were PI stained. In this case, cells were submitted to a long contact with PITC and it was expected that more cells were dead. However, some cells could develop some alternatives to survive and resist to PITC. Resazurin results indicated that cells remaining in the biofilm are viable (the samples were pink stained), especially *S. aureus*. So, *S. aureus* demonstrated greater capacity to adapt to PITC comparing with *E. coli*.

Sequence A pretends to evaluate the antimicrobial capacity of PITC on cells already exposed to PITC. With this sequence, the percentages of removal of biofilm were the highest - for *E. coli* and *S. aureus*, respectively ($P < 0.05$) - and the biofilms were not viable (the samples

were blue stained) as indicated in Figure 7b. So, new addition of PITC seems to have a positive effect against bacteria previously exposed to this phytochemical.

With sequence D, PITC was added in order to prevent biofilm formation. With this sequence, the quantities of biofilm mass formed were 55 and 53 % inferior for *E. coli* and *S. aureus* respectively. This situation can be explained by the fact that the action of PITC on sessile organisms may still involve a direct effect on adhesion, in addition to enzyme inactivation, since PITC can disrupt membrane-membrane adherence (Gomes de Saravia and Gaylarde, 1998). The resazurin results indicated lack of viability of *E. coli* cells in biofilm and a slight viability of *S. aureus* cells. This reinforces the fact that *S. aureus* can adapt more easily to PITC at this concentration.

In conclusion, results indicate that biofilm cells can adapt to PITC at 1000 µg/mL and that this compound could not avoid totally the development of the biofilm. Analyzing the results, PITC had always more effects for *E. coli* than for *S. aureus*. This fact was also according with the results in planktonic cells (Chapter 3), in which PITC had greater effects in *E. coli*. As explained before, despite Gram-negative bacteria have the asymmetric outer membrane which theoretically represents an effective permeability barrier (largely due to the presence of LPS in the outer leaflet) (Nikaido, 2005; Lai *et al.*, 2009), the reaction of PITC with activated groups in proteins and other biological molecules will result in their inactivation and membrane disruption. Moreover, it was already proved that low permeability of the outer membrane does not prevent completely influx of small molecules (Lai *et al.*, 2009). Furukawa *et al* (2010) demonstrated that *S. aureus* biofilm was more resistant to cleaning by five typical CIP cleaning agents, food additives and other compounds (EDTA, Tween20 and SDS) than the *E. coli* biofilm on stainless steel plates. This is due to the fact that biofilm formed by *S. aureus* was far denser than that formed by *E. coli*. So, an increased thickness might be the major reason for the rigidity of *S. aureus* biofilm. Other explanation is based on the fact that *S. aureus* strain could develop more mechanisms of resistance to PITC than *E. coli*, as previously mentioned.

PITC was also tested at 5000 µg/mL. Figure 8 shows the results of CV (Fig. 8a) and resazurin staining (Fig. 8b) for *E. coli* and *S. aureus* for the conditions tested.

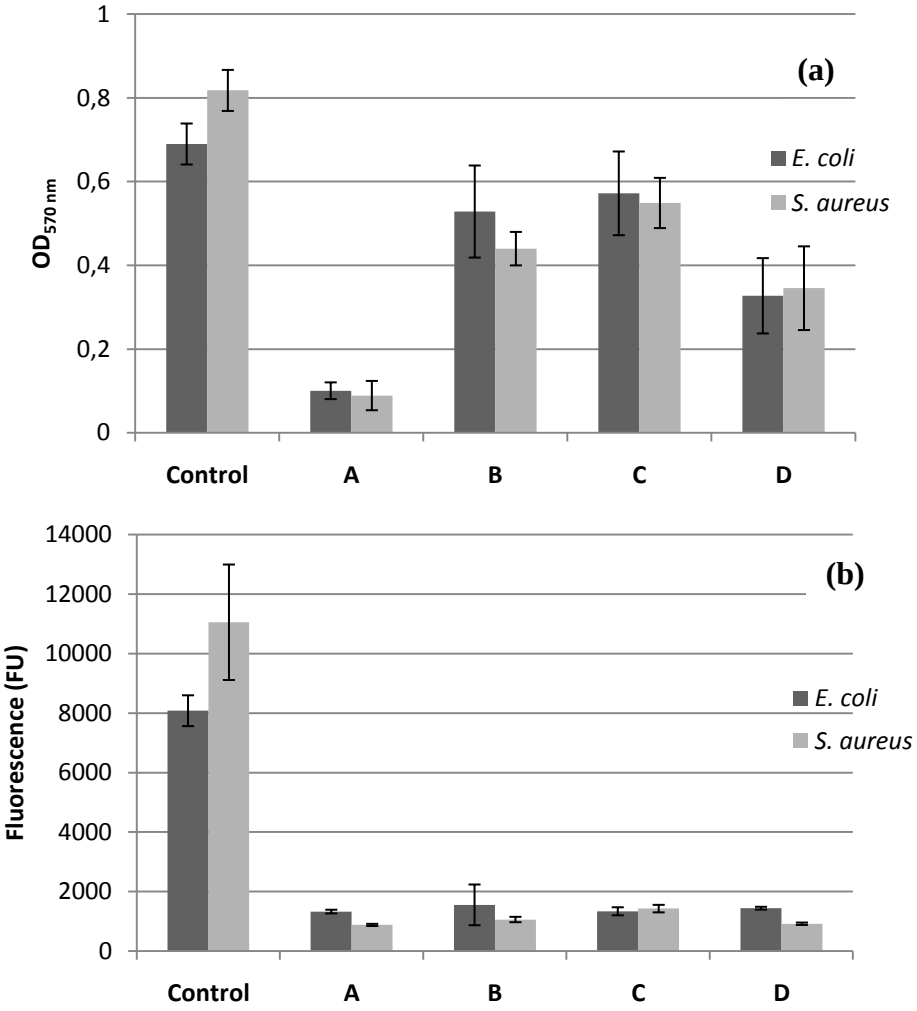


Fig. 8. $OD_{570\ nm}$ as a measure of biofilm mass (a) and fluorescence (λ_{ex}: 560 nm and λ_{em}: 590 nm) results as a measure of biofilm viability (b) for *E. coli* and *S. aureus* with the effect of PITC at 5000 µg/mL in the four sequences of treatment.

Table 9 shows the percentage of biofilm removal/prevention with PITC for the several sequences previously described.

Table 9. Biofilm removal/prevention by PITC in *E. coli* and *S. aureus* strains with PITC at 5000 µg/mL

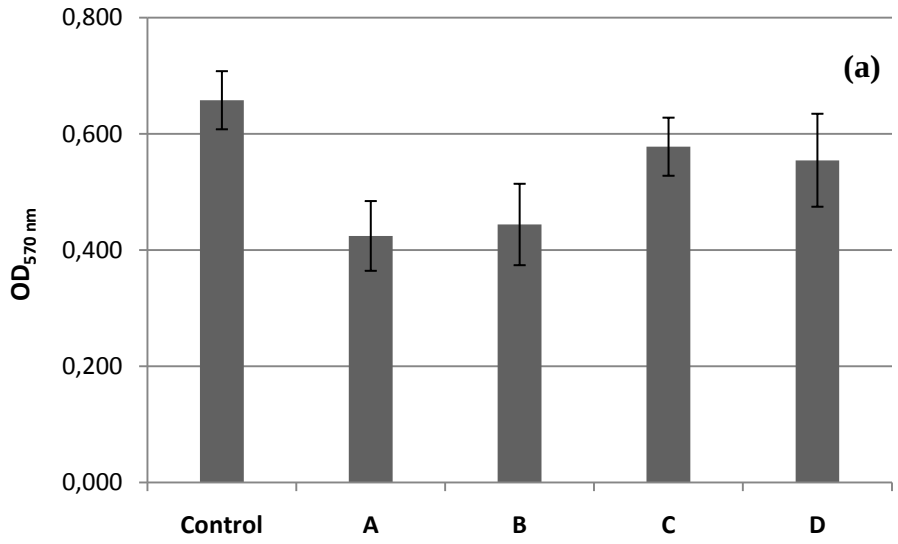
Biofilm removal by PITC (%)		
	E. coli	***S. aureus***
A	85	89
B	23	46
C	17	33
D	53	58

Table 9 shows that removal percentages with PITC at 5000 µg/mL were better in some sequences, especially for sequence A. Moreover, in this case, removal/prevention percentages in *S. aureus* were superior. However, the better improvement was obtained with resazurin results, since cells were not viable in all sequences. So, at this concentration, PITC was more effective on killing bacteria. Probably the adaption of cells previously observed was due to the use of a sub-lethal concentration of PITC.

