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Parietal structures of *Escherichia coli* can impact the D-cateslytin antibacterial activity

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17 **Abstract**

18 Bacterial resistance to conventional antibiotics is of major concern. Antimicrobial peptides
19 (AMPs) are considered as excellent alternatives. Among them, D-cateslytin (D-Ctl, derivative
20 of a host defense peptide), has shown high efficiency against a broad spectrum of bacteria.
21 The first target of AMPs is the outer membrane of the bacterium. However, the role of
22 bacterial cell-wall structures on D-Ctl mechanism of action has not yet been understood. In
23 this study, we investigated the activity of D-Ctl on two isogenic strains of *E. coli*: one is nude
24 of any parietal structures, the other constitutively overexpresses only Type 1 *fimbriae*. We
25 studied the damages caused by D-Ctl at several initial concentrations of bacteria and D-Ctl,
26 and times of exposure to D-Ctl were examined using a combination of epifluorescence
27 microscopy, atomic force microscopy (AFM), and Fourier transform infrared spectroscopy in
28 attenuated total reflectance mode (ATR-FTIR). The analysis of nanomechanical and
29 spectrochemical properties related to the antibacterial mechanism showed a concentration
30 dependent activity. Whereas the membrane permeabilization was evidenced for all
31 concentrations of D-Ctl and both mutants, no pore formation was observed. The bacterial
32 stiffness is modified dramatically concomitantly to major membrane damages and changes in
33 the spectral fingerprints of the bacteria. In case of the occurrence of Type 1 *fimbriae* only, an
34 intracellular activity was additionally detected. Our results evidenced that D-Ctl activity is
35 highly impacted by the cell-wall external structures and surface properties of the bacteria.

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37

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39 **Keywords:** parietal structures, antimicrobial peptide, D-cateslytin, mechanism of action,
40 AFM, epifluorescence microscopy, infrared spectroscopy, *Escherichia coli*

41

42 1. Introduction

43 Bacterial resistance to conventional antibiotics has been increasing at an alarming rate during
44 the last two decades, affecting dramatically our ability to treat common infections.¹ In
45 addition, insufficient investment in antimicrobial therapies resulted in a lack of the discovery
46 of new families of active molecules.¹⁻³ This has led the World Health Organization (WHO) to
47 raise the alarm against antimicrobial resistance and especially the resurgence of multi-drug
48 resistant bacteria. It is particularly true for Gram-negative bacteria.³⁻⁴ The urgent need to
49 develop new molecules as alternatives to conventional antibiotics has led to consider to use
50 peptides with antimicrobial properties.

51 Antimicrobial peptides (AMPs) have a minimum inhibitory concentration (MIC) often in the
52 range of a few μM . Despite a strong antimicrobial activity at low concentrations, their mode
53 of action in killing bacteria or in inhibiting bacterial growth remains not fully understood.⁵⁻⁶
54 Most AMPs are believed to interact with the membranes where they induce permeabilization.
55 The subsequent lysis or damage to bacteria is then observed.⁷⁻⁸ It is generally accepted that
56 AMPs can form transmembrane channels by self-aggregation or multimerization,⁹⁻¹⁰ leading
57 to membrane permeabilization and bacterial death.⁷ The AMP activity on the membrane can
58 be influenced by the parietal structures of the bacteria such as lipopolysaccharides or
59 lipoteichoic acids and the presence of *pili* and other appendages.¹¹⁻¹² Indeed, previous works
60 highlighted that external cell wall structures of bacteria act as permeability barriers allowing
61 selective passage of nutrients and exclusion of harmful substances (*e.g.* antimicrobial
62 agents).¹³⁻¹⁴ Also some non-lytic intracellular modes of action have been reported.¹⁵⁻¹⁶ Some
63 antimicrobial peptides can modify and/or inhibit bacterial metabolism, *i.e.* nucleic acids and
64 protein biosynthesis, cell division, cell wall and lipopolysaccharide biosynthesis.^{15, 17-18}
65 Among the AMPs, host defense peptides (HDPs) are emerging as excellent candidates for the
66 development of new antibiotic strategies. In addition to their antimicrobial action, they exhibit

