Activity of allylisothiocyanate and 2-phenylethylisothiocyanate on motility and biofilm prevention of pathogenic bacteria

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Isothiocyanates (ITCs) are plant secondary metabolites with a range of biological effects including antimicrobial activity. This study reports the activity of two ITCs [allylisothiocyanate (AITC) and 2-phenylethylisothiocyanate (PEITC)] on bacterial motility and prevention of biofilm formation by *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Listeria monocytogenes*. AITC caused total inhibition of swimming (*P. aeruginosa*) and swarming (*E. coli*, *P. aeruginosa*) 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. Total biofilm prevention was observed for *E. coli* with AITC. AITC and PEITC had no preventive effects in biofilm formation by *S. aureus* and *L. monocytogenes*, respectively. Significant preventive action with AITC on biofilm formation by *P. aeruginosa* (90%) and by *L. monocytogenes* (61%), and with PEITC on biofilm formation by *S. aureus* (75%) was verified. In terms of viability, AITC and PEITC promoted reductions higher than 87% for all the biofilms tested. In conclusion, these molecules demonstrated potential to inhibit bacterial motility and to prevent biofilm formation of pathogenic bacteria.

Keywords isothiocyanates; pathogenic bacteria; motility; biofilm prevention

1. Introduction

Biofilms comprise sessile microbial communities surrounded by a matrix of extracellular polymeric substances (EPS). This phenotype represents the prevalent mode of microbial life in nature, industrial process and infections [1]. Bacteria in biofilms can cause serious problems in biomedical systems [2, 3]. This attached mode of growth protects the bacteria from environmental stresses [4]. One serious problem is the faster establishment of resistance to antimicrobial agents than in planktonic state [5]. The best strategy to control or eradicate biofilms is to prevent their development [6]. In general, the transition from free-living cells to a sessile form of life begins with the transportation and attachment of microorganisms to a particular substratum. It has been shown that cell surface motility structures, such as pili, fimbrae, flagella and curli play an important role in the early attachment processes. These are structural components that serve as sensory systems for dislocation of bacteria and adhesion to a particular substrate, i.e. for the initial biofilm formation [7]. Therefore, the inhibition of bacterial motility can represent an interesting approach to prevent biofilm formation.

The emergence of resistant bacteria to conventional antimicrobials clearly shows that new biofilm control strategies are required [8]. Natural antibacterial compounds which restrict the ability of bacteria to adhere, communicate, and form biofilm complexes can represent a source of lead biofilm control molecules [9]. In this context, glucosinolates and their hydrolysis products, particularly isothiocyanates (ITCs), a group of plant secondary metabolites belonging to the *Brassicaceae* family (i.e. cabbage, broccoli, mustard, horseradish and wasabi) have long been recognized for their antimicrobial activity against clinical important microorganisms (e.g. *E. coli*, *C. albicans*, *B. subtilis*, *C. jejuni*, *H. pylori* and *V. parahaemolyticus*) [10, 11]. In addition, these compounds have other benefits for human nutrition, such as anticarcinogenic and antioxidant properties [12, 13]. In this work the activity of two selected ITCs (allylisothiocyanate and 2-phenylethylisotiocyanates) was evaluated on the prevention of biofilm formation by selected pathogenic bacteria. The assessment of ITCs on the inhibition of bacterial motility was also performed.

2. Material and methods

2.1 Bacteria and culture conditions

Escherichia coli CECT 434, *Pseudomonas aeruginosa* ATCC 10145, *Staphylococcus aureus* CECT 976 and *Listeria monocytogenes* ATCC 15313 were used in this study.

2.2 Phytochemicals

A stock solution of allylisothiocyanate (AITC) and 2-phenylethylisothiocyanate (PEITC) (Sigma-Aldrich,) at 10000 µg/mL was prepared in dimethyl sulfoxide (DMSO, Sigma) and was stored at -20 ºC until use. Phytochemicals are routinely classified as antimicrobials on the basis of susceptibility tests that produce inhibitory concentrations in the range of 100 to 1000 µg/mL [14]. In this work, each product was tested at a concentration of 1000 µg/mL. Negative controls were performed with DMSO.

2.3 Motility assays

Overnight cultures grown on Luria-Bertani broth (LBB) (Merck, Germany) were applied (15 µL of a suspension with 1×10^8 cells/mL) 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 [15, 16]. Colony spreading was assessed for *S. aureus* and twitching motility was only assessed for *P. aeruginosa*. AITC and PEITC at 1000 µg/mL were incorporated in the growth medium (tempered at 45 ºC). Plates were incubated at 30 °C and the diameter (mm) of the bacterial motility halos were measured at 24 h.