The use of sub-inhibitory concentrations of antibiotics partially reduces biofilm formation but can probably induce changes in biofilm phenotype. This situation was also reported by Cerca *et al* (2006). The changes in the bacterial phenotype and on the biofilm structure, like an increase in antibiotic resistance and biofilm matrix constitution, present a serious obstacle to the implementation of therapy using low concentrations of antibiotics (Cerca *et al*., 2006).

4.3.2. MULTI-SPECIES BIOFILM CONTROL WITH PITC

Due to the fact that mostly biofilms are constituted by multiple species, biofilms formed by the association of *E. coli* and *S. aureus* were developed. Bacterial species interact extensively with each other and these interactions determine the structure development of multispecies biofilms (Yang *et al.*, 2011). Figure 9 presents the CV (Fig. 9a) and resazurin (Fig. 9b) results for the multi-species biofilms. The sequences tested are the same that the previously ones.



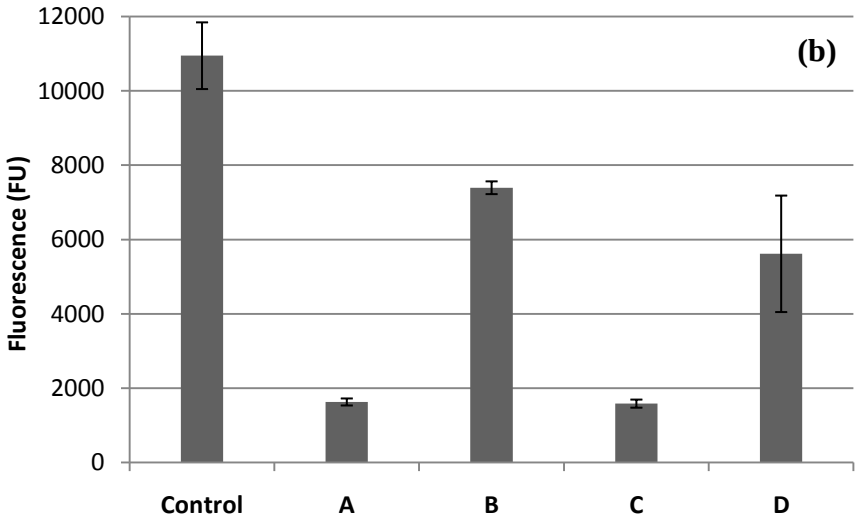


Fig. 9. $OD_{570\ nm}$ as a measure of biofilm mass (a) and fluorescence (λ_{ex}: 560 nm and λ_{em}: 590 nm) results as a measure of biofilm viability (b) for multiple strains of *E. coli* and *S. aureus* with the effect of PITC at 1000 µg/mL in the four sequences of treatment.

Table 10 shows the removal/prevention percentages caused by PITC at 1000 µg/mL.

Table 10. Biofilm removal/prevention by PITC for *E. coli* and *S. aureus* multi-species biofilms. The sequences were described previously

	Biofilm removal/prevention by PITC (%)
A	36
B	33
C	12
D	16

The quantity of biofilm formed by multiple species of *S. aureus* and *E. coli* are inferior to that obtained with single species (Fig. 7a and Fig. 9a). So, the strains are still weak biofilm producers (+) in the conditions tested. Pompermayer and Gaylarde (2000) had similar results. This indicates that, as suggested by McEldowney and Fletcher (1987), there is a competition for attachment, which reduces the numbers of each species adhering to the surface. Probably, the predominant organisms in a biofilm will be those with the highest growth rate (Pompermayer and Gaylarde, 2000). In this case, *S. aureus* strain had a greater adhesion to polystyrene surfaces comparing with *E. coli* as it is possible to observe in Fig. 7a. However, Pompermayer and Gaylard (2000) reported that usually Gram-negative cells adhere better to inert surfaces and that *E. coli* cells have a shorter generation time, which would enable them to develop and maintain

their dominance (Pompermayer and Gaylarde, 2000). So, techniques such as specific immunofluorescent or fluorescent gene probe labelling could be used to assess the proportion of each bacterium in the biofilm (Pompermayer and Gaylarde, 2000).

The structural and functional dynamics of multispecies biofilms are largely due to the interactions between different species of microorganisms. These interactions often change the physiology of biofilm species as well as the functions of the whole community (Yang *et al.*, 2011). The removal/prevention percentages by PITC obtained with multi-species biofilms were lower. The greater percentage (36%) was also obtained with sequence A ($P < 0.05$). Resazurin results also demonstrated that biofilms were viable after treatments B and C, which indicated that cells had higher capacity of adaption to the phytochemical. Probably, the biofilms formed by both strains were denser and stronger and PITC could not penetrate so efficiently. More tests would be necessary to take definitive conclusions but perhaps it would be necessary a higher concentration of PITC to kill more efficiently the bacteria.

4.3.3. ANALYSIS OF BACTERIA ADHESION

In biological systems, hydrophobic interactions are usually the strongest of all long-range non-covalent forces, and adhesion to surfaces is often mediated by these types of interaction (Cerca *et al*,. 2006). The relationship between physicochemical surface parameters - hydrophobicity and surface charge (zeta potential) - and adhesion of bacterial cells to negatively charged polystyrene was studied. Both parameters have great influence in cell adhesion. The PS microtiter plates are commonly used as the standard bioreactor system for adhesion and biofilm formation of bacteria isolated from many different environments, providing reliable comparative data (Simões *et al.*, 2010).

In chapter 3, based on the approach proposed by van Oss (van Oss, 1995; van Oss, 1997), physico-chemical characterization of bacterial surface by zeta potential and contact angle measurements indicated that the surfaces of the tested bacteria are hydrophilic ($\Delta G_{sws}^{TOT} > 0$ mJ/m^2). When incubated with PITC, the bacterial surface is less negative and more hydrophilic, probably due to the reaction with peptides and proteins in membrane, as previously explained. The PS surface was characterized in terms of surface properties - hydrophobicity and surface charge (zeta potential) by Simões *et al* (2010). This characterization is described in Appendix C (Table 16). PS surface is hydrophobic (ΔG_{sws}^{TOT} = - 44 mJ/m^2). In order to predict the ability of the microorganisms to adhere to PS surfaces, the free energy of interaction between the bacteria and the surface, when immersed in water, was calculated according to the thermodynamic

approach. Based on this approach, all the bacteria had no theoretical thermodynamic ability to adhere to PS (ΔG_{bws}^{TOT} > 0 mJ/m^2) as demonstrated in Table 11.

Table 11. Free energy of adhesion (ΔG_{bws}^{TOT}) of untreated and PITC treated cells to polystyrene

Bacteria	PITC concentration (µg/mL)	Free energy of adhesion (mJ/m^2) - ΔG_{bws}^{TOT}
E. coli	0	6.4
	1000	9.6
	5000	10.6
S. aureus	0	2.4
	1000	3.5
	5000	8.0

However, even ΔG_{bws}^{TOT} being superior to 0 mJ/m^2, bacteria adhered to PS. So, the comparison between the theoretical thermodynamic adhesion evaluation and the adhesion assays shows that adhesion was underestimated when based on thermodynamic approaches.

Moreover, as well as bacteria cells, PS is also negatively charged and, therefore, based only on the DLVO theory, the electrostatic forces between the microorganisms and the surfaces of accession would be repulsive (Simões, 2004) and could avoid cell adhesion to PS. Many authors have been observing the colonization of negatively charged supports by microorganisms with the same signal (van Loosdrecht *et al.*, 1987; Simões, 2004; Simões *et al.*, 2010).

Comparing the free energy of adhesion (ΔG_{bws}^{TOT}**)** for bacteria (Table 10), it is possible to conclude that *S. aureus* has more ability to adhere to PS (ΔG_{bws}^{TOT} is inferior) (P < 0.05). This could contribute to the higher biofilm formation of *S. aureus* comparing with *E. coli*. So, *S. aureus* is probably the dominant strain in multi-species biofilms.

With PITC, bacteria have even less ability to adhere to PS (ΔG_{bws}^{TOT} is superior). This was expected because cells surface with PITC are more hydrophobic, which is important in adhesion since hydrophobic interactions tend to increase with an increasing of nonpolar nature of one or both surfaces involved (Simões, 2005). According to Drenkard and Ausubel (2002), the ability of bacteria to attach to each other and to surfaces depends in part on the interaction of hydrophobic domains. In general, attachment will occur most readily on surfaces that are rougher, more hydrophobic and coated by surface conditioning films (Simões, 2005). Even though the correlation between cell surface hydrophobicity and bacterial adhesion has been

found by some authors (Dickson and Koohmaraie, 1989; Pompilio *et al.*, 2008), others disagree (Li and McLandsborough, 1999; O'Toole *et al.*, 2000; Simões *et al.*, 2010, Cerca *et al*., 2006). This situation explains, in part, the CV results obtained since it was demonstrated that, with PITC, adhesion of cells to PS is inferior (sequence D). So, hydrophobic differences of cells with or without PITC seem to influence the adhesion process.

