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The action of selected isothiocyanates on bacterial biofilm prevention and control

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Abstract

The activity of two selected isothiocyanates (ITCs), allylisothiocyanate (AITC) and 2-phenylethy-lisothiocyanate (PEITC) was evaluated on the prevention and control of biofilms formed by *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Listeria monocytogenes*. In addition, the effect of ITCs was also tested on planktonic cell susceptibility, bacterial motility and adhesion. Biofilm prevention and control were tested using a microtiter plate assay and the effect of ITCs was assessed on biofilm mass and metabolic activity. The minimum bactericidal concentration for *E. coli* and *P. aeruginosa* was 1000 μg mL^{-1} (AITC) and > 1000 μg mL^{-1} (PEITC), for *S. aureus* and *L. monocytogenes* was > 1000 μg mL^{-1} (for both ITCs). AITC caused total inhibition of swimming (*P. aeruginosa*) and swarming (*E. coli*) motilities. PEITC caused total inhibition of swimming (*E. coli*, *P. aeruginosa* and *L. monocytogenes*) and swarming (*E. coli* and *P. aeruginosa*) motilities. Colony spreading of *S. aureus* was completely inhibited with PEITC. Adhesion assessed in terms of free energy was less favorable when bacteria were exposed to AITC for *E. coli* and *P. aeruginosa* and PEITC for *P. aeruginosa*. Both ITCs had preventive action on biofilm formation and showed a higher potential to reduce the mass of biofilms formed by the Gram-negative bacteria. AITC and PEITC promoted reductions in biofilm activity higher than 60% for all the biofilms tested. The overall study emphasizes the potential of ITCs as emergent products to inhibit bacterial motility and prevent/control biofilms of important human pathogenic bacteria.

1. Introduction

Antimicrobial resistance is one of the major challenges for the industrial, food and biomedical sectors. The increased resistance of pathogenic microorganisms to the antibacterial agents can be directly attributed to the extreme and inappropriate use of antibiotics and disinfectants (Andersson and Levin, 1999; Guillemot, 1999; Monroe and Polk, 2000; Andersson, 2003). Some infectious diseases are almost untreatable by conventional antibiotic therapy (Dalton et al., 2012). Antimicrobial resistance is worsened when the microorganisms form biofilms (Mah and O'Toole, 2001).

Biofilms are complex multicellular microbial communities irreversibly attached to a surface, enclosed in a matrix of extracellular polymeric substances (EPSs) such as proteins, nucleic acids and polysaccharides, and represent the prevalent mode of microbial life in nature, industrial processes and some infections (Hall-Stoodley et al., 2004; Cos et al., 2010; Toté et al., 2010; Jiang et al., 2011). Biofilms are an example of physiological adaptation and are one of the most important sources of bacterial resistance to antimicrobials (Simões, 2011). Bacteria embedded in biofilms are more resistant to antimicrobial products than their planktonic counterparts (Mah and O'Toole, 2001; Stewart and Costerton, 2001; Donlan and Costerton, 2002; Jagani et al., 2009; Cos et al., 2010; Simões et al., 2011b). In addition to the conventional mechanisms of antibiotic resistance found in planktonic cells (efflux pumps, modifying enzymes, and target mutations) (Walsh, 2000; Stewart and Costerton, 2001), there are several mechanisms that protect bacteria in biofilm, particularly:(i) poor penetration or inactivation of antimicrobials in the extracellular polymeric matrix; (ii) an altered (dormant) bacterial metabolic state; (iii) the presence of persister cells; (iv) resistance induced by the antimicrobial itself following the use of sublethal concentrations and the upregulation of efflux pumps (Gilbert et al., 2003; Anderson and O'Toole, 2008). Biofilm resistance is usually multi-factorial and may vary from one organism to another (Gilbert et al., 2003; Aslam, 2008). Furthermore, the emergence of antimicrobial resistant bacteria and phenotypes clearly shows that new biofilm control strategies are required (Simões et al., 2006). A better understanding of bacterial tolerance and resistance to antimicrobial products has led to new interests in natural antibacterial products which restrict the ability of bacteria to adhere, communicate, and form complex biofilms (Al-Sohaibani and Murugan, 2012). An important strategy to combat the resistance problem involves the discovery and development of new antimicrobials capable to sup-pressing bacterial resistance mechanisms (Abreu et al., 2012).

Many of the antimicrobial drugs used to effectively treat human disease have been derived from nature (Newman and Cragg, 2007; Brown and Hampton, 2011). Dietary phytochemicals (plant secondary metabolites) are potent bioactive compounds from plant sources with a wide range of effects (Holst and Williamson, 2004). Glucosinolates (GLS) are an important group of phytochemicals present exclusively in the order Capparales and very abundant in Brassicaceae (Syn. Cruciferae) family (Halkier and Du, 1997; Grubb and Abel, 2006; Barbieri et al., 2008; Al-Gendy et al., 2010). This family includes various vegetables such as cabbage, broccoli, cauliflower, horseradish, Brussels sprouts and kohlrabi (Fahey et al., 2001; Holst and Williamson, 2004). More than 120 different GLS are known to occur naturally in plants (Fahey et al., 2003; Clarke, 2010; Berhow et al., 2012). They are grouped into aliphatic, aromatic and indole glucosinolates, based on their chemical structure (Halkier and Gershenzon, 2006). These

phytochemicals are usually broken down through hydrolysis catalyzed by myrosinase (b-thioglucosidase enzyme), released from damaged plant cells, in to numerous biologically active products such as isothiocyanates (ITCs), nitriles, epithionitriles and thiocyanates (Fahey et al., 2001; Hong and Kim, 2008; Aires et al., 2009b). GLS and their hydrolysis products (GHP), in particular ITCs, have long been known to have biological activities including various pharmaceutical benefits to human health (anticarcinogenic, antimicrobial and antioxidant properties) and plant defence (against insects, fungi and microbial infections) (Hong and Kim, 2008; D'Antuono et al., 2009; Saavedra et al., 2010). The effects of GLS on the quality of both human and animal foods, and the emerging evidence that brassica vegetables may have important anticarcinogenic effects associated with the biological activity of GHP provides a good reason for the increased interest in natural biosynthetic pathways of these compounds. In this work, the ability of two ITC's (allylisothiocyanate and 2-phenylethylisothiocyanate) to control biofilms formed by four bacterial species of potential biomedical concern (Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Listeria mono-cytogenes) was evaluated. In addition, these ITCs were also tested in planktonic growth control and in their ability to act on biofilm prevention, motility inhibition and on bacterial-surface free energy of adhesion.