2.4 Biofilm formation

Biofilms were developed according to the modified microtiter plate test proposed by Stepanović et al. [17]. 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 1×10^8 cells/mL. Negative control wells contained Mueller-Hinton broth (MHB) without bacterial cells. The plates were incubated for 24 h at 30 ºC and agitated at 150 rpm.

2.4.1 Biofilm prevention

Overnight batch cultures in MHB supplemented with AITC and PEITC at 1000 µg/mL were grown at 30 ºC and 150 rpm. Those cells were used to assess their ability to form biofilms in 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 mass reduction and inactivation.

2.4.2 Biofilm mass quantification by crystal violet staining

The biofilm mass was quantified using crystal violet (Merck) staining, according to Simões et al. [18]. The absorbance was measured at 570 nm using a Microplate reader (Spectramax M2e, Molecular Devices, Inc.). Biofilm removal was given by Eq. (1), where %BR is the percentage of biofilm removal, OD_C is the OD_{570nm} value of biofilms non-exposed to ITCs and OD_W is the OD_{570nm} value for biofilm exposed to AITC or PEITC.

$$\%BR = \frac{OD_C - OD_W}{OD_C} \times 100 \quad (1)$$

2.4.3 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. [19]. For the staining procedure, fresh MHB (190 µL) 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 with a Microplate reader. The percentage of biofilm inactivation was given by Eq. (2), where %BI is the percentage of biofilm inactivation, FI_C is the fluorescence intensity of biofilms non exposed to ITCs and FI_W is the fluorescence intensity value for biofilms exposed to AITC or PEITC.

$$\%BI = \frac{FI_C - FI_W}{FI_C} \times 100 \quad (2)$$

2.4 Statistical analysis

The data were analysed 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 analysed by the application of the non-parametric Wilcoxon test (confidence level ≥ 95%).

3. Results and discussion

In the last years microbial infections have become difficult to control using conventional antimicrobial therapy, particularly those biofilm-related [5]. This has encouraged the search for new therapeutic alternatives. Due to the safe status and history of use in traditional medicine, plant compounds are widely accepted as a source of therapeutic molecules [20]. Research on natural products as antimicrobial agents, has almost been exclusively focused on the effects against planktonic microorganisms. However, the effects of phytochemicals on biofilms remain largely unexplored [20]. In this study the effects of AITC and PEITC at 1000 µg/mL was assessed on motility inhibition and biofilm prevention of four bacteria with biomedical importance. Motility is amongst the first steps for pathogenesis and biofilm development. Three forms of surface motility, swimming, twitching and swarming, are documented for *P. aeruginosa* [21]. *P. aeruginosa* swims by means of flagella, and during biofilm formation, swimming motility is involved in initial location and adherence to solid surfaces [7]. After surface attachment, *P. aeruginosa* moves by surface motility known as twitching [21]. *E. coli* and *L. monocytogenes* has two flagella-driven motility types, swimming and swarming [22, 23]. *S. aureus* is a non-flagellated Gram-positive bacterium with a motility phenomenon defined as colony spreading [24]. Therefore, in this work, the ability of AITC and PEITC 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).

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.

		E. coli	*P. aeruginosa*	*S. aureus*	*L. monocytogenes*
Control	Swim	41 ± 1.0	17 ± 0.0	-	19 ± 0.6
	Swarm	9.0 ± 0.0	9.0 ± 0.0	-	8.0 ± 0.0
	Twitch	-	9.7 ± 0.6	-	-
	Colony spreading	-	-	20 ± 0.0	-
AITC	Swim	20 ± 1.0	8.0 ± 0.0	-	10 ± 0.6
	Swarm	8.0 ± 0.0	8.0 ± 0.0	-	8.0 ± 0.0
	Twitch	-	8.0 ± 0.0	-	-
	Colony spreading	-	-	13 ± 1.1	-
PEITC	Swim	8.0 ± 0.0	8.0 ± 0.0	-	8.0 ± 0.0
	Swarm	8.0 ± 0.0	8.0 ± 0.0	-	8.0 ± 0.0
	Twitch	-	8.0 ± 0.0	-	-
	Colony spreading	-	-	8.0 ± 0.0	-

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.