The overall hydrophobicity and/or surface charge of a bacterium are clearly important in initial cell-surface and cell-cell interactions and, as O'Toole *et al* (2000) suggested, these factors could serve as a good predictor of the surfaces that an organism might colonize. However, bacterial surfaces are heterogeneous, and, most important, they can change dramatically in response to changes in their environment; so it is difficult to determine exactly their behavior (O'Toole *et al.*, 2000). Cerca *et al*, 2006 showed that high levels of initial adherence do not necessarily lead to thick biofilm formation and that initial adhesion and biofilm maturation are two distinct phenomena. However, Pompilio *et al* (2008) found that the direct relationship between the amount of biofilm formed and the extent of the initial adhesion indicates that levels of adhesion are predictive of biofilm formation.

The bacterial components required for biofilm development and the mechanisms that regulate their production and activity are needed for a fuller understanding of this microbial phenomenon (O'Toole *et al.*, 2000). The lack of agreement between thermodynamic approaches and adhesion assays reinforces that biological mechanisms, such as the presence of extracellular biological molecules and the expression of extracellular appendages (adhesins) that mediate specific interactions with substratum at a nanometer scale during the irreversible phase of adhesion (Simões *et al.*, 2010). Pompilio *et al* (2008) refer that development of biofilm is closely associated with the generation of EPS (Pompilio *et al.*, 2008). Simões *et al* (2007b) agrees that, in addition to physicochemical surface properties of bacterium and substratum, biological factors are involved in early adhesion processes, suggesting that reliance on thermodynamic approaches alone may not accurately predict adhesion capacity.

4.3.4. MOTILITY ASSAYS

Several studies have indicated that cell surface structures such as pili, flagella and fimbriae play an integral role in enabling microorganisms to adhere initially to a surface and to form biofilms. This is possible mainly by overcoming the repulsive forces associated with the substratum and contributing to cell surface hydrophobicity (Pompilio *et al.*, 2008). The possible correlation between biofilm formation ability and cell hydrophobicity and motility was

examined. Table 12 shows motility results of bacteria not expose to PITC and in presence of PITC at 1000 and 5000 µg/mL.

Table 12. Motility results for bacteria with and without PITC

		Motility (mm) at sampling time (h)								
		Control			**PITC (1000 µg/mL)**			**PITC (5000 µg/mL)**		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
E. coli	*swim*	55±4.6	85±0.0	85±0.0	7.0±0.0	9.0±0.0	48±2.5	-	-	-
	swarm	8.0±0.0	8.0±0.0	9.0±0.0	7.0±0.0	8.0±0.0	7.0±0.0	-	-	-
	twitch	8.0±0.6	9.0±0.0	9.0±0.6	8.0±0.0	9.0±0.0	8.0±0.0	-	-	-
S. aureus	*swim*	25±0.6	60±0	65±0.6	7.0±0.0	9.0±0.0	14±0.0	8.0±0.0	8.0±0.0	8±0.0
	swarm	8.0±0.0	8.0±0.0	8.0±0.0	8.0±0.0	8.0±0.0	8.0±0.0	-	10.0±0.0	10.0±0.0
	twitch	8.0±0.0	9.0±0.0	9.0±0.0	8.0±0.0	9.0±0.0	9.0±0.0	10.0±0.0	10.0±0.0	10.0±0.0

Without PITC, both strains had an increase in swimming motility over time. This fact was not expected since *S. aureus* is non-motile. However, although streptococci do not have flagella, they can spread on solid surfaces via a mechanism called sliding (Kaito and Sekimizu, 2007). The sliding ability of these bacteria is provided by the expansive forces of a growing culture in combination with special surface properties of the cells resulting in reduced friction between the cell and its substrate. Kaito and Sekimizu (2007) founds that *S. aureus* can rapidly expand on soft agar surfaces at a speed of 100 µm/min. In addition, *E. coli* also have sliding ability that is independent of their flagella (Kaito and Sekimizu, 2007). Swarming and twitching motilities were very low for both strains.

In this study, *E. coli* has the highest motility; however, *S. aureus* is a greater biofilm producer and has more adhesion capacity to PS. So, motility does not regulate adhesion and biofilm formation by itself. Other authors conclude the same thing to several strains (Suárez *et al.*, 1992; Simoes *et al.*, 2007a; Simões *et al.*, 2007b; Pompilio *et al.*, 2008). Dickson and Koohmaraie (1989) reported that there is disagreement over the role of surface structures, since nonfimbriated and nonflagellated cells can attach at rates similar to those of cells which possess these structures. However, other reports indicate that motile bacteria attach to surfaces more rapidly than nonmotile strains (Dickson and Koohmaraie, 1989).

With PITC at 1000 µg/mL, swimming motility is very low in the first 48 hours but, in the last 24h, increased, especially for *E. coli*. This reinforces the theory that cells can adapt to

PITC after a long period of exposure, as previously observed when cells incubated overnight in presence of PITC could form biofilm (sequence B).

With PITC at 5000 µg/mL, *E. coli* did not show any motility during all experience. For *S. aureus* the results were not relevant. So, at this concentration, cells did not adapt to PITC. Moreover, it is important to highlight that PITC at 5000 µg/mL is more effective against *E. coli* than *S. aureus* since cell motility is more affected.

4.4. Conclusions

Single and multi-species biofilms of *E. coli* and *S. aureus* were developed. These bacteria were considered weak biofilm producers for the conditions. The biofilm formation assays with PITC at 1000 µg/mL demonstrated that PITC can penetrate the biofilms efficiently; cells (especially *S. aureus*) can adapt to this compound; and that bacteria can develop biofilm in presence of PITC. With PITC at 5000 µg/mL, cells could not resist to PITC. The higher removal percentages of biofilms were obtained with the addition of PITC at 5000 µg/mL to cells previously exposed to this compound: 85 and 89% for *E. coli* and *S. aureus*, respectively. The biofilm formed by multi-species of *S. aureus* and *E. coli* was inferior to that obtained with single species and the inhibition caused by PITC to biofilm formation was also inferior. *S. aureus* is probably the dominant strain.

Adhesion results indicate that adhesion was underestimated when based on thermodynamic approaches and that, with PITC, bacteria have even less ability to adhere to PS. So, hydrophobic differences of cells with or without PITC seem to have some influence on the adhesion process. Moreover, *S. aureus* had more capacity to adhere to PS than *E. coli*.

The possible correlation between biofilm formation ability and cell hydrophobicity and motility was examined. Even though *E. coli* had a greater motility, *S. aureus* is a greater biofilm producer and has more adhesion capacity to PS. So, motility of these strains tested may not regulate adhesion and biofilm formation. In presence of PITC, swim motility is very low at the beginning, but increases in the last 24 h, especially for *E. coli*. This reinforces that strains can adapt to PITC at sub-lethal concentrations after a long period of exposure. With PITC at 5000 µg/mL, motility results were insignificant during the experience.

PITC had generally more effects against *E. coli* than for *S. aureus*. Probably, *S. aureus* can develop more resistance mechanisms to PITC.

Chapter 5

Concluding remarks and perspectives for further research

5.1 General conclusions

Revealing the specific mechanisms by which microorganisms adhere to plastic surfaces may aid in treating outbreaks of these pathogens. Preventing primary adhesion would prevent biofilm formation and this may lead to better and faster eradication. To develop efficient preventive and control strategies in biofilm development, it is necessary to understand the relationship between adhesion and biofilm formation, the role of microorganisms in this process, the bacterial components required for biofilm development and the mechanisms that regulate their production and activity. With the present work, it is possible to conclude important aspects about phenyl isothiocyanate as antimicrobial and biofilm control agent:

Chapter 3:

- PITC had significant antimicrobial activities against *E. coli* and *S. aureus* and also had a good efficacy against *S. aureus* strains when combined with erythromycin and ciprofloxacin due to an additive effect;

- When incubated with PITC, the bacterial surface is less negative and more hydrophilic;
- The reaction of PITC with peptides and proteins at the membrane may be the cause of the changes on physical bacteria surface observed;
- After 1 hour of reaction with PITC at 1000 µg/mL, about 70 % of both strains were dead (as proved by Live/Dead staining).