2. Material and methods

2.1. Microorganisms and culture conditions

Two Gram-negative bacteria, *E. coli* CECT 434, *P. aeruginosa* ATCC 10145, and two Gram-positive bacteria, *S. aureus* CECT 976 and *L. monocytogenes* ATCC 15313, were used. These bacteria were previously used as model microorganisms for antimicrobial tests with phytochemical products (Simões et al., 2008a; Saavedra et al., 2010; Borges et al., 2012).

2.2. Isothiocyanates

Allylisothiocyanate (AITC) and 2-phenylethylisothiocyanate (PEITC) (Fig. 1) were obtained from Sigma-Aldrich (Portugal). Phytochemicals are routinely classified as antimicrobials on the basis of susceptibility tests that produce inhibitory concentrations in the range of 100-1000 μg mL^{-1} (Simões et al., 2009). In this work, each product was tested at a concentration of 1000 μg mL^{-1} in dimethyl sulfoxide (DMSO, Sigma), for motility, adhesion and biofilm tests. Negative controls were performed with DMSO. AITC and PEITC were selected based on their strong antimicrobial activity when compared with a panel of structurally distinct phytochemicals (Aires et al., 2009a, b; Saavedra et al., 2010).

2.3. Dose response curves

Dose response curves were performed with different concentrations (0, 100, 500 and 1000 μg mL^{-1}) of AITC and PEITC in Mueller-Hinton broth (MHB) (Merck, Germany), at 30 ^{o}C, in

96-wells flat-bottomed polystyrene (PS) tissue culture plates with a lid (Orange Scientific, USA) using a total volume of 200 μL. An inoculum of 1 x 10^8 CFU/mL of bacteria, in the log phase of growth, was used. After 1 h exposure to the ITCs, an aliquot of 50 μL of planktonic suspension was collected, according to the procedure described by Simões et al. (2008a). The number of bacteria in the samples was determined by making serial dilutions in saline solution (0.85% NaCl). Thirty μL of each dilution were plated on Mueller-Hinton agar (MHA) plates and incubated overnight, at 30 °C. Colonies were counted after 24 h incubation period. Three independent experiments were performed for each condition tested. The minimum bactericidal concentration (MBC) was taken as the lowest concentration of ITCs at which no CFU were detected on solid medium (Ferreira et al., 2011; Borges et al., 2012).

2.4. Motility assays

Overnight cultures grown on Luria-Bertani broth (LBB) (Merck, Germany), at 30 °C and under agitation (150 rpm) were used to characterize bacterial motility. Fifteen mL of these cultures were applied in the center of plates containing 1% tryptone, 0.25% NaCl, and 0.3%, 0.7% or 1.5% (w/v) agar for swimming/colony spreading, swarming and twitching motilities, respectively (Butler et al., 2010; Stickland et al., 2010). Colony spreading was assessed for *S. aureus* and twitching motility was only assessed for *P. aeruginosa*. The use of different concentrations of agar (the medium porosity directly related to the concentration of agar, so various levels of bacterial diffusion can be selected) enables the characterization of different types of bacterial motility. AITC and PEITC at 1000 μg mL^{-1} were incorporated in the growth medium (tempered at 45 °C). Negative controls were performed with medium without ITCs. Plates were incubated at 30 °C and the diameter (mm) of the motility halos were measured at 24, 48 and 72 h. Three plates were used to evaluate the motility of each bacterium.

2.5. Free energy of adhesion

The free energy of adhesion (ΔG_{iwi}^{Tot}) between the bacterial cells and PS surfaces was assessed according to the procedure described by Simões et al. (2008b). After overnight growth in MHB, the cells were centrifuged and resuspended in saline solution to obtain an OD_{640} of 0.2 ± 0.02 (1 x 10^8 CFU/mL). One hundred mL of this suspension was collected and exposed to 1000 μg mL^{-1} during 1 h. Cell suspensions without ITCs were used as controls. To ascertain the bacterial surface properties, lawns of bacteria were prepared as described by Busscher et al. (1984). PS surfaces were prepared for characterization by immersion in a solution of commercial detergent (Sonasol Pril, Henkel Ibérica S. A.) in ultrapure water for 30 min. After rising with ultrapure water, the surfaces were dried at 65 °C for 3 h.

The contact angles of the bacteria and the PS were determined by sessile drop contact angle measurements, using a model OCA 15 Plus (DATAPHYSICS, Germany) that allowed image acquisition and data analysis. The surface tension components of bacteria and PS were obtained by measuring the contact angles with three pure liquids. These measurements were carried out at room temperature (25 ± 2 °C) using water, formamide and a-bromonaphthalene (Sigma) as

reference liquids. The surface tension components of the reference liquids were taken from the literature (Janczuk et al., 1993). Contact angle data were obtained from at least 25 determinations for each liquid and for each experiment. Afterward, the hydrophobicity of bacteria and the PS surfaces was evaluated from contact angle measurements by the method of Van Oss et al.(1987, 1988). The degree of hydrophobicity of a given material (i) is expressed as the free energy of interaction between two entities of that material immersed in water (w) - ((ΔG_{iw} mJ m^{-2}). ΔG_{iw} was calculated from the surface tension components of the interacting entities, according to the equation:

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

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, respectively, of the Lewis acid-base (γ^{AB}), with $\gamma^{AB}= 2\sqrt{\gamma^+\gamma^-}$. The surface tension components were estimated by the simultaneous resolution of three equations of the type:

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

where θ is the contact angle and g$\gamma^{Tot} = \gamma^{LW} + \gamma^{AB}$. When studying the interaction (free energy of adhesion (ΔG_{iwi}^{Tot}) between substances i and I that are immersed or dissolved in water, the total interaction energy, ΔG_{iwi}^{Tot}, can be expressed as:

$$\Delta G_{iwI}^{Tot} = \gamma_{iI}^{LW} - \gamma_{iw}^{LW} - \gamma_{Iw}^{LW} + 2\left[\sqrt{\gamma_w^+}\left(\sqrt{\gamma_i^-} + \sqrt{\gamma_I^-} - \sqrt{\gamma_w^-}\right) + \sqrt{\gamma_w^-}\left(\sqrt{\gamma_i^+} + \sqrt{\gamma_I^+} - \sqrt{\gamma_w^+}\right) - \sqrt{\gamma_i^+\gamma_I^-} - \sqrt{\gamma_i^-\gamma_I^+}\right] \quad (3)$$

Thermodynamically, if ΔG_{iwi}^{Tot} < 0 mJ m^{-2} adhesion is favored, whereas adhesion is not expected to occur if ΔG_{iwi}^{Tot} > 0 mJ m^{-2}.