The application of AITC and PEITC promoted total inhibition in swimming, swarming and twitching motilities for *P. aeruginosa* ($P < 0.05$). The same result was verified for swarming of *E. coli* with both ITCs and for swimming with PEITC ($P < 0.05$). For this bacterium, swimming motility was significantly reduced by the addition of AITC ($P < 0.05$). The swimming motility of *L. monocytogenes* was completely inhibited with PEITC ($P < 0.05$); however, AITC promoted a significant reduction ($P < 0.05$). *S. aureus* colony spreading was reduced by AITC and completely inhibited with PEITC ($P < 0.05$). For most of the cases, PEITC was more efficient in motility reduction than AITC ($P < 0.05$). The inhibition of bacterial motility can represent an important strategy to control biofilms. Bacteria in a motile state undergo alterations in their morphology which distinguishes them from their planktonic state. Lai et al. [25] found increased resistance of swarming bacteria compared with their planktonic counterparts. These results may be important as changes in motility can be correlated with a decreased ability of bacteria to form biofilms. In fact, motility plays a major role in the transition from planktonic to surface-associated life-style [7]. Other reports described that many mutants with altered swarming motility were also defective in biofilm formation, indicating that it may play a key role in early biofilm development [26]. Shrout et al. [26] demonstrated that differences in surface motility could explain differences in biofilm structure at early stages of development. 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 polystyrene microtiter plates (Fig. 1a).

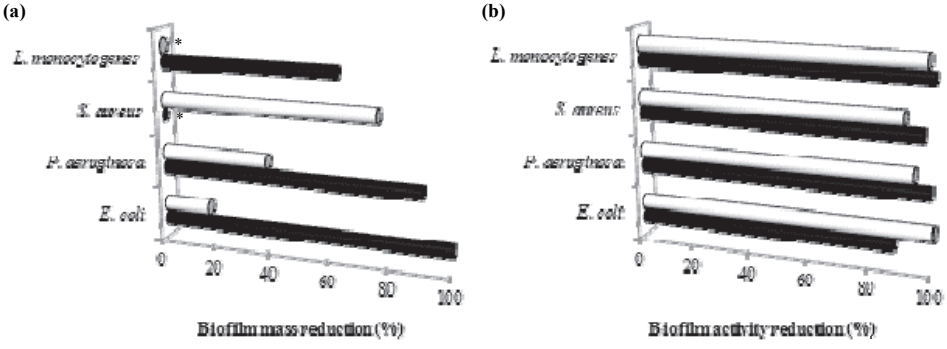

Fig. 1 Preventive action (24 h aged biofilms formed in the presence of ITCs) of AITC (■) and PEITC (□) on biomass formation (a) and metabolic activity (b) of *E. coli*, *P. aeruginosa*, *S. aureus* and *L. monocytogenes*. * - no prevention on biomass formation or metabolic activity reduction was found. Mean values ± standard deviation for at least three replicates are illustrated.

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 demonstrated for *S. aureus* with AITC (no prevention in biomass formation), and PEITC (75%) had significant preventive action in *S. aureus* biofilm formation ($P < 0.05$). Total biofilm prevention was observed only for *E. coli* with AITC. , In general, AITC had higher preventive effects on biofilm formation than PEITC ($P < 0.05$) (*E. coli* (AITC - 100%; PEITC - 16%) and *P. aeruginosa* (AITC - 90%; PEITC - 37%)). In terms of metabolic activity, the analysis of biofilms formed by planktonic bacteria grown in the presence of ITCs (Fig. 1b) shows that AITC and PEITC promoted reductions higher than 90% for all the biofilms tested, except for *E. coli* with AITC, where the biofilm activity reduction was approximately 87%. Note that, AITC reduced biofilm activity of *S. aureus*, although the chemical had no effects ($P < 0.05$) on the biomass reduction (0% biofilm mass reduction). A similar result was obtained with PEITC for *L. monocytogenes*, biofilm activity reduction was observed for this bacterium, while the ITC had no effects ($P < 0.05$) on the biomass reduction (0% biofilm mass reduction). These results are in agreement with previous study where no correlation between the biomass and metabolic activity was found [20]. In this study the comparison between the results of motility inhibition and biofilm prevention (AITC - *E. coli*, *P. aeruginosa*, *L. monocytogenes* and PEITC - *S. aureus*) suggest that the inhibition of motility can interfere with ability to form biofilms. In a study performed by Sandasi et al. [20], extracts of culinary herbs and medicinal plants had antibiofilm activity against strains of *L. monocytogenes*. Moreover, these authors also found that although most extracts were able to inhibit cell attachment and the growth inhibition of a preformed biofilm was hard to achieve. In fact, inhibit the growth of an already established biofilm (control) is more difficult to achieve than inhibit the initial stage of biofilm formation, namely cell attachment (prevention) [20].

4. Conclusions

AITC and PEITC seem to be promising products for anti-biofouling strategies. These compounds demonstrated potential to inhibit bacterial motility and to prevent biofilm formation of important pathogenic bacteria. This study also emphasizes the potential of phytochemicals as an emergent source of biofilm prevention products. Further studies are in progress to assess the mechanisms of antibacterial action of AITC and PEITC and their cytotoxicity to mammalian cells.

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References

[1] Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: From the natural environment to infectious diseases. *Nature Reviews Microbiology*. 2004;2(2):95-108.