Chapter 4:

- *E. coli* and *S. aureus* were considered weak biofilm producers;
- The biofilm formation assays with PITC demonstrated that: PITC can penetrate the biofilms efficiently since a significant number of cells were not viable; cells (especially *S. aureus*) can adapt to this compound at 1000 µg/mL; bacteria can develop biofilm in presence of PITC; with PITC at 5000 µg/mL, cells could not resist and resazurin results indicated that all biofilms were not viable; the higher removal percentages of biofilms were obtained with the addition of PITC at 5000 µg/mL to cells previously exposed to this compound;
- The adaption capacity of cells to PITC at 1000 µg/mL can probably be due to the use of a sub-lethal concentration;
- The use of sub-inhibitory concentrations of antibiotics partially reduces biofilm formation but can probably induce changes in biofilm phenotype;
- The removal percentages obtained with PITC in multi-species biofilms of *S. aureus* and *E. coli* were inferior. *S. aureus* is probably the dominant strain since it has a great capacity to adhere to PS;
- Adhesion results indicate that adhesion was underestimated when based on thermodynamic approaches and that, with PITC, bacteria have even less ability to adhere to PS. Hydrophobic differences of cells with or without PITC seem to influence the adhesion process. So, PITC can disrupt membrane-membrane adherence, which may involve a direct effect on adhesion;
- Despite *E. coli* had a greater motility, *S. aureus* is a greater biofilm producer and has more capacity to adhere to PS. So, motility of these strains does not regulate adhesion and biofilm formation;
- Strains can recover their motility in presence of PITC at 1000 µg/mL after a long period of exposure but, with PITC at 5000 µg/mL, cells did not adapt and motility was insignificant.
- PITC had generally more effects for *E. coli* than for *S. aureus*, probably because *S. aureus* could develop more mechanisms of resistant to PITC.

It is known that conventional antimicrobial products are more effective against Gram-positive bacteria compared with Gram-negative bacteria (Aires *et al*, 2009b). Consequently, antimicrobial products with significant activity against Gram-negative bacteria can be a valuable source of therapeutic agents to control infections specifically caused by those bacteria.

The overall hydrophobicity and/or surface charge of a bacterium are important to predicting what surfaces an organism might colonize. However, bacterial surfaces are heterogeneous and can change dramatically in response to changes in their environment, which reinforces the importance of biological mechanisms that mediate specific interactions with substratum. Consequently, an understanding of the structure-function relationships in microbial biofilms seems to be fundamental to interpret and predict biofilm impacts on the habitat where they are developed.

The significance of biofilms is not a well understood phenomenon because of a lack of direct observation of biofilms in their environment and a lack of research using model systems that closely simulate the environmental system (Simões, 2005).

Despite the abundant literature about the antimicrobial properties of plant extracts, none of the plant derived chemicals have successfully been used for clinical use as antibiotics (Adwan and Mhanna, 2008). The concentrations required for phytochemicals chemotherapeutic activity are too high to be clinically relevant (Simoes *et al.*, 2009). In this case, PITC is effective at 5000 µg/mL but, at this concentration, the toxicity *in vivo* could be more pronounced. In conclusion, the results of this study with phenyl isothiocyanate were encouraging; however, the apparently conflicting pro- and anti-oxidative effects of ITCs requires careful management of these compounds in human use and many more toxicological tests are necessary. This study probably suggests the possibility of concurrent use of these antimicrobial drugs and extracts in combination in treating infections caused by *S. aureus* and *E. coli* strains. However, it is hard to predict additive or synergistic effects *in vivo* on the basis of the presented *in vitro* evidence alone.

5.2 Perspectives for further research

Throughout this work, it was notice that more tests and more information would be interesting about the topic addressed in this dissertation. However, due to time constraints and material resources some tests were not possible to perform. So, more research would be necessary about the mode of action of PITC and every single reaction that may occur in the cell, the capacity of adaption of cells to PITC and the mechanisms of bacterial resistance to PITC.

This research would explain the reason for the higher resistance of *S. aureus* comparing with *E. coli* biofilms. Moreover, it would be important to evaluate the presence and extent of EPS in the biofilm communities. For this, a microscopic analysis of 24h mature biofilms of both strains could be perform using Alcian blue, a cationic dye which stains anionic polysaccharides (sulphated mucopolysaccharides, glycosaminoglycans).

Much more aspects are needed to be learned about the impact of antimicrobial agents on bacteria and their response to it. Most biofilm studies have been performed based on *in vitro* laboratory models and, sometimes, it is difficult to find out the importance and contribution of individual parameters on the process. So, to study complex biological systems, it is necessary to develop more realistic models. The evaluation of potential of bacteria for biofilm formation, the precisely mechanisms that affect cells adhesion to surfaces and interspecies interactions can provide new data to understand this phenomenon and, consequently, can facilitate the control and prevention of biofilms.

Finally, despite PITC seems to be a promising compound for antimicrobial therapy against the bacteria tested, clinical controlled studies are needed to define the real efficacy and possible toxic effects *in vivo*.

Bibliography

Adwan, G. and Mhanna, M. 2008, 'Synergistic effects of plant extracts and antibiotics on *Staphylococcus aureus* strains isolated from clinical specimens', *Middle-East Journal of Scientific Research,* vol. 3, no. 3, pp 134-139.

Aires, A., Mota, V. R., Saavedra, M. J., Monteiro, A. A., Simões, M., Rosa, E. A. S. and Bennett, R. N. 2009a, 'Initial *in vitro* evaluations of the antibacterial activities of glucosinolate enzymatic hydrolysis products against plant pathogenic bacteria', *Journal of Applied Microbiology,* vol. 106, no. 6, pp 2096-2105.

Aires, A., Mota, V. R., Saavedra, M. J., Rosa, E. A. S. and Bennett, R. N. 2009b, 'The antimicrobial effects of glucosinolates and their respective enzymatic hydrolysis products on bacteria isolated from the human intestinal tract', *Journal of Applied Microbiology,* vol. 106, no. 6, pp 2086-2095.

Alanis, A. J. 2005, 'Resistance to antibiotics: are we in the post-antibiotic era?', *Archives of medical research,* vol. 36, no. 6, pp 697-705.

Aminov, R. I. 2009, 'The role of antibiotics and antibiotic resistance in nature', *Environmental Microbiology,* vol. 11, no. 12, pp 2970-2988.

Angulo, F. J., Nargund, V. N. and Chiller, T. C. 2004, 'Evidence of an association between use of anti-microbial agents in food animals and anti-microbial resistance among bacteria isolated from humans and the human health consequences of such resistance', *Journal of Veterinary Medicine, Series B,* vol. 51, no. 8-9, pp 374-379.

Atabek, A. 2006, 'Investigation bacterial outer membrane polymers and bacterial interactions with organic molecules using atomic force microscopy', Master Thesis, Worcester Polytechnic Institute, Worcester.

Bending, G. D. and Lincoln, S. D. 2000, 'Inhibition of soil nitrifying bacteria communities and their activities by glucosinolate hydrolysis products', *Soil Biology and Biochemistry*, vol. 32, no. 8-9, pp 1261-1269.

Berney, M., Hammes, F., Bosshard, F., Weilenmann, H.-U. and Egli, T. 2007, 'Assessment and interpretation of bacterial viability by using the Live/Dead BacLight Kit in combination with flow cytometry', *Applied and Environmental Microbiology,* vol. 73, no. 10, pp 3283-3290.

Borek, V., Elberson, L. R., McCaffrey, J. P. and Morra, M. J. 1995a, 'Toxicity of aliphatic and aromatic isothiocyanates to eggs of the black vine weevil (Coleoptera: Curculionidae)', *Journal of Economic Entomology*, vol. 88, no. pp 1192-1196.

Borek, V., Elberson, L. R., McCaffrey, J. P. and Morra, M. J. 1998b, 'Toxicity of isothiocyanates produced by glucosinolates in Brassicaceae species to black vine weevil eggs', *Journal of Agricultural and Food Chemistry*, vol. 46, no. 12, pp 5318-5323.

Borek, V., Morra, M. J., Brown, P. D. and McCaffrey, J. P. 1995b, 'Transformation of the glucosinolate-derived allelochemicals allyl isothiocyanate and allylnitrile in soil', *Journal of Agricultural and Food Chemistry*, vol. 43, no. 7, pp 1935-1940.

Brehm-Stecher, B. F. and Johnson, E. A. 2003, 'Sensitization of *Staphylococcus aureus* and *Escherichia coli* to antibiotics by the sesquiterpenoids nerolidol, farnesol, bisabolol, and apritone', *Antimicrobial Agents and Chemotherapy*, vol. 47, no. 10, pp 3357-3360.