67 less side effects than other AMPs.¹⁹⁻²¹ They are able to kill a broad spectrum of
68 microorganisms with a very low cytotoxicity to mammalian cells. Owing to a general non-
69 specific action, they have a low level of induced resistance.^{12, 21} Moreover, some of them have
70 a very interesting therapeutic potential because they can activate the immune system while
71 inhibiting the inflammatory response.²²⁻²³ Peptides derived from the proteolysis of
72 chromogranin A are of particular therapeutic interest, and particularly cateslytin.²⁴⁻²⁵ This
73 fragment of chromogranin A is a linear HDP of 15 residues, and therefore very easy to
74 synthesize for a minimal cost.²⁴⁻²⁵ Previous studies demonstrated the high efficiency of D-
75 cateslytin (D-Ctl) against a broad spectrum of bacteria including *E. coli*.²⁴ It is accepted that
76 the first target of the AMP is the outer membrane of the bacterium. However, no particular
77 attention was paid to the role of bacterial cell-wall structures on D-Ctl efficiency or possible
78 modification of its mode of action from one bacterial strain to another one.

79 In the present study, we investigated the action of D-Ctl on two strains of *E. coli*: one is nude
80 of any parietal structures, the other overexpresses only Type 1 *fimbriae*.²⁶ Type 1 *fimbriae* are
81 uniformly distributed on the bacterial surface, and they are known for their surface adherence
82 on epithelial cells. The bacterial attachment allowed by these Type 1 *fimbriae* is a major
83 factor in bacterial pathogenicity.²⁷⁻²⁹ The action and the possible influence of these parietal
84 structures were analyzed with a physico-chemical point of view through morphological,
85 nanomechanical, and spectroscopic analyses. This work was performed with a combination of
86 epifluorescence microscopy, atomic force microscopy (AFM), and Fourier transform infrared
87 spectroscopy in Attenuated total reflectance mode (ATR-FTIR).³⁰⁻³¹ These techniques were
88 combined to monitor *in situ* the mechanism of action and the damages caused by D-Ctl at
89 several initial concentrations of bacteria and times of exposure. Indeed, beyond the
90 determination of MIC that gives information on the inhibition of growth, it is important to
91 investigate what can happen when a high amount of bacteria is initially present. ATR-FTIR

92 allows the investigation of changes in biochemical compounds of *E coli*, whereas AFM was
93 used to visualize the local effects of the antimicrobial peptide upon cell morphology and
94 mechanical properties. In addition, the bacterial membrane integrity was evaluated by assays
95 with the *BacLight*TM kit. Our investigations allow correlation between D-Ctl activity on both
96 nanomechanical properties and bacterial metabolism depending on the antimicrobial agent
97 concentration and the presence of cell-wall external structures.

98

99 **2. Materials and Methods**

100**2.1. Chemicals and synthetic peptide**

101 Ampicillin, kanamycin and phosphate saline buffer (PBS) were purchased from Sigma-
102 Aldrich, France. D-cateslytin is a positively charged (5+) arginine-rich peptide with the
103 following amino acid sequence: RSMRLSFRARGYGFRGPG. It was purchased from
104 ProteoGenix (Schiltigheim, France, purity > 95%). The peptides were stored at -20°C as a
105 stock solution at 1 g/L in non-pyrogenic sterile water (Aqua B-Braun, Melsungen, Germany).
106 Structures and physicochemical properties of cateslytin are reported in **Figure S1** (see
107 Supporting Information).

108

109**2.2. Bacterial strain and culture conditions**

110 The bacterial models used in this study are Gram-negative *Escherichia coli* mutants called
111 E2152 and E2146 kindly provided by Institut Pasteur from Paris.²⁶ These isogenic strains
112 were constructed from *Escherichia coli* MG1655. These mutants are non-flagellated and are
113 resistant to specific antibiotics (ampicillin, kanamycin, chloramphenicol and zeocin). Strain
114 E2152 is devoided from the main *E. coli* parietal structures while strain E2146 constitutively
115 expresses only the external Type 1 *fimbriae*. E2152 strain was selected as a reference.²⁶
116 Bacterial stocks were maintained at -80°C. Bacteria were grown in Lysogeny Broth (LB,

117 Miller, Fluka) at 25 g/L in deionized water (Purelab Option, ELGA). All cultures were grown
118 in a water bath at $37 \pm 1^\circ\text{C}$, and under continuous agitation at 160 rpm. After an overnight
119 sub-culture (16 hours, with antibiotics, *i.e.* ampicillin and kanamycin), bacteria were
120 cultivated in 25 mL of LB medium (without antibiotics) in 150 mL Erlenmeyer flasks with an
121 initial optical density at 600 nm of 0.05 ± 0.01 . The bacteria were taken at the end of the
122 exponential phase when OD_{600} reaches the value of 0.50 ± 0.02 .

