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Antibacterial activity and mode of action of selected glucosinolate hydrolysis products against bacterial pathogens

Abstract

Plants contain numerous components that are important sources of new bioactive molecules with antimicrobial properties. Isothiocyanates (ITCs) are plant secondary metabolites found in cruciferous vegetables that are arising as promising antimicrobial agents in food industry. The aim of this study was to assess the antibacterial activity of two isothiocyanates (ITCs), allylisothiocyanate (AITC) and 2-phenylethilisothiocyanate (PEITC) against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Listeria monocytogenes*. The antibacterial mode of action was also characterized by the assessment of different physiological indices: membrane integrity, intracellular potassium release, physicochemical surface properties and surface charge. The minimum inhibitory concentration (MIC) of AITC and PEITC was 100 μg/mL for all bacteria. The minimum bactericidal concentration (MBC) of the ITCs was at least 10 times higher than the MIC. Both AITC and PEITC changed the membrane properties of the bacteria decreasing their surface charge and compromising the integrity of the cytoplasmatic membrane with consequent potassium leakage and propidium iodide uptake. The surface hydrophobicity was also non-specifically altered (*E. coli* and *L. monocytogenes* become less hydrophilic; *P. aeruginosa* and *S. aureus* become more hydrophilic). This study shows that AITC and PEITC have strong antimicrobial potential against the bacteria

tested, through the disruption of the bacterial cell membranes. Moreover, phytochemicals are highlighted as a valuable sustainable source of new bioactive products.

Keywords: antibacterial activity; disinfectants; food preservatives; isothiocyanates; mechanisms of action

Introduction

The food safety is an important public health issue that continues to be a major concern to consumers, regulatory agencies and food industries worldwide. The increased incidence of food poisoning cases has been reported due to the contamination of food with pathogens and spoilage organisms (Langsrud et al. 2003; Negi 2012). This leads to the necessity of improvement of hygiene and preservative practices of food products. The presence of microorganisms in the food products frequently causes their spoilage, which sometimes can lead to the production of toxins and alteration of their organoleptic quality (Negi 2012; Tiwari et al. 2009).

Most of the traditionally used food preservation strategies (heating, refrigeration, acidification, pasteurization and addition of synthetic antimicrobial compounds), may cause adverse changes in organoleptic properties of foods and loss of nutrients, reducing the consumer acceptability (Tiwari et al. 2009). The requirement of safer foods and longer shelf-life has led to a higher frequency of disinfection (on food-contact surfaces, equipment, utensils, etc.) and to the use of preservatives (Langsrud et al. 2003).

The recurrent use of chemical disinfectants and also the inadequate disinfection strategies impose selective pressure and contribute to the emergence of resistance among microorganisms (Russell 2000). Resistant microorganisms have been responsible for the failure of many disinfection programs, and therefore for many contaminations in

52 industrial, environmental and biomedical settings (Chorianopoulos et al. 2011).
53 Combined resistance to disinfectants and other types of antimicrobials may become a
54 threat to the food processing industries. In addition, cross-resistance between
55 disinfectants and antibiotics can also lead to serious consequences for the public health
56 (Russell 2003). Therefore, new disinfection techniques and effective disinfectants are
57 required in order to ensure high levels of sanitation. In this context, substantial resources
58 have been invested in the research of effective antimicrobial compounds that preserve the
59 organoleptic properties of the products (Dufour et al. 2012; Negi 2012; Tiwari et al.
60 2009). Moreover, products that act on novel bacterial targets (e.g. bacterial ribosomal
61 subunit synthesis, fatty acid biosynthesis, aminoacyl–tRNA synthetases, two-component
62 signal transduction (2CST) systems) and circumvent the conventional mechanisms of
63 resistance to current antimicrobials are also important (Saleem et al. 2010; Sarker et al.
64 2007; Black and Hodgson, 2005). Although synthetic antimicrobials are approved in
65 many countries, the recent trend has been the use of safe natural preservatives derived
66 from microbial, animals or plants (Rahman and Kang 2009).

67 Plants are an attractive source of such compounds as they produce an enormous array
68 of secondary metabolites (phytochemicals) with medicinal properties, including
69 antimicrobial properties, which have been used traditionally for centuries (Abreu et al.
70 2012). A significant part of this diversity of phytochemicals are related to defense
71 mechanisms of plants against attack by microorganisms, insects, nematodes and even
72 other plants (Dangl and Jones 2001; Dixon 2001). Additionally, it is known that some
73 phytochemical products have an accepted safe status and distinctive properties from
74 synthetic molecules that make them perfect candidates for diverse applications (Cowan
75 1999; Lin et al. 2000a; Simões et al. 2009).

Glucosinolates (GLS) are organosulfur compounds present exclusively in the order Capparales and very abundant in the Brassicaceae (Syn. Cruciferae) family (Al-Gendy et al. 2010; Barbieri et al. 2008; Grubb and Abel 2006; Halkier and Du 1997). They occur as secondary metabolites of various vegetables such as cabbage, broccoli, cauliflower, watercress, horseradish, Brussels sprouts and kohlrabi (Fahey et al. 2001; Holst and Williamson 2004). GLS are classified as aliphatic, aromatic and indolyl, based on the amino acid from which they derive (Fahey et al. 2001; Halkier and Gershenzon 2006). Intact GLS do not show antimicrobial activity. These dietary phytochemicals are present in the cells vacuole and when tissue disruption occurs, they are hydrolyzed by the myrosinase enzyme (*β*-thioglucosidase enzyme) into numerous biologically active products such as isothiocyanates (ITCs), nitriles, epithionitriles and thiocyanates (Aires et al. 2009b; Fahey et al. 2001; Hong and Kim 2008). Glucosinolate hydrolysis products (GHP) have long been recognized for their antimicrobial activity against important pathogenic microorganisms (e.g. *Escherichia coli, Candida albicans, Bacillus subtilis*, *Campylobacter jejuni*, *Helicobacter pylori* and *Vibrio parahaemolyticus*) (Dufour et al. 2012; Fahey et al. 2001; Shin et al. 2004; Wang et al. 2010). In addition, these compounds have other pharmaceutical benefits for human health, such as anticarcinogenic, anti-inflammatory and antioxidant properties (D'Antuono et al. 2009; Hong and Kim 2008; Saavedra et al. 2010; Zhang 2012). The presence of such phytochemicals in natural foods might even contribute to the medicinal properties attributed to the consumption of cruciferous vegetables. Among GHP, ITCs are considered the most potent inhibitors of microbial activity and their properties are being actively explored (Al-Gendy et al. 2010; Cartea and Velasco 2008; Munday et al. 2008; Saavedra et al. 2010; Sofrata et al. 2011; Troncoso et al. 2005; Zhang 2012). ITCs can bind to sulfhydryl groups on active sites of important enzymes involved in the microbial growth and survival. Consequently,

reductions in the cellular levels of important thiol groups lead to the formation of oxygen and other free-radicals (Aires et al. 2009a; Jacob and Anwar 2008; Kolm et al. 1995).