Brooun, A., Liu, S. and Lewis, K. 2000, 'A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms', *Antimicrobial Agents and Chemotherapy*, vol. 44, no. 3, pp 640-646.

Busetti, A., Crawford, D. E., Earle, M. J., Gilea, M. A., Gilmore, B. F., Gorman, S. P., Laverty, G., Lowry, A. F., McLaughlin, M. and Seddon, K. R. 2010, 'Antimicrobial and antibiofilm activities of 1-alkylquinolinium bromide ionic liquids', *Green Chemistry*, vol. 12, no. 3, pp 420-425.

Busscher, H. J., Weerkamp, A. H., van der Mei, H. C., van Pelt, A. W., de Jong, H. P. and Arends, J. 1984, 'Measurement of the surface free energy of bacterial cell surfaces and its relevance for adhesion', *Applied and Environmental Microbiology*, vol. 48, no. 5, pp 980-983.

Cerca, N. M. 2006, 'Virulence aspects of *Staphylococcus epidermis*: biofilm formation and Poly-N-Acetyl-Glucosamine production', PhD thesis, University of Minho, Braga.

CHEBI. 2010a, *Ciprofloxacin (CHEBI:100241)*. Retrieved January, 2011, from http://www.ebi.ac.uk/chebi/searchId.do;6308F594F01F45299E12DAA476A413A6?chebiId=CHEBI:100241.

CHEBI. 2010b, Tetracycline (CHEBI:27902). Retrieved January, 2011, from http://www.ebi.ac.uk/chebi/searchId.do;6308F594F01F45299E12DAA476A413A6?chebiId=CHEBI:27902.

Chopra, I. and Roberts, M. 2001, 'Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance', *Microbiol. Mol. Biol. Rev.*, vol. 65, no. 2, pp 232-260.

Clinical and Laboratory Standards Institute 2005, 'NCCLS M100-S15: Performance standards for antimicrobial susceptibility testing: fifteen informational supplement', vol. 25, no. 1.

Costerton, J. W., Stewart, P. S. and Greenberg, E. P. 1999, 'Bacterial biofilms: a common cause of persistent infections', *Science*, vol. 284, no. 5418, pp 1318-1322.

Coutinho, H., Costa, J., Lima, E., Falcao-Silva, V. and Siqueira, J. 2009, 'Herbal therapy associated with antibiotic therapy: potentiation of the antibiotic activity against methicillin - resistant *Staphylococcus aureus* by Turnera ulmifolia L', *BMC Complementary and Alternative Medicine*, vol. 9, no. 13, pp 1-4.

Cowan, M. M. 1999, 'Plant products as antimicrobial agents', *Clinical Microbiology Reviews*, vol. 12, no. 4, pp 564-582.

Cramton, S. E., Gerke, C., Schnell, N. F., Nichols, W. W. and Gotz, F. 1999, 'The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation', *Infection and Immunity*, vol. 67, no. 10, pp 5427-5433.

Dantas, G., Sommer, M. O., Oluwasegun, R. D. and Church, G. M. 2008, 'Bacteria subsisting on antibiotics', *Science*, vol. 320, no. 100, pp 100-103.

Deziel, E., Comeau, Y. and Villemur, R. 2001, 'Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpiliated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities', *The Journal of Bacteriology*, vol. 183, no. 4, pp 1195-1204.

Di Bonaventura, G., Piccolomini, R., Paludi, D., D'Orio, V., Vergara, A., Conter, M. and Ianieri, A. 2008, 'Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: relationship with motility and cell surface hydrophobicity', *Journal of Applied Microbiology*, vol. 104, no. 6, pp 1552-1561.

Dickson, J. S. and Koohmaraie, M. 1989, 'Cell surface charge characteristics and their relationship to bacterial attachment to meat surfaces', *Applied and Environmental Microbiology*, vol. 55, no. 4, pp 832-836.

Donlan, R. 2001, 'Biofilms and device-associated infections', *Biofilms and Device-Associated Infections*, vol. 7, no. 2, pp 277-281.

Drenkard, E. and Ausubel, F. M. 2002, '*Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation', *Nature,* vol. 416, no. 6882, pp 740-743.

Drlica, K. 1999, 'Mechanism of fluoroquinolone action', *Current Opinion in Microbiology,* vol. 2, no. 5, pp 504-508.

Drug Bank. 2005a, *Spectinomycin.* Retrieved January, 2011, from http://www.drug bank. ca/ drugs/DB00919.

Drug Bank 2005b, *Streptomycin,* Retrieved January, 2011, from http://www.drugbank. ca/drugs/DB01082.

Drug Bank. 2005, *Erythromycin,* Retrieved January, 2011, from http://www.drugbank. ca/drugs/DB00199.

Emerson IV, R. J. 2006, 'A nanoscale investigation of microbial adhesion to biomedical implant devices', PhD thesis, Worcester Polytechnic Institute, Worcester.

Extremina, C. I., Costa, L., Aguiar, A. I., Peixe, L. and Fonseca, A. P. 2011, 'Optimization of processing conditions for the quantification of enterococci biofilms using microtitre-plates', *Journal of Microbiological Methods,* vol. 84, no. 2, pp 167-173.

Fahey, J. W., Zalcmann, A. T. and Talalay, P. 2001, 'The chemical diversity and distribution of glucosinolates and isothiocyanates among plants', *Phytochemistry,* vol. 56, no. 1, pp 5-51.

Fantin, B. and Carbon, C. 1992, '*In vivo* antibiotic synergism: contribution of animal models', *Antimicrobial Agents and Chemotherapy,* vol. 36, no. 5, pp 907-912.

Ferreira, C., Pereira, A. M., Pereira, M. C., Melo, L. F. and Simões, M. 2011, 'Physiological changes induced by the quaternary ammonium compound benzyldimethyldodecylammonium chloride on *Pseudomonas fluorescens*', *Journal of Antimicrobial Chemotherapy,* vol. 66, no. 6, pp 1-8.

Ferreira, C., Rosmaninho, R., Simoes, M., Pereira, M. C., Bastos, M. M., Nunes, O. C., Coelho, M. and Melo, L. F. 2010, 'Biofouling control using microparticles carrying a biocide', *Biofouling,* vol. 26, no. 2, pp 205-212.

Fux, C. A., Costerton, J. W., Stewart, P. S. and Stoodley, P. 2005, 'Survival strategies of infectious biofilms', *Trends in Microbiology,* vol. 13, no. 1, pp 34-40.

Gilbert, P., Das, J. and Foley, I. 1997, 'Biofilm susceptibility to antimicrobials', *Advances in Dental Research,* vol. 11, no. 1, pp 160-167.

Gilbert, P., Maira-Litran, T., McBain, A. J., Rickard, A. H. and Whyte, F. W. 2002, 'The physiology and collective recalcitrance of microbial biofilm communities', in *Advances in Microbial Physiology*. Academic Press,pp. 203-256.

Gomes de Saravia, S. G. and Gaylarde, C. C. 1998, 'The antimicrobial activity of an aqueous extract of Brassica negra', *International Biodeterioration & Biodegradation,* vol. 41, no. 2, pp 145-148.

Gould, I. M. 1999, 'A review of the role of antibiotic policies in the control of antibiotic resistance', *Journal of Antimicrobial Chemotherapy,* vol. 43, no. 4, pp 459-465.

Halkier, B. A. and Gershenzon, J. 2006, 'Biology and biochemistry of glucosinolates', *Annual Review of Plant Biology,* vol. 57, no. 1, pp 303-333.

Hammer, B. K. and Bassler, B. L. 2003, 'Quorum sensing controls biofilm formation in *Vibrio cholerae*', *Molecular Microbiology,* vol. 50, no. 1, pp 101-104.

Hoffman, S. B. 2001, 'Mechanisms of antibiotic resistance', *Compendium,* vol. 23, no. 5, pp 464-473.

Holst, B. and Williamson, G. 2004, 'A critical review of the bioavailability of glucosinolates and related compounds', *Natural Product Reports,* vol. 21, no. 3, pp 425-447.

Inoue, T., Shingaki, R. and Fukui, K. 2008, 'Inhibition of swarming motility of *Pseudomonas aeruginosa* by branched-chain fatty acids', *FEMS Microbiology Letters,* vol. 281, no. 1, pp 81-86.

Jacob, C. 2007, 'A scent of therapy: pharmacological implications of natural products containing redox-active sulfur atoms', *ChemInform,* vol. 38, no. 14, pp 851-863.

Janczuk, B., Chibowski, E., Bruque, J. M., Kerkeb, M. L. and Caballero, F. G. 1993, 'On the consistency of surface free energy components as calculated from contact angles of different liquids: an application to the cholesterol surface', *Journal of Colloid and Interface Science,* vol. 159, no. 2, pp 421-428.