123

124 **2.3. Growth curves and minimum inhibitory concentration (MIC) estimation**

125 The growth curves were obtained by measuring the optical density at 600 nm (OD_{600}) with a
126 Tecan Infinite M200 spectrometer during 24 hours on bacterial suspensions (volume 200 μL)
127 put in a 96-well plate (Nunc) in the presence or not of D-Ctl in LB medium. The bacterial
128 suspension was used either at OD_{600} value of 0.50 or diluted to values at 0.10 and 0.001
129 corresponding to $3.18 \pm 0.27 \times 10^8$, $1.05 \pm 0.18 \times 10^8$ and $2.64 \pm 0.33 \times 10^6$ CFU/mL,
130 respectively. The measurements were performed at least in triplicate. The MIC of strain
131 E2152 was estimated in LB medium by the two-fold serial dilution assay as described
132 elsewhere.²⁴ Briefly, the bacterial suspension was diluted to $\text{OD}_{600} = 0.001$ in a sterile 96-well
133 plate (Nunc, suspension volume 200 μL). After 24 h of incubation at 37°C without or with D-
134 Ctl at different concentrations, the bacterial growth was assessed by the measurement of
135 OD_{600} using a Tecan Infinite M200.

136

137 **2.4. Suspensions for epifluorescence microscopy, infrared spectroscopy and AFM**

138 For epifluorescence and infrared spectroscopy analyses, the antimicrobial assays against
139 planktonic *E. coli* were performed in duplicate in sterile 96-well plates (Nunc) in a final
140 volume of 200 μL . When the optical density of the bacterial culture reached an OD_{600} value
141 of 0.50 ± 0.02 , the suspension was diluted in LB to give an $\text{OD}_{600} = 0.10 \pm 0.01$. The

142 necessary volumes of the stock solution of the peptide at 1 g/L was spotted in the bacterial
143 suspension to a final volume of 200 μ L. Sterility and growth controls were sterile LB, and a
144 bacterial suspension without peptide, respectively. The plate was incubated for 1, 4 and 14
145 hours at $23 \pm 1^\circ\text{C}$ in an air conditioned room.

146 For AFM *in-situ* experiments, the required volume of D-Ctl mother solution at 537 μM (1
147 g/L) was added to 1 mL of bacterial suspensions with an OD_{600} of 0.50 ± 0.02 to reach final
148 concentrations of 0, 8, 40 and 80 $\mu\text{g/mL}$. Then suspensions were deposited into a 24-well
149 plates (Nunc) containing gold disks coated with amino-terminated thiol ($\text{HS-C}_{11}\text{-NH}_3\text{Cl}$)
150 purchased from Prochimia (Prochimia surfaces, Poland). Samples were incubated at $22 \pm 1^\circ\text{C}$
151 for 1, 4 and 14 hours exposure times.

152

152.5. Fluorescence optical microscopy and membrane status assays

154 The bacteria shape and the integrity of the bacterial membrane were assessed using the
155 *BacLight*TM stain kit (L7012, Molecular Probes, Eugene, USA). The cell-wall permeability in
156 the absence and presence of the D-Ctl was examined according to a methodology described in
157 previous works.^{24, 31} With this kit, bacteria with intact membranes exhibit green fluorescence
158 (Syto 9), while bacteria with damaged membranes show red fluorescence (propidium iodide,
159 PI). The bacterial suspensions were stained with the *BacLight*TM kit during 20 minutes in the
160 dark. Then the suspension was filtered onto a 0.22 μm membrane, and the membrane was
161 rinsed with non-pyrogenic sterile water to eliminate excess dyes. The sample was mounted in
162 *BacLight*TM mounting oil as described by the instructions provided by the manufacturer.
163 Images were acquired with the $\times 100$ oil immersion objective of an Olympus BX51
164 microscope equipped with an Olympus XC50 camera. Both fluorescences were observed
165 using fluorescence filter cube U-MWIB3 (Olympus, excitation filter: BP 460-495 nm,
166 emission filter: LP 510 nm).