The aim of this work was to investigate the antibacterial activity and some aspects of the mode of action of two selected ITCs against strains of *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. These bacteria are reference microorganisms for antimicrobial studies (EN-1276, 1997; Jones and Stilwell, 2013). Also, some of these species are important foodborne or spoilage microorganisms commonly found in food industries, being important causal agents of foodborne diseases (McCabe-Sellers and Beattie 2004; Rahman and Kang 2009).

Materials and methods

Bacterial strains and growth medium

The following strains were used in this study: *Escherichia coli* CECT 434, *Pseudomonas aeruginosa* ATCC 10145, *Staphylococcus aureus* CECT 976 and *Listeria monocytogenes* ATCC 15313. These bacteria were already used as model microorganisms for antimicrobial tests with phytochemical products (Abreu et al. 2013; Borges et al. 2012; Saavedra et al. 2010; Simões et al. 2008). *E. coli*, *P. aeruginosa* and *S. aureus* are reference microorganisms to be used in the development of disinfection strategies (EN-1276, 1997). Also, the strains used in this study are commonly used as routine quality control strains, and as reference for antimicrobial testing and for bacterial resistance testing (Ananou et al. 2004; Diab et al. 2012; Tabata et al. 2003; UNE-CEN ISO/TS 11133, 2006). All microbial strains were stored at -80 ºC in cryovial, 30% (v/v) glycerol, and subcultured in Mueller-Hinton Agar (MHA) (Merck, Darmstadt-Germany) at 30 ºC, before testing.

Isothiocyanates

Allylisothiocyanate (AITC) and 2-phenylethylisothiocyanate (PEITC) (Fig. 1) were obtained from Sigma-Aldrich (Sintra-Portugal). 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 (Simões et al. 2009; Tegos et al. 2002). Therefore, in this study, each product was tested at a concentration of 100, 500 and 1000 μg/mL prepared in dimethyl sulfoxide (DMSO) (99%, v/v) (Sigma-Aldrich, Sintra-Portugal).

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of ITCs was determined by the microdilution broth method (Borges et al. 2013). Briefly, overnight culture growth in Mueller-Hinton Broth (MHB), was adjusted to an OD_{640nm} of 0.2 ± 0.02 (1×10^8 cells/mL). Subsequently, for each bacterium, a sterile 96-well polystyrene microtiter plate (Orange Scientific, Braine-L'Alleud-Belgium) was filled with bacteria (180 μL) and phytochemicals (20 μL). These were tested at three different concentrations (100, 500 and 1000 μg/mL). Cell suspensions with DMSO and cell suspensions without phytochemicals were used as controls. The microtiter plates were covered with a lid that was sealed with parafilm (to avoid the volatilization of ITCs) and then incubated for 24 h at 30 ºC in an orbital shaker (150 rpm). The absorbance was measured at 640 nm using a Microplate Reader (Spectramax M2e, Molecular Devices, Inc.). The MIC was recorded as the lowest concentration of ITCs at which no growth was detected (Borges et al. 2013). All tests were performed in triplicate with three repeats.

152

153 Minimum bactericidal concentration

154 Bacterial cells were grown overnight in batch culture using MHB at 30 ºC and 150 rpm.
155 After the overnight growth, the bacterial suspension was centrifuged (3772 g, 6 min),
156 washed two times with saline solution (0.85% NaCl) and resuspended in saline solution
157 to obtain an OD_{640nm} of 0.2 ± 0.02 (1×10^8 cells/mL). Then, an aliquot of this suspension
158 was collected and maintained 30 min in contact with different concentrations of the ITCs
159 (100, 500 and 1000 µg/mL). Subsequently, bacterial suspensions were diluted to an
160 adequate cellular concentration (from 10^7 to 10^0) in saline solution. A volume of 100 µL
161 of each suspension (dilution 10^7 to 10^4) was transferred onto MHA plates and incubated
162 at 30 ºC. Colony enumeration was carried out after 24 h. Cell suspensions without
163 phytochemical were used as controls. The minimum bactericidal concentration (MBC)
164 was taken as the lowest concentration of phytochemicals at which no colony forming
165 units (CFU) were detected on solid medium (Borges et al. 2013). All experiments were
166 performed in triplicate with three repeats.

167

168 Physicochemical characterization of the bacterial surfaces

169 Bacterial suspensions were prepared in ultrapure water (Milli-Q®) (pH 6). No significant
170 osmotic pressure effects were found when comparing the planktonic bacterial viability in
171 water and in saline solution (0.85% NaCl), for a period of up to 150 min ($P > 0.05$).
172 Afterward, their physicochemical properties were determined by the sessile drop contact
173 angle measurement on bacterial lawns, prepared as described by Busscher *et al*. (1984).
174 Contact angles were determined automatically using an OCA 15 Plus (DATAPHYSICS,
175 Germany) video-based optical measuring instrument, allowing image acquisition and data

analysis. Contact angle measurements were carried out according to Simões et al. (2007). Hydrophobicity was evaluated after contact angle measurement, following the van Oss approach (van Oss et al. 1987; van Oss et al. 1988; van Oss et al. 1989), where the degree of hydrophobicity of a given surface (*s*) is expressed as the free energy of interaction between two entities of that surface, when immersed in water (*w*) – (ΔG_{sws} mJ/m^2). If the interaction between the two entities is stronger than the interaction of each entity with water, $\Delta G_{sws} < 0$, 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:

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

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, respectively, of the Lewis acid-base component (γ^{AB}), with $\gamma^{AB} = 2\sqrt{\gamma^+\gamma^-}$. The surface tension components, of a solid material, can be obtained by measuring the contact angles of the three liquids (l): the apolar α-bromonaphthalene; the polar formamide and water. The liquid surface tension components reference values were obtained from the literature (Janczuk et al. 1993). Once the values are obtained, three equations of the type below can be solved:

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

where θ is the contact angle and $\gamma^{Tot} = \gamma^{LW} + \gamma^{AB}$. At least three independent experiments were performed for each condition tested.