2.6. Biofilm formation

Biofilms were developed according to the modified microtiter plate test proposed by Stepanovic et al. (2000). Briefly, a sterile 96-wells flat-bottomed PS tissue culture plates with a lid were filled with 200 µL of bacterial suspension with a density of 1 x 10^8 cells mL^{-1}. Negative control wells contained MHB without adding any bacterial cells. The plates were incubated for 24 h at 30 °C and agitated at 150 rpm.

2.6.1. Biofilm prevention

Overnight cultures in MHB supplemented with AITC and PEITC at 1000 µg mL^{-1} were grown at 30 °C and 150 rpm. Those cells were used to assess their ability to form biofilms in the microtiter

plates, as previously described. Biofilms (24 h aged) were characterized in terms of biomass formation and metabolic activity. Final results are presented as percentage of biofilm reduction and inactivation.

The ability of bacteria to form biofilms was also ranked according to the scheme proposed by Stepanovic et al. (2000) as follow: non biofilm producer (0): OD ≤ ODc; weak biofilm producer (+): ODc < OD ≤ 2 x ODc; moderate biofilm producer (++): 2 x ODc < OD ≤ 4 x ODc; strong biofilm producer (+++): 4 x ODc < OD. This classification was based upon the cut-off of the optical density (ODc) values, from biomass quantification (crystal violet staining) defined as three standard deviation (SD) values above the mean OD of the negative control.

2.6.2. Biofilm control

To determine whether the ITCs had effects on biofilm control, microtiter plates with 24 h aged biofilms were exposed to 1000 μg mL^{-1} of AITC or PEITC, according to Simões et al. (2010b). One hour after exposure, the biofilms were analyzed in terms of biomass and metabolic activity and the results are presented as percentage of biofilm reduction and inactivation.

2.6.3. Biofilm mass quantification by crystal violet staining

The biofilm mass was quantified using crystal violet (Merck, Portugal) staining, according to Simões et al. (2010b). The absorbance was measured at 570 nm using a Microplate Reader (Spectramax M2e, Molecular Devices, Inc.). All tests were performed in triplicate with three repeats.

Biofilm removal was given by:

$$\%BR = \frac{OD_C - OD_W}{OD_C} \times 100$$

where %BR is the percentage of biofilm removal, OD_C is the OD_{570nm} value of biofilms non exposed to ITCs and ODW is the OD_{570nm} value for biofilm exposed to AITC or PEITC.

2.6.4. Biofilm metabolic activity quantification by alamar blue assay

The modified alamar blue (7-hydroxy-3H-phenoxazin-3-one-10-oxide) (Sigma-Aldrich) microtiter plate assay was applied to determine the bacterial activity of the cells as reported by Sarker et al. (2007). Alamar blue staining was described as a reliable and reproducible method for evaluating biofilm susceptibility (Pettit et al., 2009). For the staining procedure, fresh MHB (190 mL) was added to the plates. To each well 10 μL of alamar blue (400 μM) indicator solution was added. Plates were incubated during 20 min in darkness and room temperature (RT). Fluorescence was measured at $\lambda_{excitation}$ = 570 nm and $\lambda_{emission}$ = 590 nm using a Microplate Reader (Spectramax M2e, Molecular Devices, Inc.).

The percentage of biofilm inactivation was given by:

$$\%BI = \frac{FI_C - FI_W}{FI_C} \times 100$$

where %BI is the percentage of biofilm inactivation, FI_C is the fluorescence intensity of biofilms non exposed to ITCs and F_{IW} is the fluorescence intensity value for biofilms exposed to AITC or PEITC.

2.7. Statistical analysis

The data were analyzed using the statistical program SPSS version 17.0 (Statistical Package for the Social Sciences). The mean and standard deviation within samples were calculated for all cases. At least three independent experiments were performed for each condition tested. All data were analyzed by the application of the non-parametric Wilcoxon test (confidence level ⍰ 95%).

3. Results

The action of two selected ITCs, AITC and PEITC, was assessed on biofilm prevention and control of four pathogenic bacteria. Anti-microbial tests with planktonic bacteria were performed in order to understand the antimicrobial potential of AITC and PEITC. In addition, the effect of ITCs was also tested on bacterial motility and adhesion.

The MBC for *E. coli* and *P. aeruginosa* was 1000 μg mL^{-1} for AITC and >1000 μg mL^{-1} for PEITC (Fig. 2). Both ITCs had MBC > 1000 μg mL^{-1} for *S. aureus* and *L. monocytogenes*. The dose response behavior is only statistically similar ($P > 0.05$) for *S. aureus* and *L. monocytogenes* when exposed to AITC (Fig. 2a).

The ability of AITC and PEITC at 1000 μg mL^{-1} to interfere with swimming, swarming and twitching motilities of *P. aeruginosa*, swimming and swarming of *E. coli* and *L. monocytogenes* and colony spreading of *S. aureus* was investigated (Table 1). The swimming motility of *E. coli*, *L. monocytogenes* and *P. aeruginosa* increased from 24 to 48 h ($P < 0.05$) as well as the twitch motility of *P. aeruginosa*. The same effect was observed for colony spreading of *S. aureus* from 24 to 72 h. A significant time-increase in swimming motility was verified for *E. coli*, the bacteria with the highest swimming motility values for all the sampling times. The application of AITC and PEITC promoted total inhibition in swimming, swarming and twitching motilities of *P. aeruginosa*, 24 h after inoculation and a significant decrease was observed with AITC and PEITC in swimming motility 48 and 72 h later ($P < 0.05$). The more complex type of surface motility, i.e. swarming, was completely inhibited for *E. coli* and *P. aeruginosa* with both AITC and PEITC for all sampling times ($P < 0.05$). The same result was verified for swimming motility of *E. coli* with application of PEITC ($P < 0.05$). In addition, the swimming motility of *E. coli* and *L. monocytogenes* was reduced by AITC ($P < 0.05$). PEITC caused total inhibition of the swimming motility of *L. monocytogenes* 24, 48 and 72 h after inoculation ($P < 0.05$). For S. aureus, colony spreading was reduced by AITC and completely inhibited with PEITC for all the sampling times ($P < 0.05$). In general, PEITC was more efficient in motility reduction than AITC ($P < 0.05$).