167

168 **2.6. ATR-FTIR spectroscopy**

169 ATR-FTIR spectra were recorded on a Bruker Vertex70v spectrometer equipped with a KBr
170 beam splitter and a DTGS detector. Spectra recording and data processing were performed
171 using the Bruker OPUS 7.5 software. The resolution of the single beam spectra was 4 cm^{-1} .
172 Two hundred scans were collected per spectrum corresponding to a 2-minutes accumulation
173 time. All interferograms were Fourier processed using the Mertz phase correction and a
174 Blackman-Harris three-term apodization function. A nine-reflection diamond ATR accessory
175 (DurasamplIR™, SensIR Technologies, incidence angle: 45°) was used for acquiring spectra.
176 No ATR correction was performed. ATR-FTIR spectra are shown with an absorbance scale
177 corresponding to $\log(R_{\text{reference}}/R_{\text{sample}})$, where R is the internal reflectance of the device.
178 Measurements were performed at $22 \pm 1^\circ\text{C}$ in an air-conditioned room. One drop of the
179 bacterial suspension in LB medium was put on the ATR crystal. The spectral background was
180 removed by recording the spectrum of the LB medium free of bacteria (obtained by
181 centrifugation at 7000 g during 5 minutes). Water vapor subtraction was performed when
182 necessary. All spectra were baseline corrected at 1800 and 900 cm^{-1} . FTIR measurements
183 were performed at $22 \pm 1^\circ\text{C}$ in an air-conditioned room.

184

185 **2.7. Bacteria drying procedure for AFM imaging**

186 Critical point drying (CPD) with CO_2 was used for sample preparation in order to get images
187 at the nanoscale of the bacterial cells subjected or not to D-Ctl at various concentrations and
188 for several exposure times. Indeed, this technique is one of the most accurate to preserve the
189 highly fragile three-dimensional structure of biological samples especially when they are
190 difficult to observe in aqueous media.³²⁻³⁴ Gold slides incubated with bacterial samples were
191 rinsed three times in baths of PBS for 5 minutes each. Then samples were dehydrated by

192 immersion into 8 successive baths of water/ethanol mixtures (ratios of 90/10, 80/20, 70/30,
193 50/50, 30/70, 20/80, 10/90, 0/100) for 10 minutes each and stored in pure ethanol before CPD
194 treatment. The CPD procedure is carried out using a homemade device³⁵, and according to 5
195 successive steps: (1) samples are placed into an autoclave with 250 μL of pure ethanol onto
196 each samples; (2) the autoclave is filled and pressurized with liquid CO_2 for 30 minutes up to
197 90 bars at 30°C ; (3) the autoclave is heated for 20 minutes up to 45°C ; (4) water and ethanol
198 residues were extracted from the samples through the supercritical CO_2 (5) depressurization
199 and decrease of temperature from 45°C to the ambient conditions during 30 minutes. At the
200 end of these steps, the samples were taken out of the autoclave and the samples were stored in
201 petri dishes tightly closed to prevent absorption of water in the samples.

202

202.8. Atomic Force Microscopy characterizations

204 Morphology images (dried samples) and *in-situ* monitoring of bacterial cells mechanical
205 properties (living bacteria) were performed using the PeakForce tappingTM mode, with a
206 Bioscope Resolve (Bruker Nano Surface, Bruker France SAS, Palaiseau, France). Silicon
207 nitride cantilevers with conical tips were purchased from Bruker (NPG-10, Bruker France
208 SAS, Palaiseau, France) with a spring constant of about 0.35 nN/nm. The applied force
209 between the tip and the samples surface was carefully controlled and minimized at ~ 0.250
210 nN. All images were acquired with a resolution of 512 pixels by 512 pixels and a scan rate of
211 1.0 Hz. AFM images with scan sizes of $10\ \mu\text{m} \times 10\ \mu\text{m}$ and $5\ \mu\text{m} \times 5\ \mu\text{m}$ were then analyzed
212 with Nanoscope Analysis for extracting roughness, thickness, and general morphology.