Bacterial surface charge - zeta potential

199 The zeta potential of bacterial suspensions, before and after the contact with different
200 AITC and PEITC concentrations (100, 500 and 1000 μg/mL), was determined using a
201 Nano Zetasizer (Malvern Instruments, UK). Cell suspensions in ultrapure water (pH 6),
202 without phytochemical, were used as controls. The zeta potential was measured by
203 applying an electric field across the bacterial suspensions. Bacteria in the aqueous
204 dispersion with non-zero zeta potential migrated towards the electrode of opposite charge,
205 with a velocity proportional to the magnitude of the zeta potential. The experiments were
206 repeated at least three times.
207
208 Assessment of membrane integrity due to propidium iodide uptake
209 The Live/Dead *Bac*Light™ kit (Invitrogen/Molecular Probes, Leiden, Netherlands)
210 assesses membrane integrity by selective stain exclusion (Simões et al. 2005). This fast
211 method was applied to estimate both viable and total counts of bacteria. *Bac*Light is
212 composed of two nucleic acid-binding stains: SYTO 9™ and propidium iodide (PI).
213 SYTO 9™ penetrates bacterial membranes, staining the cells green; PI only penetrates
214 cells with damaged membranes, binding to single and double-stranded nucleic acids. The
215 combination of these two stains generates red fluorescing cells. After overnight growth,
216 the cells were centrifuged (3772 g, 10 min) and washed one time with saline solution
217 (0.85%). Afterwards, bacteria were resuspended in saline solution to obtain an OD_{640nm}
218 of 0.2 ± 0.02 (1×10^8 cells/mL). Then, an aliquot of 1 mL of this suspension was collected
219 and different concentrations of the ITCs were tested (100, 500 and 1000 μg/mL) for 30
220 min in contact with the bacteria. Cell suspensions without phytochemicals were used as
221 controls. Afterwards, bacteria were transferred to saline solution and diluted 1:10. Three
222 hundred microliters of each diluted suspension were filtered through a Nucleopore®
223 (Whatman, Middlesex, UK) black polycarbonate membrane (pore size 0.22 μm) and

224 stained with 250 mL of diluted SYTO 9^{TM} and 250 mL of diluted component PI. The dyes
225 were left to react for 15 min in the dark, at 27 ± 3 ºC. The membrane was then mounted
226 on *Bac*Light mounting oil, as described in the manufacturer's instructions. The
227 microscope used for the observation of stained bacteria was a LEICA DMLB2 with a
228 mercury lamp HBO/100W/3, incorporating a CCD camera to acquire images using IM50
229 software (LEICA) and a 100× oil immersion fluorescence objective. The optical filter
230 combination for optimal viewing of stained mounts consisted of a 480–500 nm excitation
231 filter in combination with a 485 nm emission filter (Chroma 61000-V2 DAPI/
232 FITC/TRITC). A program path (Scan Pro 5) involving object measurement and data
233 output was used to obtain the total number of cells (both stains) and the number of PI-
234 stained cells (damaged cells). Both the total number of cells and the number of PI-stained
235 cells on each membrane was estimated from counts of ≥ 20 fields of view. The total
236 number of cells counted per field of view ranged from 50 to 200 cells. Three independent
237 experiments were performed for each condition tested.
238
239 Potassium (K+) leakage
240 Flame emission and atomic absorption spectroscopy were used for K^+ titration in bacteria
241 suspensions treated with 1000 µg/mL of each ITC. The samples were filtrated after
242 contact with the phytochemicals, using a sterile cellulose nitrate membrane filter (pore
243 size 0.22 µm) (Whatman, Maidstone-England), and then the filtrates were analyzed in a
244 GBC AAS 932plus device using GBC Avante 1.33 software. The experiments were
245 repeated three times.
246
247 Statistical analysis

248 The data were analysed using the statistical program SPSS (Statistical Package for the
249 Social Sciences) version 20.0 (IBM® SPSS® Statistics Corporation). The mean and
250 standard deviation within samples were calculated for all cases. One-way Anova with
251 Bonferroni test was used to assess the statistical significance value (confidence level ≥
252 95%).
253
254 **Results**
255 Inhibitory and bactericidal concentration of isothiocyanates
256 The MIC is the lowest concentration that inhibits visible microbial growth, while the
257 MBC is the lowest concentration at which no CFU were detected on solid medium. In
258 this study, the MIC of both ITCs against the four bacterial strains was 100 μg/mL (Table
259 1). The MBC for *S. aureus* and *L. monocytogenes* was > 1000 μg/mL for AITC and
260 PEITC (Table 1). *E. coli* and *P. aeruginosa* had MBC of 1000 μg/mL for AITC and >
261 1000 μg/mL for PEITC.
262
263 Effects of isothiocyanates on bacterial physicochemical surface properties
264 The physicochemical cell surface properties were determined using the van Oss approach,
265 which allows the assessment of the total degree of hydrophobicity of any surface in
266 comparison with their interaction with water (Table 2). All the bacteria used in this study
267 had hydrophilic properties ($\Delta G^{TOT} > 0$ mJ/m^2), before exposure to the ITCs. It is possible
268 to observe changes in the bacterial membrane physicochemical character with the
269 application of ITCs, particularly with PEITC ($P < 0.05$). *E. coli* cell surface (31.3 mJ/m^2)
270 became less hydrophilic in the presence of AITC (at 500 μg/mL - 30.9 mJ/m^2 and 1000
271 μg/mL - 28.3 mJ/m^2) and PEITC (at 100 μg/mL - 31.0 mJ/m^2 and 1000 μg/mL - 21.9
272 mJ/m^2) ($P < 0.05$). The application of both ITCs promoted the increase of hydrophilic