Regarding to the adhesion results (Table 2) the Gram-positive bacteria tested and *E. coli* had no theoretical thermodynamic ability to adhere to PS ($\Delta G_{iwi}^{Tot} > 0$ mJ m^{-2}). Only for *P. aeruginosa* the free energy of adhesion was < 0 mJ m^{-2}. *L. monocytogenes* had the highest ΔG_{iwi}^{Tot} value (less prone to adhere to PS). Only for *E. coli* with AITC and *P. aeruginosa* with both ITCs, it was observed increase of the ΔG_{iwi}^{Tot} values, i.e. there was a decreased in the thermodynamic adhesion potential. For the other bacteria the ΔG_{iwi}^{Tot} values decreased due to ITCs. This decrease was only significant for *E. coli* treated with PEITC and *L. monocytogenes* with both ITCs

($\Delta G_{iwi}^{Tot} < 0$ mJ m^{-2}). In general, the application of ITCs on Gram-positive bacteria increased the thermodynamic adhesion potential, while the opposite occurred for these Gram-negative (except for *E. coli* with PEITC).

In order to ascertain the potential of AITC and PEITC on biofilm prevention, planktonic bacteria were grown in the presence of ITCs and used to form biofilms on PS microtiter plates (Fig. 3a). PEITC had no preventive effects in biofilm formation by *L. monocytogenes* ($P > 0.05$). However, significant prevention in biofilm formation was verified for this bacterium with AITC (61%) ($P < 0.05$). The opposite effect was found for *S. aureus* with AITC. This product had no prevention on biomass formation, and PEITC (75%) had significant preventive action in *S. aureus* biofilm formation ($P < 0.05$). Total biofilm prevention was only observed for *E. coli* with AITC. AITC had higher preventive effects than PEITC ($P < 0.05$) on biofilm formation by *E. coli* (AITC e 100%; PEITC e 16%) and *P. aeruginosa* (AITC - 90%; PEITC e 37%).

A ranking of biofilm formation was produced according to Stepanovic et al. (2000) classifying bacteria as non-biofilm producers (0), weak biofilm producers (+), moderate biofilm producers (++), or strong biofilm producers (+++) (Table 3). *E. coli* and *P. aeruginosa* showed a strong biofilm producing ability. *L. monocytogenes* presented moderate biofilm formation ability and *S. aureus* formed weak biofilms. When the biofilms were formed in the presence of ITCs, a different biofilm preventive behavior was observed (Table 3). AITC had a complete preventive action on *P. aeruginosa* and *L. monocytogenes* biofilms (from strong and moderate biofilm producers to non-biofilm producers, respectively); a preventive action on *E. coli* biofilms was observed (strong to weak); and no preventive action on *S. aureus* biofilms was observed. On the other hand, PEITC had preventive action only on *S. aureus* biofilms (weak to non-biofilm producer) and no preventive effect on biofilm formation by the other bacteria.

In terms of metabolic activity, the analysis of biofilms formed by planktonic bacteria grown in the presence of ITCs (Fig. 3b) shows that AITC (87% for *E. coli*; 99% for *P. aeruginosa* and *L. monocytogenes*; 96% for S. aureus) and PEITC (100% for *E. coli*; 93% for *P. aeruginosa*; 90% for *S. aureus* and 97% for *L. monocytogenes*) had high effects on metabolic activity reduction for all the biofilms tested.

The ability of AITC and PEITC to control 24 h aged biofilms was analyzed based on their effects on biomass (Fig. 4a) and metabolic activity (Fig. 4b). Total biofilm mass removal was not achieved with AITC or PEITC. Both ITCs promoted biofilm mass reduction lower than 45%, for all the biofilms tested. The highest reduction in bio-mass was found for *E. coli* with both ITCs ($P < 0.05$). PEITC was more active than AITC to remove *E. coli*, *P. aeruginosa* and *S. aureus* bio-films ($P < 0.05$). In terms of metabolic activity, AITC and PEITC promoted reductions higher than 80% for all the biofilms tested, except for *P. aeruginosa* (reduction of approximately 70%). PEITC had a

moderately higher effect on metabolic inhibition than AITC for all the biofilms ($P < 0.05$), except for these of *L. monocytogenes*.

4. Discussion

The interest in antimicrobials derived from natural sources has increased in the last years due to the accepted safe status of these compounds (Lin et al., 2000b). It is recognized that some of these molecules benefit health by preventing the risk of some diseases (Prior and Cao, 2000; Aires et al., 2009a,b; Saavedra et al., 2010). ITCs occur naturally in plants and are released during consumption of cruciferous vegetables. These compounds have been demonstrated to possess health-beneficial effects (Conaway et al., 2005; Troncoso et al., 2005; Cartea and Velasco, 2008; Munday et al., 2008; Al-Gendy et al., 2010; Sofrata et al., 2011; Zhang, 2012). The antimicrobial activity of the AITC and PEITC has been predominantly demonstrated against bacteria in planktonic states (Lin et al., 2000a; Masuda et al., 2001; Jang et al., 2010; Saavedra et al., 2010; Chen et al., 2012). In fact, the antimicrobial effects on the biofilm control potential by ITCs are scarce (De Saravia et al., 2003; Guiamet and Gómez De Saravia, 2005; Zou et al., 2012). The antimicrobial mode of action of ITCs is attributed to the binding to sulfhydryl groups on active sites of enzymes important to microbial growth and survival. This leads to reductions in the cellular levels of important thiol groups leading to formation of oxygen and other free-radicals (Kolm et al., 1995; Jacob and Anwar, 2008; Aires et al., 2009a).

In this study, the analysis of dose response curves shows that the MBC for *E. coli* and *P. aeruginosa* was 1000 $\mu g\ mL^{-1}$ with AITC and > 1000 $\mu g\ mL^{-1}$ with PEITC and for *S. aureus* and *L. monocytogenes* was > 1000 $\mu g\ mL^{-1}$, with both ITCs. In a previously study performed by Jang et al. (2010), PEITC had growth inhibitory effects against several bacterial pathogens, namely *P. aeruginosa*, *S. aureus* and *L. monocytogenes*. ITCs from seeds of Sinapis alba L. (white mustard), containing phenethyl, benzyl and benzoyl groups demonstrated significant antimicrobial activity against harmful intestinal bacteria (Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium longum, Clostridium difficile, Clostridium perfringens, *E. coli*, Lactobacillus acidophilus, and Lactobacillus casei) (Kim and Lee, 2009). Others reports (Lin et al., 2000a; Rhee et al., 2003) shown that AITC had high bactericidal activity against many food-borne pathogens, including *L. monocytogenes*, S. aureus, Salmonella enterica serovar Typhimurium, and *E. coli* O157:H7. Moreover, strong activity was obtained by Shin et al. (2004), with AITC from roots of Korean and Japanese wasabi against six foodborne pathogenic bacteria, including *E. coli* O157:H7 ATCC 43889 (MBC of 660 $\mu g\ mL^{-1}$) and *S. aureus* ATCC 25923 (MBC of 5210 $\mu g\ mL^{-1}$). Lin et al. (2000b) evaluated the activity of AITC against species of *E. coli* and *L. monocytogenes* and found that 500 $\mu g\ mL^{-1}$ and 2500 $\mu g\ mL^{-1}$, respectively, were required to inhibit the bacterial growth.