213 Mechanical properties of bacterial cells were investigated in LB medium using a MFP3D-BIO
214 instrument (Asylum Research Technology, Oxford Instrument, Manheim, Germany) using
215 silicon nitride cantilevers with conical tips (OMCL-TR400PSA-3, Olympus, Japan) with a
216 spring constant of about 10-15 pN/nm. The bacterial stiffness was calculated by analyzing the

217 force-indentation curves according to the Sneddon model.³⁶⁻³⁷ In this model, the Young
218 modulus is related to the applied force according to the equation given below:

$$219 \quad F = \frac{2E \cdot \tan(\alpha)}{\pi(1-\nu^2)} R^{1/2} \delta^2 \cdot f_{\text{BECC}} \quad (1)$$

220 where δ is the indentation depth, ν the Poisson coefficient, α the semi-top angle of the tip and
221 f_{BECC} is the bottom effect cone correction function that take into account the presence of the
222 substrate stiffness.³⁷ All mechanical measurements were determined using an automatic
223 Matlab algorithm described elsewhere,³⁸ and the average values given in this work were
224 calculated from at least 2000 force curves.

225

226 **2.9. Bacterial trapping and monitoring by fast scanning microscopy**

227 Bacterial suspensions were injected through a syringe to a homemade nanofluidic chip
228 containing bacterial traps³⁹ and directly mounted on a custom integration of a Dimension Fast
229 Scan AFM on an inverted optical microscope as described elsewhere.⁴⁰ By creating a pressure
230 difference across the nanofluidic traps, the bacteria were attracted and physically immobilized
231 into the traps for further AFM analyses. Morphology as well as mechanical properties of
232 immobilized bacteria were obtained in LB medium using PeakForce QNM® mode using a
233 ScanAsyst®-Fluid cantilever with $1.6 \text{ nN}\cdot\text{nm}^{-1}$ spring constant. During the bacterial
234 monitoring procedure, the pressure in the microfluidic chamber underneath the membrane
235 was kept between 20 and 100 mbar below atmospheric pressure.

236

237 **3. Results and Discussion**

238 **3.1. Effect of D-Ctl on the bacterial growth of *E. coli* strains**

239 The minimal inhibitory concentration (MIC) was determined at $8 \mu\text{g}/\text{mL}$ for the E2146
240 strain.²⁴ The MIC of E2152 was estimated in the same range, *i.e.* between 5 and $10 \mu\text{g}/\text{mL}$ in

241 this work. Hereafter, MIC refers to the minimal inhibitory concentration for E2146. The
242 growth of both bacteria at different initial OD₆₀₀ was monitored by measuring the OD₆₀₀ as a
243 function of time for 24 hours (**Figure 1**). In absence of D-Ctl the middle of exponential
244 growth phase is reached after 13-14, 9 and 9 hours for both strains at initial OD₆₀₀ set at
245 0.001, 0.1 and 0.5, respectively. The stationary growth phase begins after 24 hours for an
246 initial OD₆₀₀ set at 0.001, and it was not reached for suspensions with initial OD₆₀₀ set at 0.1
247 and 0.5, only pseudo-plateaus are observed. The growth curves of bacteria incubated for 24
248 hours with D-Ctl at the MIC show several features as a function of the initial concentration of
249 bacteria (**Figure 1**). At initial OD₆₀₀ set at 0.001, no growth of E2152 was recorded during 23
250 hours, whereas a significant bacterial growth started after 10 hours for strain E2146. At initial
251 OD₆₀₀ set at 0.01, bacteria grew during ~ 6 hours and then a slightly descending plateau was
252 reached, suggesting the stop of the multiplying process. When the initial number of bacteria
253 was higher, *i.e.* DO₆₀₀ = 0.50, the OD₆₀₀ after 24 hours of culture were below those of control
254 cultures. The bacterial growth continued at a slower rate than in control culture for strain
255 E2152. In presence of D-Ctl at 5×MIC and 10×MIC, no significant bacterial growth was
256 measured during 24 hours whatever the initial concentration of bacteria. These results
257 demonstrated the existence of a concentration threshold from which D-Ctl totally inhibits the
258 bacterial growth for a defined number of cells. Besides, the inhibitory and even the
259 bactericidal activity of D-Ctl also depend on the initial number of bacterial cells exposed. To
260 sum-up, these results strongly suggested that E2146 seems to tolerate D-Ctl better than 2152.
261