273 character of *P. aeruginosa* (particularly with PEITC) and *S. aureus* ($P < 0.05$). However,
274 for *P. aeruginosa* with AITC a decrease of hydrophilic character was verified with the
275 increase of phytochemical concentration ($P < 0.05$). The same behavior was observed for
276 *S. aureus* with PEITC ($P < 0.05$). The opposite effect was observed for *L. monocytogenes*,
277 *i.e.* AITC and PEITC induced a cell surface hydrophobic character ($P < 0.05$), except
278 with AITC at 100 μg/mL. The values of the surface tension components demonstrated
279 that the *E. coli* and *L. monocytogenes* acquired polar character after treatment with ITCs
280 (except for *E. coli* with PEITC at 500 and 1000 μg/mL), as reflected by an increase in γ^{AB}
281 ($P < 0.05$). However, *P. aeruginosa* and *S. aureus* acquired apolar properties after
282 exposure to AITC and PEITC ($P < 0.05$). The apolar and polar components (γ^{LW} and γ^{AB})
283 of *L. monocytogenes* was almost unaffected by the exposure to AITC at 100 μg/mL ($P >$
284 0.05). The electron acceptor component (γ^{+}), increased with ITCs application for *E. coli*
285 (except with PEITC at 500 and 1000 μg/mL) and *L. monocytogenes* ($P < 0.05$) and
286 decreased for *P. aeruginosa* and *S. aureus* ($P < 0.05$).
287
288 Effects of isothiocyanates on bacterial surface charge
289 The assessment of zeta potential is based on the mobility of cells in the presence of an
290 electrical field under defined pH and salt concentrations and allows the determination of
291 the surface charge of cells. The results obtained from the zeta potential measurements
292 (Fig. 2) allowed a better understanding on the cellular changes induced by AITC and
293 PEITC. The bacteria tested had a negative surface charge: -14.4 mV for *E. coli*, -12.5 mV
294 for *P. aeruginosa*, -20.2 mV for *S. aureus* and -34.9 mV for *L. monocytogenes*. The
295 exposure of *S. aureus* and *L. monocytogenes* to ITCs changed the surface charge of cells
296 to less negative values ($P < 0.05$). In contrast, for the Gram-negative bacteria, no
297 significant changes were caused by AITC and PEITC on the surface charge ($P > 0.05$).

298

299 Effects of isothiocyanates on bacterial membrane integrity

300 The PI uptake results suggest that AITC and PEITC compromise the integrity of the
301 cytoplasmatic membrane (Fig. 3). It is possible to observe that the percentage of cells
302 with damaged membrane increased considerably with ITCs concentration. For AITC and
303 PEITC at 100 µg/mL the percentages of PI stained cells of *E. coli* (AITC – 11%; PEITC
304 – 12%), *P. aeruginosa* (AITC – 32%; PEITC – 34%), *S. aureus* (AITC – 26%; PEITC –
305 7%) and *L. monocytogenes* (AITC – 12%; PEITC – 3%) were low. A concentration of
306 500 µg/mL increased significantly the membrane damage of *E. coli* for PEITC ($P < 0.05$),
307 and *P. aeruginosa* for both ITCs ($P < 0.05$). For AITC at 1000 µg/mL, the percentage of
308 cells of *E. coli* and *S. aureus* stained with PI was higher than 90%. However with PEITC,
309 this percentage was 68% and 67%, respectively. For *P. aeruginosa* exposed to AITC and
310 PETIC at 1000 µg/mL the damage in cytoplasmatic membrane was about 64% and 58%,
311 respectively, of the total cells. Although the MBC for this bacterial strain is 1000 µg/mL,
312 the results obtained for PI uptake at this concentration can be due to the presence of viable
313 but not cultivable cells.
314 *L. monocytogenes* was the microorganism less sensitive to both ITCs with 44% and 18%
315 of the cells with cytoplasmatic membrane damaged for ATIC and PEITC, respectively.

316

317

318

319 Effects of isothiocyanates in intracellular potassium release

320 The results of intracellular release of K^+ by *E. coli*, *P. aeruginosa*, *S. aureus* and *L.*
321 *monocytogenes* after exposure to 1000 µg/mL of AITC and PEITC during 30 min are
322 presented in Table 3. It is possible to observe that, when compared to the control

323 experiments, the K^+ leakage occurred due to the action of phytochemicals ($P < 0.05$).
324 However, no K^+ release was found for *P. aeruginosa* due to phytochemicals exposure (P
325 > 0.05). Moreover, the release of K^+ by Gram-positive bacteria was considerably higher
326 than for the Gram-negative ($P < 0.05$).
327
328 **Discussion**
329 Foodborne infections resulting from consumption of food contaminated with pathogenic
330 bacteria has been widely reported and constitutes an enormous public health problem.
331 Moreover, some foodborne bacteria that cause human diseases are less susceptible to the
332 existing treatments, rising the need of using different disinfection methods, with new
333 products, in order to successfully eliminate these contaminants (Oussalah et al. 2007). To
334 reduce health hazard due to foodborne microorganisms, natural products from plants have
335 gained importance as antibacterial compounds (Burt 2004; Luciano and Holley 2009;
336 Tiwari et al. 2009). The antimicrobial activity of some dietary phytochemicals produced
337 by cruciferous vegetables such as ITCs has been demonstrated against diverse bacteria
338 (Chen et al. 2012; Jang et al. 2010; Lin et al. 2000a; Masuda et al. 2001; Saavedra et al.
339 2010). However, their antimicrobial mode of action is still unknown.
340 In the present study, the antimicrobial activity and mode of action of AITC and
341 PEITC against *E. coli*, *P. aeruginosa*, *S. aureus*, and *L. monocytogenes* were
342 characterized. With this aim, the MIC and MBC were assessed followed by the
343 characterization of physiological changes induced by ITCs on the bacterial cells. The
344 analysis of antimicrobial activity showed that AITC and PEITC display a MIC of 100
345 μg/mL against all bacteria tested. The MICs obtained are in the range of those described
346 in other studies. Kyung and Fleming (1997) tested the antimicrobial activity of various
347 sulfur compounds including AITC, against 15 species of bacteria, namely *L.*