Due to the different susceptibilities of planktonic bacteria to AITC and PEITC, a concentration of 1000 $\mu g\ mL^{-1}$ was selected to test in bacterial motility, adhesion and biofilm studies (prevention and control). In fact, phytochemical products are routinely classified as antimicrobials on the basis of susceptibility tests that pro-duce growth inhibitory effects in the range of 100-1000 $\mu g\ mL^{-1}$ (Tegos et al., 2002).

Three forms of surface motility, swimming, twitching and swarming, are documented for *P. aeruginosa* (Rashid and Kornberg, 2000; Déziel et al., 2001; Kearns et al., 2001; Overhage et al., 2008). *P. aeruginosa* swims by means of flagella, and during biofilm formation, swimming motility is involved in initial location and adherence to solid surfaces (O'Toole and Kolter, 1998). After surface attachment, *P. aeruginosa* moves by surface motility known as twitching (Kearns et al., 2001). *E. coli* has two flagella-driven motility types, swimming and swarming (Harshey, 2003; Gómez-Gómez et al., 2007; Inoue et al., 2007). *L. monocytogenes* can also swimming by means of flagella-based motility to access nutrient sources. Swarming motility in *L. monocytogenes* is a phenotype that has been shown to contribute to the ability to adhere to intestinal epithelial cells (Gründling et al., 2004; Gray et al., 2006; Roberts et al., 2009). Although *S. aureus* is a non-flagellated Gram-positive bacterium, they can spread on solid surfaces by a motility phenomenon defined as colony spreading (Henrichsen, 1972; Tsompanidou et al., 2011). This bacterial movement is independent of flagella or pili and is apparently similar to sliding (a form of passive bacterial movement). Generally, the ability to spread is evaluated on soft agar plates (Kaito and Sekimizu, 2007; Tsompanidou et al., 2011). In this work it was found that AITC and PEITC affected the motility of the tested bacteria and, in general, PEITC was more efficient in motility reduction than AITC. Motility inhibition was not apparently due to bactericidal effects. In fact, motility tests were also performed, for all the bacteria, with AITC and PEITC at the MBC and at 100 $\mu g\ mL^{-1}$. The results obtained were similar ($P > 0.05$) when the effects of the different concentrations were compared (data not shown). Therefore, AITC and PEITC affected bacterial motility to the same extent for concentrations below and at the MBC, reinforcing that inhibition of viability and motility are different phenomena. This motility effect is apparently related with the cell state of the bacteria. A motile cell senses stimuli and alters the functioning of its motility machinery to improve its chances of migrating to a better location (Jarrell and McBride, 2008). In fact, motility plays a major role in the transition from planktonic to surface-associated life-style (O'Toole and Kolter, 1998). Therefore, the inhibition of bacterial motility can represent an important strategy to control biofilms. Furthermore bacteria in a motile state undergo alterations in their morphology which distinguishes them from their planktonic state (Julkowska et al., 2004). Lai et al. (2009) found increased resistance of swarming bacteria compared with their planktonic counterparts.

In addition to motility it is necessary to understand other factors that are involved in biofilm development, such as the initial adhesion process. Bacterial adhesion to surfaces has been studied extensively over the past decades in many diverse areas (Simões et al., 2010a, 2011a). Adhesion is a complex process that is affected by many factors including the physicochemical characteristics of bacteria, the material surface properties, and the environmental factors. The biological properties of bacteria, such as the presence of fimbriae and flagella and the production of extracellular polymeric substances, also influence attachment to surfaces (An and Friedman, 1998; Machado et al., 2011). In this work, the effects of AITC and PEITC at 1000 $\mu g\ mL^{-1}$, on the adhesion potential of bacteria to PS was determined by the characterization of the free energy of adhesion according to a thermodynamic approach (Van Oss et al., 1987, 1989). Similarly to previous studies, PS was used as a model surface for adhesion and biofilm formation (Simões et al., 2007, 2010a). Only for *P. aeruginosa* the free energy of adhesion was $< 0\ mJ\ m^{-2}$, in the absence of ITCs. When the bacteria were exposed to ITCs, an increase in the ΔG_{iwi}^{Tot} value was found for *P. aeruginosa* (exposed to AITC and PEITC) and *E. coli* (exposed to AITC). A

significant decrease in the ΔG_{iwi}^{Tot} value was found for E coli (exposed to PEITC) and *L. monocytogenes* (exposed to AITC and PEITC). These distinct values of ΔG_{iwi}^{Tot} found after exposure to AITC and PEITC was apparently related to changes in the physicochemical cell surface properties as result of the presence of ITCs (data not shown). The electrophilic reactivity of ITCs (Cejpek et al., 2000; Verma, 2003; Luciano et al., 2008), seems to depend on the bacteria tested and on the molecule used. The electrophilic character of AITC and PEITC resulted in opposite effects when comparing the ΔG_{iwi}^{Tot} t for Gram-positive (the free energy of adhesion decreased) and Gram-negative bacteria (the free energy of adhesion increased), except for *E. coli* exposed to PEITC. Therefore, it is expected that the ITCs may hinder the adhesion of bacteria to PS, particularly *L. monocytogenes* and S. aureus. Lee et al. (2012) demonstrated that AITC interferes with the expression of genes related to adhesion of S. aureus. Guiamet and Gómez De Saravia (2005), found that aqueous extracts of Brassica nigra, where the main component is AITC, had good activity to reduce planktonic cell growth and the number of adhered cells of Pseudomonas sp. Similar results were obtained by Gómez De Saravia and Gaylarde (1998).In fact, ITCs have long been known to bind to the external proteins of cell membranes, thus causing growth inhibition and consequently the cell death, with poor or no penetration to the cell cytoplasm (Gómez De Saravia and Gaylarde, 1998; Troncoso et al., 2005).

AITC and PEITC had preventive action on biofilm formation in both biofilm formation and metabolic activity. This preventive action is apparently due to interference with bacterial viability, motility and surface properties. This is evident for *E. coli*, *P. aeruginosa* and *L. monocytogenes* exposed to AITC and *S. aureus* exposed to PEITC. The transition from a planktonic to a surface-associated lifestyle, initiates with the transportation (motility) and attachment (adhesion) of microorganisms to a particular sub-stratum (O'Toole and Kolter, 1998). Once in contact with the sub-stratum, bacterial adhesion can be mediated by forces such as hydrophobic interaction (Gilbert et al., 1991). Bacterial motility has also influence on adhesion and biofilm formation processes. For this reason motility inhibition can be correlated with a decreased ability of bacteria to form biofilms. Shrout et al. (2006) demonstrated that differences in surface motility could explain differences in biofilm structure at the early stages of development.