263.2. Bacterial membrane status and morphology under D-Ctl treatment

263 E2152 and E2146 bacteria were stained with the *BacLight*[™] kit at the D-Ctl concentrations
264 and exposure times previously described. **Figure 2** and **Figure 3** show representative images
265 for E2152 and E2146, respectively. Except for few of them, the bacteria of both strains grown

266 without D-Ctl were green showing rod-shaped bacteria with non-damaged membranes. The
 267 average lengths were estimated and are gathered in **Table 1**. Both bacteria strains have the
 268 same range of average length, *i.e.* ~2.3 to ~2.7 μm whatever the time of culture. Many
 269 bacteria were dividing, and here we did not discriminate bacteria just divided (length ~1.5
 270 μm) from those that were dividing (lengths from ~2.0 to ~3 μm).

271

272 **Table 1:** Average length (μm) of bacteria in presence or not to antimicrobial peptide D-Ctl
 273 (red: average length for red bacteria in the images; green: average length for green bacteria in
 274 the images).

	Time	Without D-Ctl	MIC	5×MIC	10×MIC
E2152	1 hr	2.7 ± 0.6	2.9 ± 0.5	2.3 ± 0.3	2.6 ± 0.4
	4 hrs	2.7 ± 0.6	3.6 ± 1.0 (red) 2.6 ± 0.4 (green)	2.9 ± 0.8	2.5 ± 0.4
	14 hrs	2.6 ± 0.4	3.3 ± 0.8 (red) 2.6 ± 0.5 (green)	3.2 ± 0.5	-
E2146	1 hr	2.3 ± 0.4	2.7 ± 0.2	2.9 ± 0.4	2.6 ± 0.4
	4 hrs	2.4 ± 0.4	2.9 ± 0.7 (red) 2.3 ± 0.3 (green)	2.6 ± 0.4	2.6 ± 0.3
	14 hrs	2.3 ± 0.4	4.1 ± 2.7 (red) 3.1 ± 1.7 (green)	2.6 ± 0.4	2.6 ± 0.4

275

276 When *E. coli* E2152 was exposed to D-Ctl at the MIC, almost all bacteria appeared red, and
 277 their average size slightly increased at all the contact times. Only a few of them were green
 278 showing intact membranes (**Figures 2b, f, j**), and their shape and size did not change
 279 significantly with respect to bacteria not exposed to D-Ctl (control experiment). The average
 280 size was increased after 4 and 14 hours of exposure to D-Ctl at the MIC for red bacteria. The
 281 higher standard deviation for length measurements showed a non-homogenous behavior of the
 282 bacteria in front of D-Ctl (**Table 1**). The occurrence of a few filamentous cells can be noticed,
 283 and they were up to ~ 25 μm long after 14 hours of contact with D-Ctl. In addition to the
 284 increase of the permeability to PI, some bacteria were highly stressed showing it by the

285 inhibition of the division of the cells. For concentrations of D-Ctl at 5×MIC and 10×MIC, all
286 bacteria were red, showing damaged membranes. At 10×MIC and after 14 hours of contact,
287 no bacteria were observed. This result suggested that almost all the bacteria were lysed by the
288 action of D-Ctl or they were too much damaged to resist to the mechanical filtration. For
289 5×MIC and 10×MIC of D-Ctl, few bacteria become filamentous with lengths up to 18 µm, the
290 average length of the remaining bacteria were close to those in absence of the peptide.