348 *monocytogenes* (F 5069 and ATCC 19115), *S. aureus* (B 31) and *E. coli* (ATCC 33625)
349 and found a MIC of 200 µg/mL, 100 µg/mL and 50 µg/mL, respectively. Other study
350 demonstrated that MIC values of AITC against *E. coli* O157:H7 ranged between 25.5
351 µg/mL to 510 µg/mL with the raising of pH (Luciano and Holley 2009). In a study
352 performed by Pang et al. (2013), AITC demonstrated to be an effective antimicrobial
353 agent against a cocktail of *P. aeruginosa* (ATCC 15442, 10145 and 27853), extending
354 the shelf life of fresh catfish fillets. A mixture of ITCs (AITC, benzylisothiocyanate and
355 PEITC) was tested by Conrad et al. (2013) against clinical important bacterial
356 (*Haemophilus influenzae*, *Moraxella catarrhalis*, *Serratia marcescens, Proteus vulgaris*,
357 *S. aureus*, *S. pyogenes*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *E. coli* and *P.*
358 *aeruginosa*) and fungal (*Candida* spp.) pathogens including antimicrobial resistant
359 isolates. The results obtained showed positive inhibitory activity.

360 The MBC of both ITCs was > 1000 µg/mL for the Gram-positive bacteria. The same
361 result was obtained for *E. coli* and *P. aeruginosa* with PEITC. These bacteria were the
362 most susceptible to AITC, with a MBC of 1000 µg/mL. The bactericidal effect was found
363 at a concentration ten times higher than that needed for the bacteriostatic effect (10 ×
364 MIC). The result of MIC and MBC determinations proposes that AITC and PEITC exert
365 non-specific antimicrobial effects on both Gram-negative and –positive bacteria. In fact,
366 the presence of an outer membrane, in addition to the cytoplasmic membrane, in Gram-
367 negative bacteria, did not increase antimicrobial resistance of *E. coli* and *P. aeruginosa*.
368 In a study performed by Lin *et al*. (2000b), AITC demonstrated bactericidal activity
369 against strains of *E. coli* and *L. monocytogenes* at a concentration of 500 µg/mL and 2500
370 µg/mL, respectively. Moreover, strong activity was obtained by Shin *et al*. (2004) with
371 AITC from roots of Korean and Japanese wasabi against six foodborne pathogenic
372 bacteria, including *E. coli* O157:H7 ATCC 43889 (MBC of 660 µg/mL) and *S. aureus*

373 ATCC 25923 (MBC of 5210 μg/mL). Others reports showed that AITC had high
374 bactericidal activity against many foodborne pathogens, including *L. monocytogenes*, *S.*
375 *aureus*, *Salmonella enterica* serovar Typhimurium, and enterohemorrhagic *E. coli*
376 O157:H7 (Lin et al. 2000a; Park et al. 2000; Rhee et al. 2003).

377 It is known that phytochemicals may inhibit the bacterial growth using different
378 mechanisms than those of the presently used antibiotics, providing an interesting
379 approach to drug-resistant microorganisms (Cowan 1999). Although there are numerous
380 studies reporting the antimicrobial properties of ITCs, the specific mechanisms of their
381 action are not completely understood. Hence, more studies are needed in order to know
382 the exact target of these phytochemicals in the bacterial cells. Zsolnai (1966)
383 hypothesized that the antimicrobial activity of ITCs may be linked to intracellular
384 inactivation of sulphydryl-enzymes through oxidative cleavage of disulfide bonds. Other
385 researchers found that ITCs can react with amino acids and microbial proteins forming
386 reactive thiocyanate radicals (Cejpek et al. 2000; Delaquis and Mazza 1995; Luciano et
387 al. 2008; Verma 2003).

388 The tested ITCs, in particular PEITC, had the ability to change bacterial
389 hydrophobicity of the bacteria used in this study. The differences verified relative to the
390 chemical properties and biological activity among ITCs are generally dependent on the
391 chemical structure and on the bacteria tested (Aires et al. 2009b; Borges et al. 2014a; Kim
392 and Lee 2009). The smallest effect detected for AITC can be explained by its less
393 chemical reactivity comparatively to PEITC, which have electron donating benzene rings
394 that increase the reactivity of their –N=C=S groups. Also, AITC has a higher water
395 solubility and higher volatility (Saavedra et al. 2010). It was also verified that ITCs
396 changed the polar, apolar and the electron acceptor (γ^+) components of the bacterial cells.
397 The electron acceptor ability, after exposure to AITC and PEITC, increased for *E. coli*

398 and *L. monocytogenes* and decreased for *P. aeruginosa* and *S. aureus*. This result
399 demonstrates that AITC and PEITC are products with electrophilic potential that appears
400 to interact significantly with the bacterial surface components, modifying its
401 physicochemical properties. So, it is possible to hypothesize that the alteration of
402 hydrophobicity of bacterial membranes, after exposure to ITCs, can lead to perturbation
403 of the amphiphilic nature of lipid bilayer and eventually affect the integrity of
404 cytoplasmatic membrane of Gram-positive bacteria. Given that the hydrophobicity of
405 Gram-negative bacteria was also changed, these compounds may also have affected the
406 hydrophobic character of lipopolysaccharides (LPS) of their outer membrane in addition
407 to cytoplasmatic membrane. Consequently, this can lead to inactivation and/or dead of
408 both Gram-negative and -positive bacteria. Moreover, ITCs are well known to bind to
409 the external proteins of cell membranes, and penetrate to the cell cytoplasm (Gómez De
410 Saravia and Gaylarde 1998; Troncoso et al. 2005). Some researchers have shown the
411 ability of AITC to cross the membrane and achieve the cytoplasm of prokaryotic (Ahn et
412 al. 2001) and eukaryotic cells (Tang and Zhang 2005). Therefore, this interaction can
413 cause growth inhibition and, consequently, the cell death.