For the control experiments, total biofilm removal was not achieved with AITC and PEITC. PEITC was more active than AITC in biofilm removal of *E. coli*, and both ITCs had similar effects in biofilm removal for the other bacteria. Also, biofilm activity inhibition occurred at higher levels than removal. The comparison of prevention and control results indicates that AITC and PEITC seem to prevent biofilm formation to a higher extent than the control effects (except for *S. aureus* grown in the presence of AITC and for *E. coli* and *L. monocytogenes* grown in the presence of PEITC). The higher preventive effects were also found for the biofilm activity of cells grown in the presence of AITC and PEITC, for all the bacteria. The comparison of biofilm removal and inactivation (in both biofilm prevention and control experiments) shows that these are distinct phenomena. Biofilms can be inactivated (with dead cells and/or dormant) but remain attached to the surface. Previous studies also demonstrated that biofilm removal and killing are distinct phenomena (Chen and Stewart, 2000; Simões et al., 2003, 2005; Sandasi et al., 2010; Borges et al., 2012). In fact, to our knowledge, the biofilm community has never assumed that inactivation of bacteria (either by killing or metabolic inactivation) signify that biofilm was removed, this

include both bacteria and extracellular matrix (Borges et al., 2012). Moreover, other authors also found that although some compounds were able to inhibit cell attachment, the growth inhibition of a preformed biofilm was hard to achieve (Sandasi et al., 2010). To inhibit the growth of an already established biofilm (control) is more difficult to achieve than to inhibit the initial stage of biofilm formation, namely cell attachment (prevention) (Sandasi et al., 2010).

Volatile substances, like ITCs, do not influence processed food and can be safe preservatives (Al-Gendy et al., 2010). Additionally, these compounds have a strong antibacterial activity against foodborne pathogens (Aires et al., 2009a). These properties and the potential use of ITCs as natural preservatives in food industry can be an attractive alternative to synthetic products (Delaquis and Mazza, 1995; Jang et al., 2010). Also, in a recent report about the safety of AITC for the use as a food additive, the European Food Safety Authority (EFSA) Panel on Food Additives and Nutrient Sources added to Food (ANS) concluded that no significant safety concerns are expected with its use as anti-spoilage agent (EFSA, 2010). However, relatively little research has been carried out on the effect of ITCs on the growth of microbial biofilms. In this study, it was demonstrated that AITC and PEITC, in addition to planktonic antimicrobial action, had effects in the prevention and control of biofilms of *E. coli*, *L. monocytogenes*, *P. aeruginosa* and S. aureus. These effects are seemingly related to antimicrobial action, interference with bacterial motility and alteration of the cell surface properties.

5. Conclusions

AITC and PEITC demonstrated potential to inhibit the planktonic bacterial growth, cell motility and to change the cell surface properties. Also, the ITCs acted on biofilm prevention and control. To our knowledge this is the first study that demonstrates the preventive and biofilm control potential of AITC and PEITC on biofilms of *E. coli*, *L. monocytogenes*, *P. aeruginosa* and S. aureus. In fact, ITCs can be an eco-innovative intervention strategy to prevent and control biofilms, with immediate potential practical application in the food sector. Studies are in progress to evaluate the cytotoxicity of AITC and PEITC to mammalian cells and to ascertain the potential of these molecules to be used as antimicrobial therapeutic agents.

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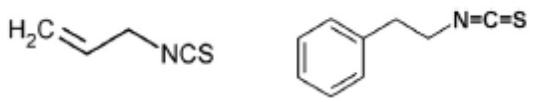

A B

Fig. 1. Chemical structures of AITC (A) and PEITC (B).

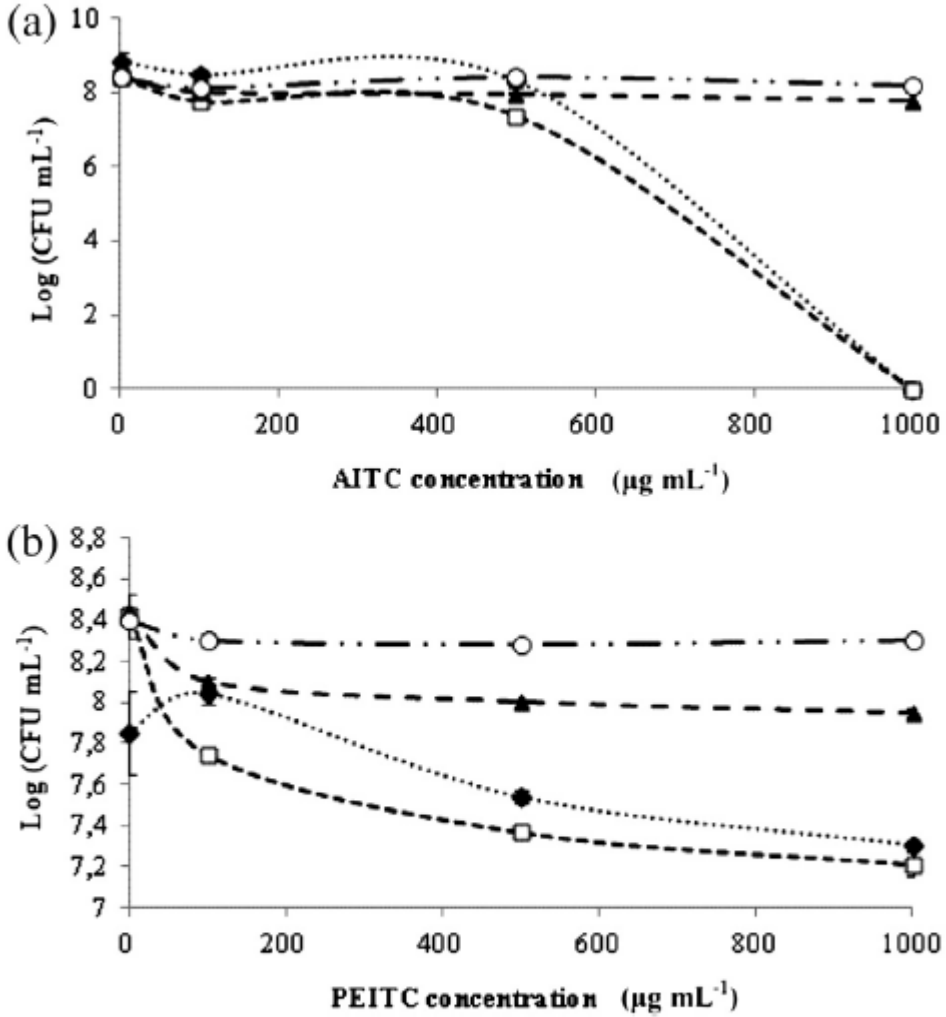

Fig. 2. Log CFU mL^{-1} for *E. coli* (◆), *P. aeruginosa* (□), *S. aureus* (▲) and *L. monocytogenes* (○) as function of AITC (a) and PEITC (b) concentration (0, 100, 500, and 1000 µg mL^{-1}) after an 1 h exposure period. The means ± SD for at least three replicates are illustrated.