[2] Davey ME, O'Toole GA. Microbial biofilms: From ecology to molecular genetics. *Microbiology and Molecular Biology Reviews*. 2000;64(4):847-867.
[3] Simões M. Antimicrobial strategies effective against infectious bacterial biofilms. *Current Medicinal Chemistry*. 2011;18(14):2129-2145.
[4] Lewis K. Riddle of biofilm resistance. *Antimicrobial Agents and Chemotherapy*. 2001;45(4):999-1007.
[5] Mah TFC, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology*. 2001;9(1):34-39.
[6] Johnson LR. Microcolony and biofilm formation as a survival strategy for bacteria. *Journal of Theoretical Biology*. 2008;251(1):24-34.
[7] O'Toole GA, Kolter R. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molecular Microbiology*. 1998;30(2):295-304.
[8] Simões M, Simões LC, Machado I, Pereira MO, Vieira MJ. Control of flow-generated biofilms with surfactants: Evidence of resistance and recovery. *Food and Bioproducts Processing*. 2006;84(4 C):338-345.
[9] Al-Sohaibani S, Murugan K. Anti-biofilm activity of *Salvadora persica* on cariogenic isolates of *Streptococcus mutans*: in vitro and molecular docking studies. *Biofouling*. 2012;28(1):29-38.
[10] Fahey JW, Zalcmann AT, Talalay P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry*. 2001;56(1):5-51.
[11] Kim MG, Lee HS. Growth-inhibiting activities of phenethyl isothiocyanate and its derivatives against intestinal bacteria. *Journal of Food Science*. 2009;74(8):M467-M471.
[12] Saavedra MJ, Borges A, Dias C, Aires A, Bennett RN, Rosa ES, Simões M. Antimicrobial activity of phenolics and glucosinolate hydrolysis products and their synergy with streptomycin against pathogenic bacteria. *Medicinal Chemistry*. 2010;6(3):174-183.
[13] Zhang Y. The molecular basis that unifies the metabolism, cellular uptake and chemopreventive activities of dietary isothiocyanates. *Carcinogenesis*. 2012;33(1):2-9.
[14] Simões M, Bennett RN, Rosa EA. Understanding antimicrobial activities of phytochemicals against multidrug resistant bacteria and biofilms. *Natural Product Reports*. 2009;26(6):746-757.
[15] Butler MT, Wang Q, Harshey RM. Cell density and mobility protect swarming bacteria against antibiotics. *Proceedings of the National Academy of Sciences of the United States of America*. 2010;107(8):3776-3781.
[16] Stickland HG, Davenport PW, Lilley KS, Griffin JL, Welch M. Mutation of nfxB causes global changes in the physiology and metabolism of *Pseudomonas aeruginosa*. *Journal of Proteome Research*. 2010;9(6):2957-2967.
[17] Stepanović S, Vuković D, Dakić I, Savić B, Švabić-Vlahović M. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods*. 2000;40(2):175-179.
[18] Simões LC, Simões M, Vieira MJ. Influence of the diversity of bacterial isolates from drinking water on resistance of biofilms to disinfection. *Applied and Environmental Microbiology*. 2010;76(19):6673-6679.
[19] Sarker SD, Nahar L, Kumarasamy Y. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. *Methods*. 2007;42(4):321-324.
[20] Sandasi M, Leonard CM, Viljoen AM. The in vitro antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocytogenes*. *Letters in Applied Microbiology*. 2010;50(1):30-35.
[21] Déziel E, Comeau Y, Villemur R. 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. *Journal of Bacteriology*. 2001;183(4):1195-1204.
[22] Harshey RM. Bacterial Motility on a Surface: Many Ways to a Common Goal. *Annual Review of Microbiology*. 2003;57(249-273.
[23] Roberts AJ, Williams SK, Wiedmann M, Nightingale KK. Some *Listeria monocytogenes* outbreak strains demonstrate significantly reduced invasion, inlA transcript levels, and swarming motility in vitro. *Applied and Environmental Microbiology*. 2009;75(17):5647-5658.
[24] Tsompanidou E, Sibbald MJJB, Chlebowicz MA, Dreisbach A, Back JW, Van Dijl JM, Buist G, Denham EL. Requirement of the agr locus for colony spreading of *Staphylococcus aureus*. *Journal of Bacteriology*. 2011;193(5):1267-1272.
[25] Lai S, Tremblay J, Déziel E. Swarming motility: A multicellular behaviour conferring antimicrobial resistance. *Environmental Microbiology*. 2009;11(1):126-136.
[26] Shrout JD, Chopp DL, Just CL, Hentzer M, Givskov M, Parsek MR. The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* biofilm formation is nutritionally conditional. *Molecular Microbiology*. 2006;62(5):1264-1277.