414 The charge properties of the cell surfaces can play a vital role in the microbial
415 homeostasis and resistance to antimicrobial agents (Ferreira et al. 2011). Under
416 physiological conditions, bacterial cells have normally negative surface charge, due to
417 the presence of anionic groups (e.g. carboxyl and phosphate) in their membranes (Gilbert
418 et al. 1991; Lerebour et al. 2004; Palmer et al. 2007). However, the magnitude of the
419 charge varies from species to species and can be influenced by various conditions, namely
420 age of the culture, ionic strength and pH (Ahimou et al. 2002; Palmer et al. 2007). Zeta
421 potential measurements demonstrated that after ITCs exposure, the cells become less
422 negatively charged. This surface charge alteration was particularly verified for the Gram-

positive bacteria. The results of the alteration of electrostatic potential of membrane corroborate previous studies, where the Gram-negative bacteria were less sensitive than Gram-positive to various ITCs (Aires et al. 2009b; Jang et al. 2010; Saavedra et al. 2010). This can be attributed to the presence of an outer membrane, in addition to the cytoplasmic membrane in Gram-negative bacteria (Simões et al. 2008). In Gram-negative bacteria, the passage through the outer membrane is regulated by the presence of hydrophilic channels (porins) that usually exclude the entry of hydrophobic compounds such as ITCs. Moreover, the outer membrane of these bacteria lacks phosphoglycerides and, hence, lacks the effective channels for hydrophobic diffusion (Bos et al. 2007; Cohen 2011; Liu and Yang 2010). However, the results obtained with the zeta potential measurements are not correlated with the antimicrobial susceptibility tests. Both Gram-negative and Gram-positive bacteria had similar susceptibilities to AITC (aliphatic molecule) and PEITC (aromatic molecule). This result proposes once more that the presence of an outer membrane for the Gram-negative *E. coli* and *P. aeruginosa* was not relevant for antimicrobial resistance.

Cytoplasmic membrane permeabilization was observed based in the uptake of PI, a nucleic acid stain to which cell membrane is usually impermeable. The results obtained demonstrate that ITCs compromise the integrity of the cytoplasmatic membrane. The percentage of cells with damaged membranes can be correlated with ITCs concentration. It was also possible to verify that *L. monocytogenes* was the bacterium less susceptible to both ITCs, with the minor percentage of cells with damaged membrane. The exact mechanism of bacterial resistance to ITCs is not completely understood (Dufour et al. 2012; Tajima et al. 1998). Dufour et al. (2012) have proposed that the efficacy of the ITCs may depend on both the rate of spontaneous degradation of ITC-thiol conjugates and of

447 the detoxification mechanisms of the bacterial isolate. The addition of exogenous thiol
448 groups can also suppress the antimicrobial effect of ITC.

449 The cytoplasmatic membrane of bacteria acts as a barrier between cytoplasm and
450 extracellular medium. The internal ionic environment of prokaryotic and eukaryotic cells
451 is generally rich in potassium and, therefore, leakage of this ion has been used to monitor
452 the membranolytic events in bacteria. On the other hand, K^+ leakage is usually the
453 primary indicator of membrane damage in microorganisms (Lambert and Hammond
454 1973). According to Carson et al. (2002), the marked leakage of cytoplasmatic material
455 is considered indicative of gross and irreversible cytoplasmatic membrane damage. In
456 this work, significant release of K^+ was verified particularly for *S. aureus* and *L.*
457 *monocytogenes*. So, the antimicrobial effects promoted by ITCs can be related with their
458 ability to react with cytoplasmatic membrane. This result together with those related from
459 PI uptake, zeta potential and contact angles assessment demonstrate that AITC and
460 PEITC interacted with the surface of Gram-negative and -positive bacteria, promoting
461 membrane damage, release of intracellular content and the consequent cell death. This
462 effect was dependent on the bacterial species.

463 Considering the results obtained in this study, it seems that ITCs have antimicrobial
464 activity, targeting mainly the bacterial membranes. It is possible to hypothesize that the
465 antimicrobial activity of AITC and PEITC is associated with their interaction with cell
466 surface constitutes, especially proteins and other critical biological macromolecules
467 necessary for microbial growth and survival, forming a monolayer around the cell that
468 changes the electrostatic potential, hydrophobicity and so disturbs the membrane
469 integrity.

470 It has been estimated that as many as 30% of people in industrialized countries suffer
471 from a foodborne disease each year (Burt 2004). Hence, it is also important to refer that

ITCs are frequently used as safe natural preservatives in food industry due to their recognized antimicrobial activity against foodborne pathogens (Aires et al. 2009a; Delaquis and Mazza 1995; EFSA 2010). In addition, these products are promising food preservative candidates because they do not influence the organoleptic properties of processed food (Al-Gendy et al. 2010). This is in part due to their higher volatility (Saavedra et al. 2010; Sun et al. 2011). In a previously report, AITC was proposed as a potential industrial disinfectant, due to its relatively simple and economical synthesis, and also due to its rapid degradation in the environment (Gómez De Saravia and Gaylarde 1998). AITC is easily decomposed due to its electrophilic character. This relatively immediate aqueous degradation of AITC is an advantage when considering it as a disinfectant because it will not persist in the environment (Liu and Yang 2010; Mushantaf et al. 2012). Moreover, in a study 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).

For the design and development of effective antimicrobial strategies, it is crucial to understand the mechanisms of action of antimicrobial agents as well as the mechanisms of bacterial resistance. Phytochemical products can be a new attractive source of environmentally friendly antimicrobials. The present work showed that ITCs may have capacity to control the growth and proliferation of common foodborne microorganisms, with pathogenic potential. It is also important to conclude that the electrophilic nature of ITCs disrupt bacterial cell membranes and cause breakdown of the transmembrane potential with leakage of important cytoplasmatic constituents. AITC and PEITC are not promising molecules for clinical antimicrobial therapy due to their high cytotoxicity (Borges et al. 2014b). However, these products can be promising alternatives or

497 synergists/complements to synthetic antimicrobials for disinfection in the food industry.
498 Their green status can contribute to the reduction of the environmental and health risks
499 associated with the intensified use of synthetic antimicrobial chemicals (Heidler et al.
500 2006; Wu et al. 2010). At this moment, additional studies are required to validate their
501 disinfectant potential, particularly the tests with adhered cells using standard protocols
502 (EN 13697, 2001). In fact, AITC and PEITC already demonstrated a significant potential
503 to prevent and control biofilm formation on polystyrene surfaces (Borges et al. 2014a).
504

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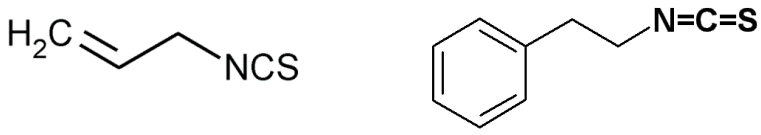
725