Javale, P. and Sabnis, S. 2010, 'Antimicrobial properties and phytochemical analysis of *Emblica officinalis*', *Asian Journal of Experimental Biological Sciences,* vol. 1, no. (Special Issue), pp 91-95.

Jayaraman, P., Sakharkar, M. K., Lim, C. S., Tang, T. H. and Sakharkar, K. R. 2010, 'Activity and interactions of antibiotic and phytochemical combinations against *Pseudomonas aeruginosa in vitro*', *International Journal of Biological Sciences,* vol. 6, no. 6, pp 556-568.

Kähkönen, M. P., Hopia, A. I., Vuorela, H. J., Rauha, J.-P., Pihlaja, K., Kujala, T. S. and Heinonen, M. 1999, 'Antioxidant activity of plant extracts containing phenolic compounds', *Journal of Agricultural and Food Chemistry,* vol. 47, no. 10, pp 3954-3962.

Kaito, C. and Sekimizu, K. 2007, 'Colony spreading in *Staphylococcus aureus*', *Journal of Bacteriology,* vol. 189, no. 6, pp 2553-2557.

Kim, J. H., Park, E.-S., Shim, J. H., Kim, M.-N., Moon, W.-S., Chung, K.-H. and Yoon, J.-S. 2004, 'Antimicrobial activity of p-hydroxyphenyl acrylate derivatives', *Journal of Agricultural and Food Chemistry,* vol. 52, no. 25, pp 7480-7483.

Kolari, M. 2003, 'Attachment mechanisms and properties of bacterial biofilms on non-living surfaces', Master thesis, University of Helsinki, Helsinki.

Komnick, H., Stockem, W. and Wohlfarth-Bottermann, K. E. 1973, 'Cell motility: mechanisms in protoplasmic streaming and ameboid movement', in *International Review of Cytology*. Academic Press pp. 169-249.

Lai, S., Tremblay, J. and Déziel, E. 2009, 'Swarming motility: a multicellular behaviour conferring antimicrobial resistance', *Environmental Microbiology,* vol. 11, no. 1, pp 126-136.

Levy, S. B. 1997, 'Antibiotic resistance: an ecological imbalance', in *Antibiotic Resistance: Origins, Evolution, Selection and Spread*. pp. 1-14.

Levy, S. B. 2001, 'Antibiotic resistance: consequences of inaction', *Clinical Infectious Diseases,* vol. 33, no. 3, pp 124-129.

Lewis, K. and Ausubel, F. M. 2006, 'Prospects for plant-derived antibacterials', *Nature Biotechnology,* vol. 24, no. 12, pp 1504-1507.

Li, J. and McLandsborough, L. A. 1999, 'The effects of the surface charge and hydrophobicity of *Escherichia coli* on its adhesion to beef muscle', *International Journal of Food Microbiology,* vol. 53, no. 2-3, pp 185-193.

Lila, M. A. and Raskin, I. 2005, 'Health-related interactions of phytochemicals', *Journal of Food Science,* vol. 70, no. 1, pp 20-27.

Mah, T.-F. C. and O'Toole, G. A. 2001, 'Mechanisms of biofilm resistance to antimicrobial agents', *Trends in Microbiology,* vol. 9, no. 1, pp 34-39.

Manici, L., Lazzeri, L. and Palmieri, S. 1997, '*In vitro* fungi-toxic activity of some glucosinolates and their enzyme-derived products toward plant pathogenic fungi', *Journal of Agricultural and Food Chemistry,* vol. 45, no. 1, pp 2768–2773.

Mayer, G. 2010, *Antibiotics - Protein Synthesis, Nucleic Acid Synthesis and Metabolism*. Retrieved June, 2011, from http://pathmicro.med.sc.edu/mayer/ antibiot.htm.

Morra, M. J. and Kirkegaard, J. A. 2002, 'Isothiocyanate release from soil-incorporated Brassica tissues', *Soil Biology and Biochemistry*, vol. 34, no. 11, pp 1683-1690.

Nascimento, G. G., Locatelli, J., Freitas, P. C. and Silva, G. L. 2000, 'Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria', *Brazilian Journal of Microbiology*, vol. 31, no. pp 247-256.

Newman, D. J., Cragg, G. M. and Snader, K. M. 2000, 'The influence of natural products upon drug discovery', *Natural Product Reports*, vol. 17, no. 3, pp 215-234.

Nikaido, H. 2005, 'Restoring permeability barrier function to outer membrane', *Chemistry & Biology*, vol. 12, no. 5, pp 507-509.

Norsworthy, J. K. and Meehan, J. T. 2005, 'Use of isothiocyanates for suppression of Palmer amaranth (*Amaranthus palmeri*), pitted morningglory (*Ipomoea lacunosa*), and yellow nutsedge (*Cyperus esculentus*)', *Weed Science*, vol. 53, no. 6, pp 884 - 890.

O'Toole, G., Kaplan, H. B. and Kolter, R. 2000, 'Biofilm formation as microbial development', *Annual Review of Microbiology*, vol. 54, no. 1, pp 49-79.

Olson, M., Ceri, H., Morck, D., Buret, A. and Read, R. 2002, 'Biofilm bacteria: formation and comparative susceptibility to antibiotics', *Canadian Journal of Veterinary Research*, vol. 66, no. 1, pp 86 - 92.

Osbourn, A. E. 1996, 'Preformed antimicrobial compounds and plant defense against fungal attack', *Plant Cell*, vol. 8, no. 10, pp 1821-1831.

Peeters, E., Nelis, H. J. and Coenye, T. 2008, 'Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates', *Journal of Microbiological Methods*, vol. 72, no. 2, pp 157-165.

Pompermayer, D. M. C. and Gaylarde, C. C. 2000, 'The influence of temperature on the adhesion of mixed cultures of *Staphylococcus aureus* and *Escherichia coli* to polypropylene', *Food Microbiology*, vol. 17, no. 4, pp 361-365.

Pompilio, A., Piccolomini, R., Picciani, C., D'Antonio, D., Savini, V. and Di Bonaventura, G. 2008, 'Factors associated with adherence to and biofilm formation on polystyrene by *Stenotrophomonas maltophilia*: the role of cell surface hydrophobicity and motility', *FEMS Microbiology Letters*, vol. 287, no. 1, pp 41-47.

Pratt, L. A. and Kolter, R. 1998, 'Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili', *Molecular Microbiology,* vol. 30, no. 2, pp 285-293.

Purushotham, K. H., Arun, P., Jayarani, J. J., Vasnthakumari, R., Sankar, L. and Reddy, B. R. 2010, 'Synergism *in vitro* antibacterial activity of *Tectona grandis* leaves with tetracycline', *International Journal of PharmTech Research,* vol. 2, no. 1, pp 519 - 523.

Saavedra, M. J., Borges, A., Dias, C., Aires, A., Bennett, R. N., Rosa, E. S. and Simões, M. 2010, 'Antimicrobial activity of phenolics and glucosinolate hydrolysis products and their synergy with streptomycin against pathogenic bacteria', *Medicinal Chemistry* vol. 6, no. 3, pp 174-183.

Sakharkar, M. K., Jayaraman, P., Soe, W. M., Chow, V. T., Sing, L. C. and Sakharkar, K. R. 2009, '*In vitro* combinations of antibiotics and phytochemicals against *Pseudomonas aeruginosa.*', *Journal of Microbiology, Immunology and Infection,* vol. 42, no. 5, pp 364-370.

Sarwar, M., Kirkegaard, J. A., Wong, P. T. W. and Desmarchelier, J. M. 1998, 'Biofumigation potential of brassicas', *Plant and Soil,* vol. 201, no. 1, pp 103-112.

Schultz, T. W., Yarbrough, J. W. and Woldemeskel, M. 2005, 'Toxicity to *Tetrahymena* and abiotic thiol reactivity of aromatic isothiocyanates', *Cell Biology and Toxicology,* vol. 21, no. 3, pp 181-189.

Schultz, T. W., Yarbrough, J. W. and Pilkington, T. B. 2007, 'Aquatic toxicity and abiotic thiol reactivity of aliphatic isothiocyanates: Effects of alkyl-size and -shape', *Environmental Toxicology and Pharmacology,* vol. 23, no. 1, pp 10-17.

Sibanda, T. and Okoh, A. I. 2007, 'The challenges of overcoming antibiotic resistance: Plant extracts as potential sources of antimicrobial and resistance modifying agents', *African Journal of Biotechnology,* vol. 6, no. 25, pp 2886 - 2896.