291 When *E. coli* E2146 was submitted to D-Ctl at the MIC, most of bacteria were red, but a non-
292 negligible number of bacteria remained green meaning that their membrane was not damaged
293 (**Figure 3b, f, j**). The average shape and length of the green bacteria were close to each other
294 and close to those observed for the bacteria in the control experiment whatever the time of
295 exposure to D-Ctl. After 4 and 14 hours of contact with D-Ctl, red bacteria (*i.e.* with damaged
296 membranes) had a higher average length with a high distribution of lengths, especially after
297 14 hours of contact with D-Ctl (4.1 ± 2.7 µm, **Table 1, Figure 3f, j**). A few bacteria
298 developed long filamentous shapes, their length reached up to ~ 9 µm after 4 h and 24 µm
299 after 14 hours of contact with D-Ctl indicating that the stress induced by the presence of D-Ctl
300 was very high. Bacteria submitted to D-Ctl at 5×MIC and 10×MIC were almost all red, only a
301 few of them were observed green for an exposure of 4 hours at 5×MIC. The average length
302 increased slightly (less than 0.6 µm). In contrast of all the other conditions of D-Ctl exposure,
303 the PI staining of E2146 at D-Ctl concentrations of $5 \times \text{MIC}$ and $10 \times \text{MIC}$ was not
304 homogenous along the cell (**Figure 3c,d,g,h,k,l**). The fluorescence was highly concentrated at
305 specific areas of the bacteria making to appear bright orange-red dots inside the bacteria.
306 Propidium iodide stains both RNA and DNA. This non-homogenous staining through the
307 whole bacteria implies that nucleic acids were probably aggregated upon the presence of D-
308 Ctl.

309 A closer look on the bacterial morphology was performed using AFM to decipher possible
310 cell wall damages at the nanoscale. Several areas of 10×10 and $5 \times 5 \mu\text{m}^2$ were investigated
311 and representative images were collected, and they are reported in **Figure 4** and **Figure 5**. In
312 absence of D-Ctl, E2152 exhibits a smooth and rod-shaped structure with an average length of
313 about $1.6 \pm 0.4 \mu\text{m}$ for a width of $0.8 \pm 0.1 \mu\text{m}$, which are in accordance with epifluorescence
314 images. The height profiles (**Figures S2 – S9**) showed bacterial diameter of *c.a.* 650 nm.
315 When E2152 cells are exposed to D-Ctl, the rod-shaped structure is maintained but the cell
316 size was increased. Indeed, the average length and width are increased up to $2.3 \pm 0.6 \mu\text{m}$ and
317 $1.0 \pm 0.2 \mu\text{m}$, respectively. Such increase in size is also confirmed by the height profiles that
318 evidenced bacterial diameters reaching from 650 nm up to 750 nm (**Figure 6a**). D-Ctl activity
319 seems to inflate bacterial cells but no major damages as pores or important roughness have
320 been observed with AFM even though the *BacLight*TM assays evidenced that almost all
321 bacteria exposed to D-Ctl exhibited damaged membranes.

322 In absence of D-Ctl, E2146 exhibits a smooth and rod-shaped structure with an average length
323 of about $1.4 \pm 0.2 \mu\text{m}$ for a width of $0.7 \pm 0.1 \mu\text{m}$ that are in accordance with epifluorescence
324 images. The height profiles (**Figure 6b**) showed bacterial diameter of *c.a.* 600 nm. When
325 E2146 cells are exposed to D-Ctl, an evolution of bacterial morphology similar to E2152 was
326 observed. The rod-shaped structure is maintained but the cell size was increased with average
327 length and width reached up to $2.5 \pm 0.6 \mu\text{m}$ and $1.0 \pm 0.3 \mu\text{m}$, respectively. The analyses of
328 height profiles (**Figure 6**) revealed an increase of the bacterial diameter from 600 nm up to
329 850 nm. As previously observed for E2152, bacterial cells seem to be also inflated without
330 major detectable damages.

331 One can emphasize that permeabilization activity of D-Ctl is increased with concentration and
332 exposure time but the rod-shaped structure of non-lysed treated cells is maintained whatever
333 exposure time and D-Ctl concentration. In addition, the damages revealed by AFM could be

334 associated to high membrane weakening that maybe enhanced by the drying process (**Figure**
335 **S10**). Non-lysed treated cells are inflated upon D-Ctl exposure and the bacterial swelling is
336 much higher in presence of Type 1 *fimbriae*. The membrane permeability and bacterial shape
337 are intimately connected to the cell-wall mechanical features. In this way, the
338 permeabilization or membrane activity of D-Ctl can be investigated through the elastic
339 properties of the bacterial cell wall by force spectroscopy.