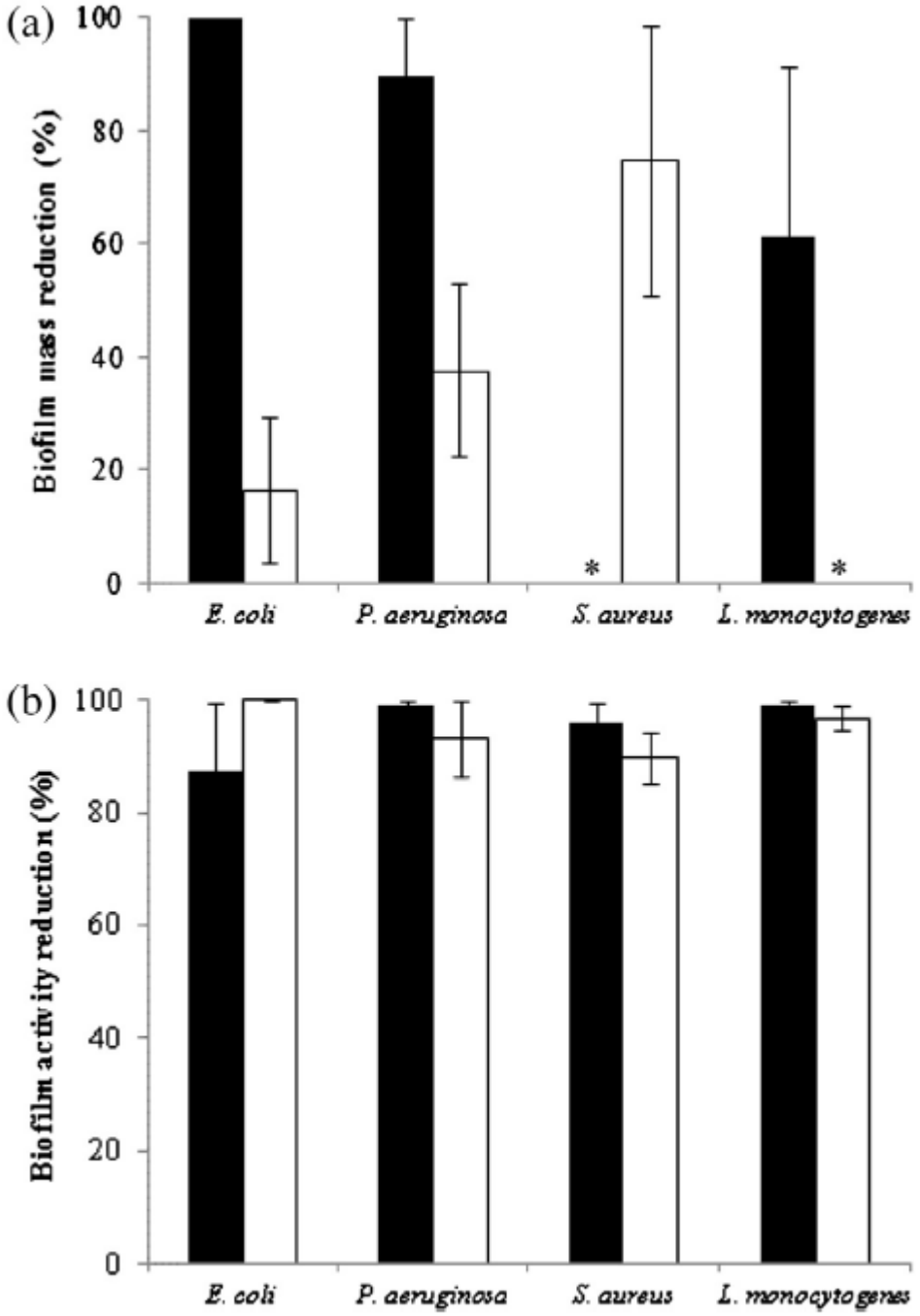

Fig. 3. Preventive action of AITC (■) and PEITC (□) on biomass formation (a) and metabolic activity (b) of *E. coli*, *P. aeruginosa*, *S. aureus* and *L. monocytogenes*. *no prevention on biofilm formation was found. Mean values ± standard deviation for at least three replicates are illustrated.