726 **Figures and tables**

727



a b

728

729 **Fig. 1** Chemical structures of allylisothiocyanate (a) and 2-phenylethylisothiocyanate (b)

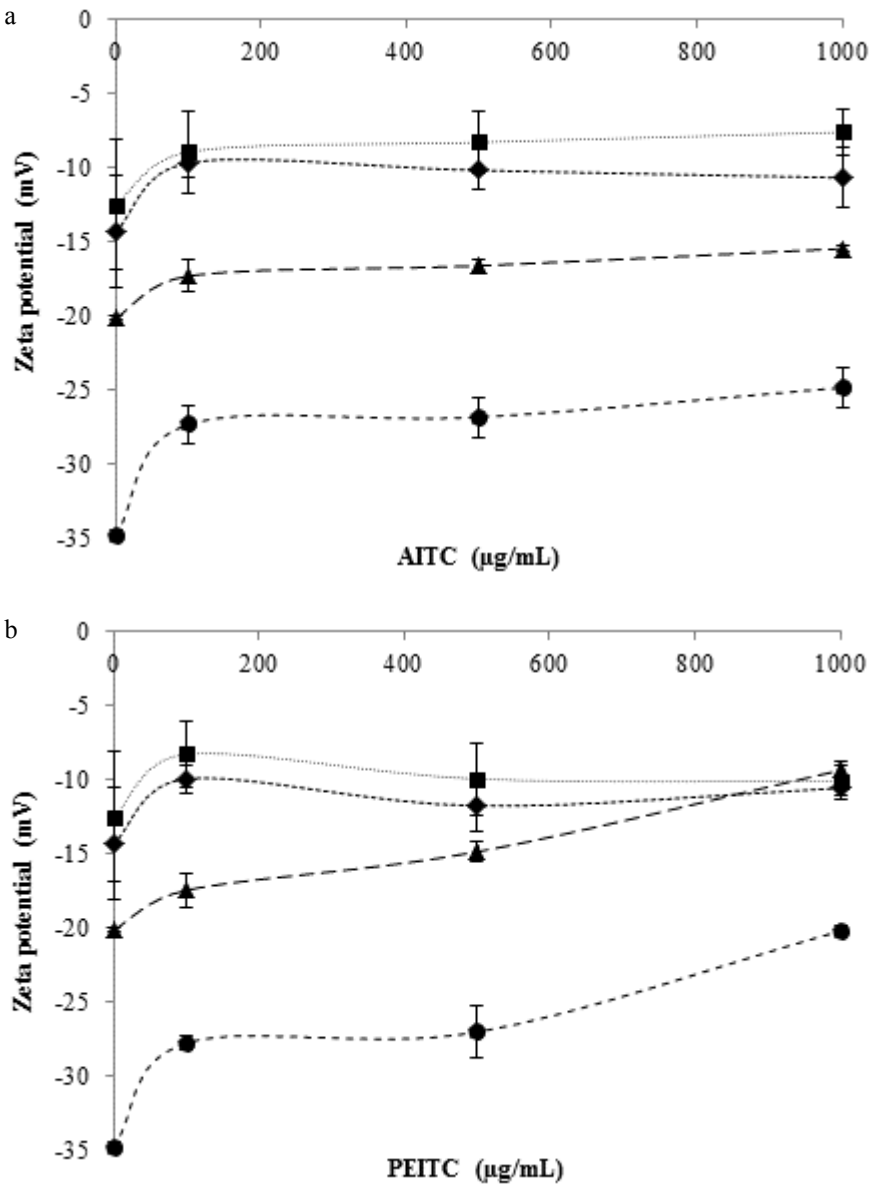


Fig. 2 Zeta potential values (mV) of suspensions of *E. coli* (♦), *P. aeruginosa* (■), *S. aureus* (▲) and *L. monocytogenes* (●) when exposed to different concentrations (0, 100, 500 and 1000 µg/mL) of AITC (a) and PEITC (b) for 30 min. The means ± SD for at least three replicates are illustrated