Sigma-Aldrich 2011, *Phenyl isothiocyanate solution,* Retrieved June, 2011, from http://www.sigmaaldrich.com/catalog/ProductDetail.do?lang=pt&N4=P7587|SIGMA&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC.

Simões, L. 2004, 'Estudo da dinética de formação de biofilmes em superfícies em contacto com água potável', Master thesis, University of Minho, Braga.

Simões, L., Simões, M. and Vieira, M. 2010, 'Adhesion and biofilm formation on polystyrene by drinking water-isolated bacteria', *Antonie van Leeuwenhoek,* vol. 98, no. 3, pp 317-329.

Simões, L. C., Simões, M., Oliveira, R. and Vieira, M. J. 2007b, 'Potential of the adhesion of bacteria isolated from drinking water to materials', *Journal of Basic Microbiology,* vol. 47, no. 2, pp 174-183.

Simoes, L. C., Simoes, M. and Vieira, M. J. 2007a, 'Biofilm interactions between distinct bacterial genera isolated from drinking water', *Applied and Environmental Microbiology,* vol. 73, no. 19, pp 6192-6200.

Simões, M. 2005, 'Use of biocides and surfactants to control *Pseudomonas fluorescens* biofilms: role of the hydrodynamic conditions', PhD, University of Minho, Braga.

Simoes, M., Bennett, R. N. and Rosa, E. A. S. 2009, 'Understanding antimicrobial activities of phytochemicals against multidrug resistant bacteria and biofilms', *Natural Product Reports,* vol. 26, no. 6, pp 746-757.

Simões, M., Rocha, R., Coimbra, M. A. and Vieira, M. 2008, 'Enhancement of *Escherichia coli* and *Staphylococcus aureus* antibiotic susceptibility using sesquiterpenoids', *Medicinal Chemistry,* vol. 4, no. pp 616-623.

Simões, M., Simões, L. C., Cleto, S., Machado, I., Pereira, M. O. and Vieira, M. J. 2007, 'Antimicrobial mechanisms of ortho-phthalaldehyde action', *Journal of Basic Microbiology,* vol. 47, no. 3, pp 230-242.

Smith, A. W. 2005, 'Biofilms and antibiotic therapy: Is there a role for combating bacterial resistance by the use of novel drug delivery systems?', *Advanced Drug Delivery Reviews,* vol. 57, no. 10, pp 1539-1550.

Smolinska, U., Morra, M. J., Knudsen, G. R. and James, R. L. 2003, 'Isothiocyanates produced by Brassicaceae species as inhibitors of *Fusarium oxysporum*', *Plant Disease,* vol. 87, no. 4, pp 407-412.

Speijers, G. J. A., Danse, L. H. J. C., van Leeuwen, F. X. R. and Loeber, J. G. 1985, 'Four-week toxicity study of phenyl isothiocyanate in rats', *Food and Chemical Toxicology,* vol. 23, no. 11, pp 1015-1017.

Stepanović, S., Vukovic, D., Dakic, I., Savic, B. and Svabic-Vlahovic, M. 2000, 'A modified microtiter-plate test for quantification of staphylococcal biofilm formation', *Journal of Microbiological Methods,* vol. 40, no. 2, pp 175-179.

Stewart, P. 1996, 'Theoretical aspects of antibiotic diffusion into microbial biofilms', *Antimicrob. Agents Chemother.,* vol. 40, no. 11, pp 2517-2522.

Stewart, P. S. 2002, 'Mechanisms of antibiotic resistance in bacterial biofilms', *International Journal of Medical Microbiology,* vol. 292, no. 2, pp 107-113.

Suárez, B., Ferreirós, C. M. and Criado, M.-T. 1992, 'Adherence of psychrotrophic bacteria to dairy equipment surfaces', *Journal of Dairy Research,* vol. 59, no. 1, pp 381-388.

Taylor, P. W., Stapleton, P. D. and Paul Luzio, J. 2002, 'New ways to treat bacterial infections', *Drug Discovery Today,* vol. 7, no. 21, pp 1086-1091.

Teuber, M. 2001, 'Veterinary use and antibiotic resistance', *Current Opinion in Microbiology,* vol. 4, no. 5, pp 493-499.

Toté, K., Berghe, D. V., Deschacht, M., de Wit, K., Maes, L. and Cos, P. 2009, 'Inhibitory efficacy of various antibiotics on matrix and viable mass of *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms', *International Journal of Antimicrobial Agents,* vol. 33, no. 6, pp 525-531.

Toté, K., Berghe, D. V., Maes, L. and Cos, P. 2008, 'A new colorimetric microtitre model for the detection of *Staphylococcus aureus* biofilms', *Letters in Applied Microbiology,* vol. 46, no. 2, pp 249-254.

Valle, J., Toledo-Arana, A., Berasain, C., Ghigo, J.-M., Amorena, B., Penadés, J. R. and Lasa, I. 2003, 'SarA and not σB is essential for biofilm development by *Staphylococcus aureus*', *Molecular Microbiology,* vol. 48, no. 4, pp 1075-1087.

Van Bambeke, F., Glupczynski, Y., Plesiat, P., Pechere, J. and Tulkens, P. 2003, 'Antibiotic efflux pumps in prokaryotic cells: occurrence, impact on resistance and strategies for the future of antimicrobial therapy', *Journal of Antimicrobial Chemotherapy,* vol. 51, no. pp 1055 - 1065.

van Loosdrecht, M. C., Lyklema, J., Norde, W., Schraa, G. and Zehnder, A. J. 1987, 'Electrophoretic mobility and hydrophobicity as a measured to predict the initial steps of bacterial adhesion', *Applied and Environmental Microbiology,* vol. 53, no. 8, pp 1898-1901.

van Oss, C. J. 1995, 'Hydrophobicity of biosurfaces - Origin, quantitative determination and interaction energies', *Colloids and Surfaces B: Biointerfaces,* vol. 5, no. 3-4, pp 91-110.

van Oss, C. J. 1997, 'Hydrophobicity and hydrophilicity of biosurfaces', *Current Opinion in Colloid & Interface Science,* vol. 2, no. 5, pp 503-512.

van Oss, C. J., Chaudhury, M. K. and Good, R. J. 1987, 'Monopolar surfaces', *Advances in Colloid and Interface Science,* vol. 28, no. pp 35-64.

van Oss, C. J., Good, R. J. and Chaudhury, M. K. 1988, 'Additive and nonadditive surface tension components and the interpretation of contact angles', *Langmuir,* vol. 4, no. 4, pp 884-891.

van Oss, C. J., Ju, L., Chaudhury, M. K. and Good, R. J. 1989, 'Estimation of the polar parameters of the surface tension of liquids by contact angle measurements on gels', *Journal of Colloid and Interface Science,* vol. 128, no. 2, pp 313-319.

Verstraeten, N., Braeken, K., Debkumari, B., Fauvart, M., Fransaer, J., Vermant, J. and Michiels, J. 2008, 'Living on a surface: swarming and biofilm formation', *Trends in Microbiology,* vol. 16, no. 10, pp 496-506.

Walker, S. L., Redman, J. A. and Elimelech, M. 2004, 'Role of cell surface lipopolysaccharides in *Escherichia coli* K12 adhesion and transport', *Langmuir,* vol. 20, no. 18, pp 7736-7746.

WHO 2002, *World Health Organization: Antimicrobial resistance,* Retrieved June, 2011, from http://www.who.int/mediacentre/factsheets/fs194/en/.

Wood, M. J. and Moellering, R. C. 2003, 'Microbial resistance: bacteria and more', *Clinical Infectious Diseases,* vol. 36, no. Supplement 1, pp S2-S3.

Xiao, D., Srivastava, S. K., Lew, K. L., Zeng, Y., Hershberger, P., Johnson, C. S., Trump, D. L. and Singh, S. V. 2003, 'Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer cells by causing G2/M arrest and inducing apoptosis', *Carcinogenesis,* vol. 24, no. 5, pp 891-897.

Xu, K. D., McFeters, G. A. and Stewart, P. S. 2000, 'Biofilm resistance to antimicrobial agents', *Microbiology,* vol. 146, no. 3, pp 547-549.

Yang, L., Liu, Y., Wu, H., Høiby, N., Molin, S. and Song, J. 2011, 'Current understanding of multi-species biofilms', *International Journal of Oral Science,* vol. 3, no. 1, pp 74-81.

Yim, G. 2009, *Attack of the superbugs: antibiotic resistance,* Retrieved January, 2011, from http://www.scq.ubc.ca/attack-of-the-superbugs-antibiotic-resistance/.