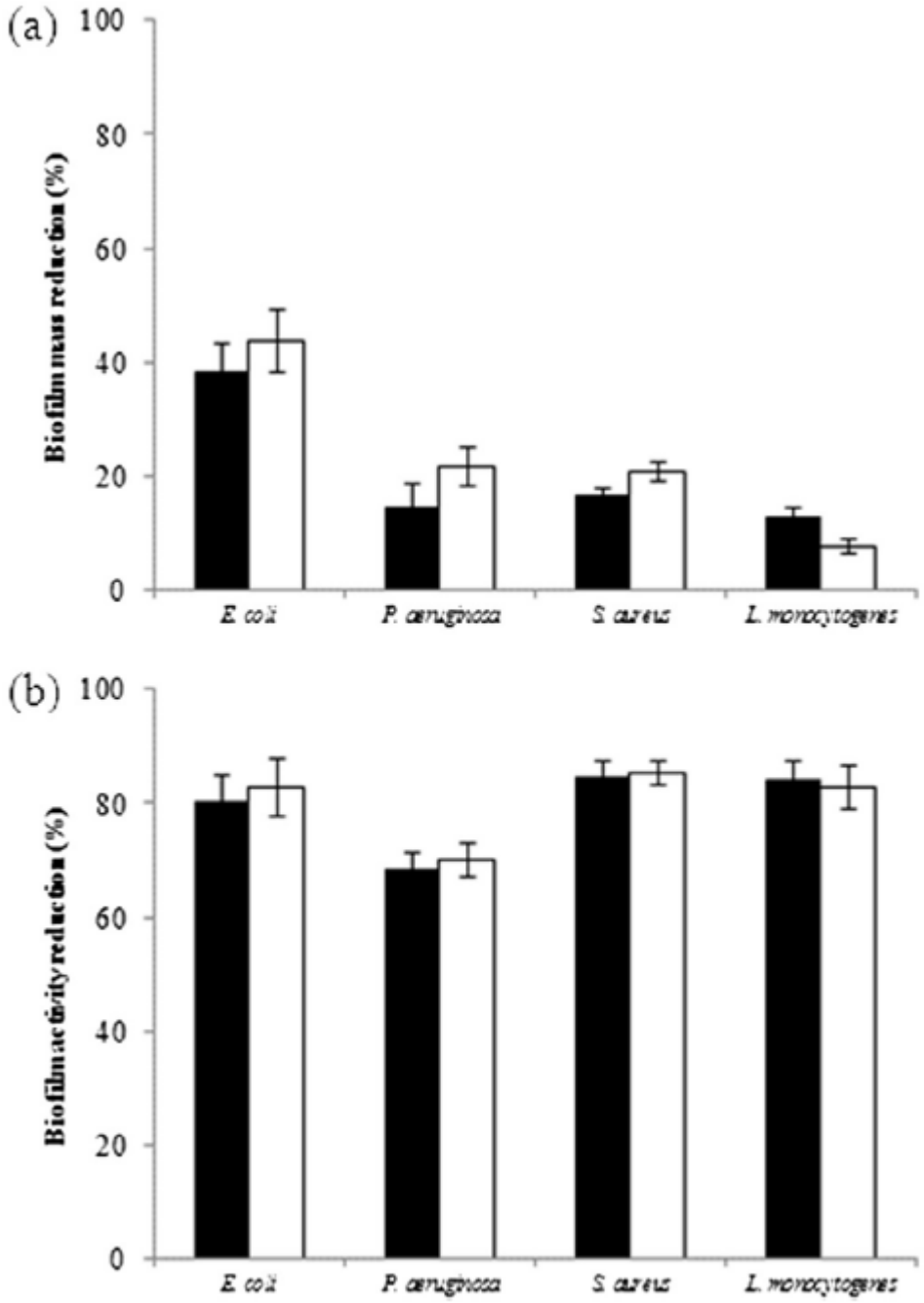

Fig. 4. Percentage of biomass reduction (a) and inactivation (b) of *E. coli*, *P. aeruginosa*, *S. aureus* and *L. monocytogenes* biofilms treated with AITC (■) and PEITC (□) for 1 h. Mean values ± standard deviation for at least three replicates are illustrated.

Table 1
Motility (swimming, swarming, twitching and colony spreading) (mm) of *E. coli*, *P. aeruginosa*, *S. aureus* and *L. monocytogenes* in the absence (control) and presence of AITC and PEITC.

		Control				AITC				PEITC			
		Swim	Swarm	Twitch	Colony spreading	Swim	Swarm	Twitch	Colony spreading	Swim	Swarm	Twitch	Colony spreading
24 h	*E. coli*	41 ± 1.0	9.0 ± 0.0	–	–	20 ± 1.0	8.0 ± 0.0	–	–	8.0 ± 0.0	8.0 ± 0.0	–	–
	P. aeruginosa	17 ± 0.0	9.0 ± 0.0	9.7 ± 0.6	–	8.0 ± 0.0	8.0 ± 0.0	8.0 ± 0.0	–	8.0 ± 0.0	8.0 ± 0.0	8.0 ± 0.0	–
	S. aureus	–	–	–	20 ± 0.0	–	–	–	13 ± 1.1	–	–	–	8.0 ± 0.0
	L. monocytogenes	19 ± 0.6	8.0 ± 0.0	–	–	10 ± 0.6	8.0 ± 0.0	–	–	8.0 ± 0.0	8.0 ± 0.0	–	–
48 h	*E. coli*	85 ± 0.0	9.0 ± 0.0	–	–	52 ± 1.1	8.0 ± 0.0	–	–	8.0 ± 0.0	8.0 ± 0.0	–	–
	P. aeruginosa	30 ± 0.6	11 ± 0.0	12 ± 0.0	–	17 ± 1.1	8.0 ± 0.0	8.0 ± 0.0	–	15 ± 3.2	8.3 ± 0.6	8.7 ± 0.6	–
	S. aureus	–	–	–	28 ± 0.0	–	–	–	21 ± 2.7	–	–	–	8.0 ± 0.0
	L. monocytogenes	27 ± 0.0	8.0 ± 0.0	–	–	17 ± 1.1	8.0 ± 0.0	–	–	8.0 ± 0.0	8.0 ± 0.0	–	–
72 h	*E. coli*	85 ± 0.0	9.3 ± 0.6	–	–	85 ± 0.0	8.0 ± 0.0	–	–	8.0 ± 0.0	8.0 ± 0.0	–	–
	P. aeruginosa	64 ± 1.1	11 ± 0.0	12 ± 0.0	–	42 ± 8.0	8.0 ± 0.0	8.0 ± 0.0	–	18 ± 2.6	8.3 ± 0.6	8.7 ± 0.6	–
	S. aureus	–	–	–	35 ± 0.0	–	–	–	29 ± 0.6	–	–	–	8.0 ± 0.0
	L. monocytogenes	33 ± 0.6	8.0 ± 0.0	–	–	26 ± 1.7	8.0 ± 0.0	–	–	8.0 ± 0.0	8.0 ± 0.0	–	–

Note: Results are shown as mean ± standard deviation of at least three independent experiments. The 15 μL of bacterial culture produced an 8 mm (baseline) spot on the agar.

Table 2
Adhesion potential of bacteria to PS.

	ΔG_{iWI}^{TOT} (mJ m^{-2})			
	E. coli	*P. aeruginosa*	*S. aureus*	*L. monocytogenes*
Control	4.7 ± 0.5	−2.9 ± 0.3	3.1 ± 0.7	5.4 ± 1.2
AITC	7.8 ± 0.3	6.9 ± 0.6	1.5 ± 0.4	−12.0 ± 0.2
PEITC	−2.0 ± 1.3	9.4 ± 0.9	2.3 ± 0.8	−43.4 ± 1.4

Note: $\Delta G_{iWI}^{TOT} < 0$ mJ m^{-2} – thermodynamic favorable adhesion; $\Delta G_{iWI}^{TOT} > 0$ mJ m^{-2} – thermodynamic unfavorable adhesion.

Table 3
Biofilm formation ability according to the classification proposed by Stepanović et al. (2000) and used in this study to characterize the biofilm preventive action of AITC and PEITC.

	Control	AITC	PEITC
E. coli	+++	+	+++
P. aeruginosa	+++	0	+++
S. aureus	+	+	0
L. monocytogenes	++	0	++

(0) non-biofilm producer; (+) weak biofilm producer; (++) moderate biofilm producer; (+++) strong biofilm producer.