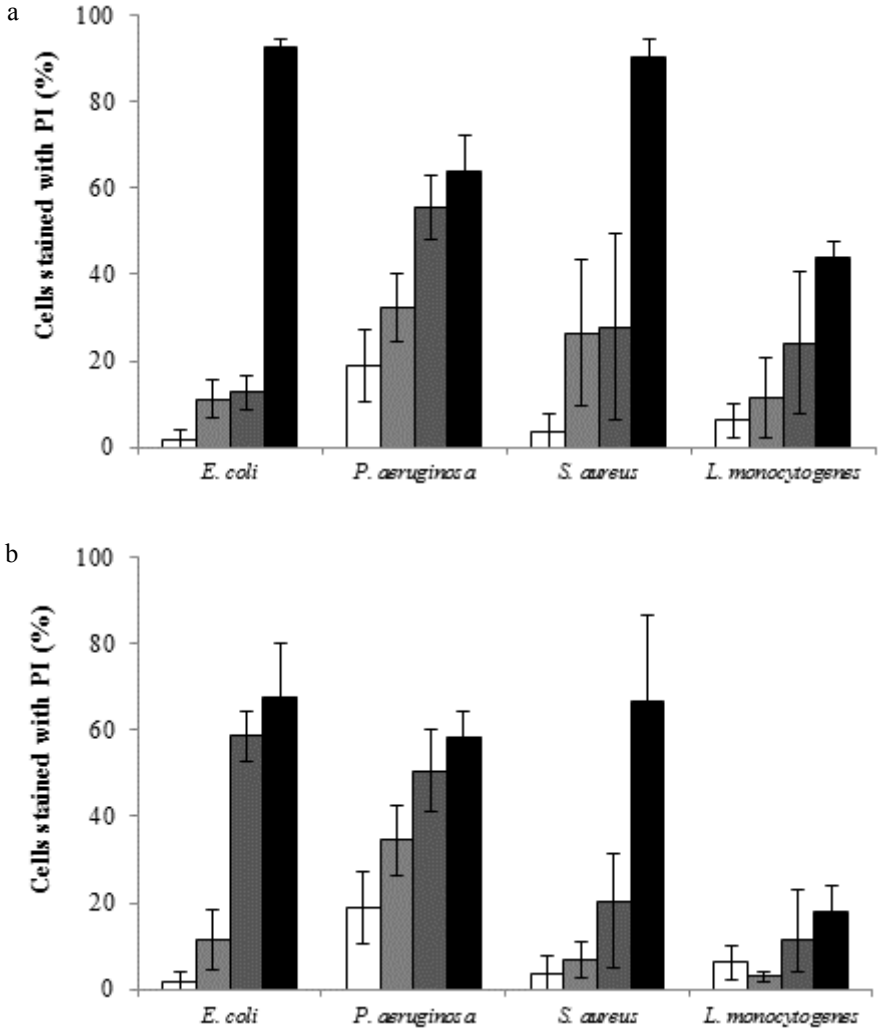


Fig. 3 Permeability of *E. coli*, *P. aeruginosa*, *S. aureus* and *L. monocytogenes* to PI after treatment with AITC (a) and PEITC (b) at different concentrations, 0 (□), 100 (■) , 500 (■) and 1000 (■) µg/mL for 30 min. The percentage of cells non-stained with PI corresponds to the fraction of viable cells. The means ± SD for at least three replicates are illustrated

777 **Table 1** MIC and MBC of AITC and PEITC for *E. coli*, *P. aeruginosa*, *S. aureus* and *L.*

778 *monocytogenes*

	MIC (μg/mL)		MBC (μg/mL)	
	AITC	PEITC	AITC	PEITC
E. coli	100	100	1000	> 1000
P. aeruginosa	100	100	1000	> 1000
S. aureus	100	100	> 1000	> 1000
L. monocytogenes	100	100	> 1000	> 1000

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780 **Table 2** Hydrophobicity (ΔG_{sws}^{TOT}), apolar (γ^{LW}) and polar (γ^{AB}) components of the surface

781 tension of untreated and ITCs-treated bacteria[a]

			Surface tension parameters (mJ/m^2)				ΔG^{TOT} (mJ/m^2)[b]
		[Phytochemical; μg/mL]	γ^{LW}	γ^{AB}	γ^{+}	γ^{-}	
E. coli	Control	0	36.4±1.2	18.6±0.3	1.6±1.2	54.3±0.8	31.3±0.5
	AITC	100	33.8±0.9	21.2±0.5	2.02±0.4	55.2±1.7	31.8±0.9
		500	33.7±0.8	21.5±1.1	2.13±0.7	54.4±0.4	30.9±0.2
		1000	29.9±0.3	25.8±1.5	3.12±1.1	53.4±1.6	28.3±0.9
	PEITC	100	35.1±1.3	20.1±1.5	1.86±0.2	54.3±0.5	31.0±0.3
		500	29.2±0.4	12.0±0.7	0.71±1.0	50.5±0.9	33.5±.1.1
		1000	25.2±0.9	14.1±0.5	1.19±0.2	41.6±0.4	21.9±0.9
P. aeruginosa	Control	0	13.6±0.7	45.2±0.7	10.36±0.3	49.2±0.7	12.5±1.7
	AITC	100	31.0±0.3	16.4±0.2	1.20±1.5	55.9±0.5	36.7±1.4
		500	28.0±0.7	24.3±0.8	2.72±0.7	54.5±0.8	30.9±0.4
		1000	28.2±1.3	25.1±0.6	3.07±0.8	51.4±0.2	27.1±0.9
	PEITC	100	31.2±1.2	0.0±0.0	0.0±0.0	68.6±1.3	63.6±1.6
		500	32.6±0.5	0.0±0.0	0.0±0.0	70.5±0.7	65.4±0.8
		1000	33.6±0.8	0.0±0.0	0.0±0.0	67.9±1.4	61.9±0.4
S. aureus	Control	0	29.1±1.6	24.2±1.9	3.16±0.9	46.4±1.0	22.1±0.7
	AITC	100	33.7±0.3	19.1±1.3	1.87±0.2	48.4±0.3	25.5±0.2
		500	34.4±0.5	18.3±1.0	1.73±1.1	48.0±0.5	25.2±0.6
		1000	35.1±1.0	16.4±0.7	1.35±0.5	49.8±0.8	28.0±1.3
	PEITC	100	38.0±1.2	14.0±0.7	1.0±1.3	49.0±0.9	27.0±0.5
		500	33.1±1.1	19.0±0.5	1.88±0.4	47.8±0.4	25.1±1.0
		1000	32.7±0.9	19.6±0.3	1.93±0.6	49.5±1.4	26.9±1.3
L. monocytogenes	Control	0	34.5±0.9	0.0±1.4	0.0±0.1	61.9±0.9	54.0±1.0
	AITC	100	25.5±0.6	0.0±0.5	0.0±0.7	70.0±0.1	66.8±0.6
		500	33.9±0.8	9.27±0.9	0.94±0.5	22.7±1.7	-7.32±1.9
		1000	32.0±0.2	12.2±0.1	1.15±1.3	32.1±0.3	7.89±0.3
	PEITC	100	25.6±1.2	11.5±1.3	0.65±0.3	50.9±1.4	35.0±1.2
		500	22.9±0.7	7.74±0.5	0.65±0.6	22.8±0.8	-4.7±1.9
		1000	26.8±1.0	4.22±0.8	0.71±0.5	6.23±1.1	-43.5±1.7

782 [a]The means ± SD for at least three replicates are given.

783 [b]$\Delta G^{TOT} > 0$ mJ/m^2 – Hydrophilic; $\Delta G^{TOT} < 0$ mJ/m^2 – Hydrophobic.

784 **Table 3** K^+ concentration (μg/mL) in the solution after contact of *E. coli*, *P. aeruginosa*,

785 *S. aureus* and *L. monocytogenes* with AITC and PEITC at 1000 μg/mL[a]

	K^+ in solution (μg/mL)			
	E. coli	*P. aeruginosa*	*S. aureus*	*L. monocytogenes*
Control	0.30 ± 0.0	0.61 ± 0.0	0.78 ± 0.01	0.99 ± 0.0
AITC	0.64 ± 0.0	0.56 ± 0.0	1.14 ± 0.0	1.41 ± 0.02
PEITC	0.45 ± 0.0	0.61 ± 0.0	0.92 ± 0.0	1.26 ± 0.0

786 [a]The means ± SD for at least three replicates are illustrated.