Zago, J. A. A., Ushimaru, P. I., Barbosa, L. N. and Fernandes, J. 2009, 'Sinergismo entre óleos essenciais e drogas antimicrobianas sobre linhagens de *Staphylococcus aureus* e *Escherichia coli* isoladas de casos clínicos humanos', *Revista brasileira de farmacognosia,* vol. 19, no. 4, pp 828-833.

Zahin, M., Aqil, F., Khan, M. S. A. and Ahmad, I. 2010, 'Ethnomedicinal plants derived antibacterials and their prospects', in *Ethnomedicine: a source of complementary therapeutics*. Research Signpost,Kerala, India, pp. 149-178.

Zhang, Y. 2001, 'Molecular mechanism of rapid cellular accumulation of anticarcinogenic isothiocyanates', *Carcinogenesis,* vol. 22, no. 3, pp 425-431.

Zhang, Y., Yao, S. and Li, J. 2006, 'Vegetable-derived isothiocyanates: anti-proliferative activity and mechanism of action', *Proceedings of the Nutrition Society*, vol. 65, no. 1, pp 68-75.

Appendix

A. Security Data

This section pretends to report the dangers associated to PITC and to ensure all the security when handling the product. Figure 10 presents several pictograms for PITC.

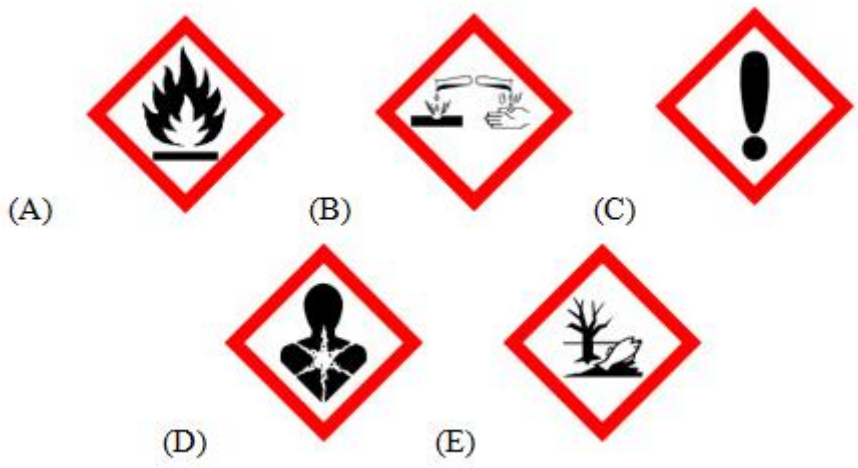


Fig. 10. Pictograms of PITC: (A) Flammable liquid, (B) Skin corrosion/irritation; (C) Acute oral toxicity, Skin sensitization, Specific target organ toxicity (single exposure), Narcotic effects; (D) Aspiration hazard; (E) Hazardous to the aquatic environment (long-term hazard) (Sigma-Aldrich, 2011).

Table 13 describes several hazard and precautionary statements of PITC.

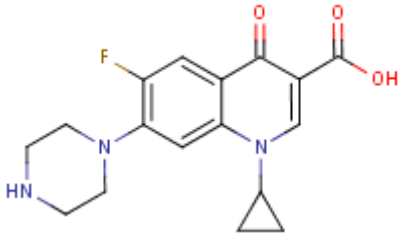
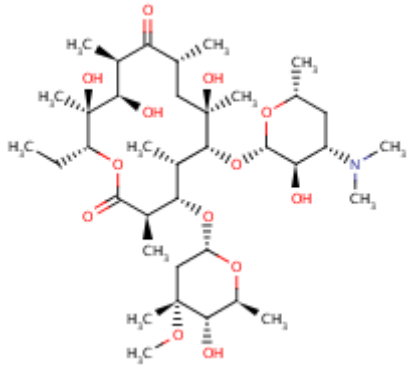
Table 13. Hazard and precautionary statements of PITC (Sigma-Aldrich, 2011)

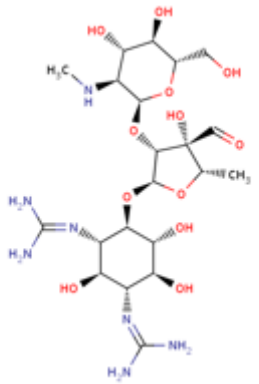
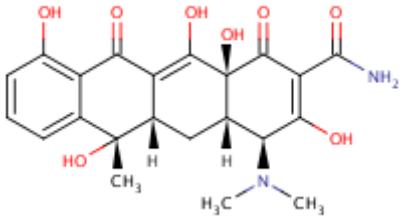
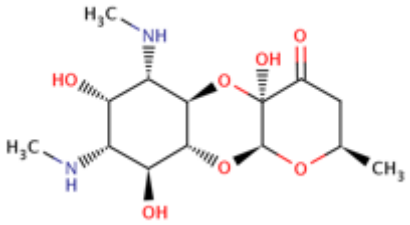
Pictogram	Hazard statements	Precautionary statements
A	Highly flammable liquid and vapor	- Keep away from heat/ sparks/ open flames/ hot surfaces; No smoking; - Avoid breathing dust/ fume/ gas/ mist/ vapors/ spray; - Avoid release to the environment - Wear protective gloves/ protective clothing/ eye protection/ face protection; - If swallowed: Immediately call a poison center or doctor/physician - If case of contact with eyes: Rinse cautiously with water for several minutes; Remove contact lenses, if present and easy to do. Continue rinsing.
B	Causes severe skin burns and eye damage	
C	Harmful if swallowed; may cause drowsiness or dizziness	
D	May be fatal if swallowed and enters airways; may cause allergy or asthma symptoms or breathing	
E	Very toxic to aquatic life with long lasting effect	

B. Antibiotic Characterization

Table 14 describes some fundamental aspects about the antibiotics used in this study as antimicrobial class, spectrum of activity, main targets in cell and the chemical structure.

Table 14. Characterization of antibiotics used in this study

Antibiotics (abbr)	Antimicrobial class	Spectrum of Activity	Targets	Structure	Reference
Ciprofloxacin (CIP)	Fluoroquinolone	Gram-positive cocci (in urinary tract infections), some Gram-negative bacteria	DNA gyrase and DNA topoisomerase IV enzymes and prevent supercoiling of DNA, thereby inhibiting DNA synthesis		(Drlica, 1999; CHEBI., 2010a; Jayaraman *et al.*, 2010; Mayer, 2010)
Erythromycin (ERY)	Macrolide	Gram-positive bacteria, *Mycoplasma*, *Legionella*	Inhibit translocation of the peptidyl tRNA from the A to the P site on the ribosome by binding to the 50S ribosomal 23S RNA		(Drug Bank., 2005; Mayer, 2010)

Streptomycin (STR)	Aminoglycosides	Many Gram-negative and some Gram-positive bacteria	Irreversibly bind to the 30S ribosome and freeze the 30S initiation complex (30S-mRNA-tRNA)		(Drug Bank, 2005b; Mayer, 2010)
Tetracycline (TET)	Polyketide	Broad spectrum	30S ribosome; inhibit binding of aminoacyl-t-RNA to the acceptor site on the 70S ribosome		(Chopra and Roberts, 2001; CHEBI., 2010b; Jayaraman *et al.*, 2010; Mayer, 2010)
Spectinomycin (SPT)	Aminoglycosides	Treatment of penicillin-resistant *Neisseria gonorrhoeae*	Reversibly interferes with mRNA interaction with the 30S ribosome		(Drug Bank, 2005a; Mayer, 2010)

C. Other experimental data

Table 15 shows the diameters of inhibition zones (mm) obtained with the combination of PITC with antibiotics.

Table 15. Antimicrobial activity of dual combinations (PITC – antibiotics) on bacteria observed by disc diffusion assay. The means (mm) ± SD for at least three replicates are illustrated

	Diameter of inhibition zone (mm)				
	ERY	**STR**	**CIP**	**TET**	**SPT**
E.coli	19.3±1.2	22.0±1.7	52.3±2.1	34.7±0.6	27.7±1.5
S.aureus	45.0±1.2	21.0±1.0	48.0±2.8	45.7±1.5	16.7±0.6

Table 16 describes the physico-chemical characteristics of polystyrene in order to predict cells adhesion to this surface.

Table 16. Physico-chemical characterization of polystyrene (PS)

	Contact Angle (º)			**Surface tension parameters (mJ/m^2)**			**Hydrophobicity (mJ/m^2)**	**Zeta potential (mV)**
	θ_w	θ_F	θ_B	γ^{LW}	γ^+	γ^-	ΔG_{sws}^{TOT}	
PS	83±3	71±2	28±1	39	0.0	9.9	-44	-32±2