340

343.3. Evolution of bacterial stiffness during D-Ctl treatment

342 Bacterial stiffness of E2152 and E2146 was monitored during 24 hours under D-Ctl
343 concentrations of 0 and 40 $\mu\text{g/mL}$ ($5 \times \text{MIC}$) and reported in **Figure 7a, b**. In absence of D-
344 Ctl, **Figure 7** shows that the bacterial stiffness of E2152 and E2146 remain very stable during
345 24 hours with values of *c.a.* 600 and 300 kPa, respectively. These values are in agreement
346 with previous mechanical analyses of the two bacterial strains.²⁶ When the bacterial cells are
347 exposed to D-Ctl at 40 $\mu\text{g/mL}$, the bacterial stiffness shows two different behaviors according
348 to the bacterial strains. Indeed, the bacterial stiffness of E2152 slightly increased from *c.a.*
349 600 kPa up to 900 kPa while it decreased from *c.a.* 300 kPa down to 40 kPa for E2146. Here,
350 the impact of D-Ctl on the bacterial stiffness is highly influenced by the presence of the of
351 Type 1 *fimbriae* structures. In the absence of Type 1 *fimbriae*, a stiffening of about 30% is
352 observed over 24 hour-treatment whereas a dramatic softening of about 85% when Type 1
353 *fimbriae* are expressed.

354 The mechanical behavior of E2152 under D-Ctl treatment seemed counterintuitive in respect
355 with the membrane permeabilization supported by the *BacLight*TM assays. However,
356 mechanical stiffening under AMP has been already reported for huge peptides accumulation
357 at bacterial envelope concomitantly to its permeabilization.^{31, 41-42} The mechanical behavior of
358 E2146 under D-Ctl treatment is in good agreement with previous observations²⁴ that have

359 evidenced a reduction of bacterial stiffness by a factor of 3 with respect to the untreated
360 bacteria. This dramatic evolution of bacterial stiffness was explained by membrane
361 permeabilization as supported by the *BacLight*TM assays and should reflect the loss of cytosol
362 that led to bacterial lysis and death.^{24, 43-44} Here, we clearly evidenced unexpected different
363 mechanical effects of D-Ctl on *E. coli* cells constitutively expressing or not the Type 1
364 *fimbriae*. How such opposite mechanical effects can be explained, and what is the influence
365 of the bacterial external structures on the D-Ctl activity?

366 To address these questions, and to understand the molecular origin in such differences of
367 mechanical effects, the evolution of the biochemical fingerprints of D-Ctl and the two
368 bacterial strains were monitored by Fourier transform infrared spectroscopy (ATR-FTIR).
369 Indeed, ATR-FTIR allows the investigation of physiological, biochemical and conformational
370 changes of D-Ctl.

371

372 **3.4. Infrared spectral fingerprints of the bacteria submitted or not to D-Ctl**

373 IR-ATR spectra from both *E. coli* suspensions in presence or not of D-Ctl are shown in
374 **Figure 8** and **Figure 9**. One should notice that the background for every spectrum presented
375 here is the supernatant free of bacteria corresponding to the suspension under study.
376 Therefore, the spectra are representative for the remaining bacteria only, the contribution of
377 the medium being removed.

378 The spectra of the bacteria cultured without D-Ctl showed the specific features of Gram-
379 negative bacteria.^{24, 30, 45} Usual signatures of the biochemical components of bacterial cells,
380 i.e. proteins (P), nucleic acids (NA), phospholipids (PL) and polysaccharides (PS) are present
381 (main assignments are given in **Figures 8a** and **Figure 9a**). The overall intensities of the
382 whole spectra increased in as a function of the increase of the time of culture in accordance
383 with the growth curves recorded with the same conditions (**Figure 1**).

384 When E2152 was submitted to D-Ctl for 1 and 4 hours, the general spectral signatures of the
385 spectra were preserved with respect to those of the bacteria of the control experiment for all
386 three concentrations of D-Ctl. However, the spectra stayed at the same level of intensities
387 showing, in accordance with growth curves, that at least the growth was very quickly stopped
388 (**Figure 8**). After 14 hours of D-Ctl exposure to bacteria, the signal-to-noise ratio of the
389 spectra were very low, suggesting a very low concentration of bacteria in those suspensions.
390 After 14 hours of D-Ctl exposure, the intensities of the bands at ~ 1240 and 1085 cm^{-1} mainly
391 assigned to PO_2 stretchings of nucleic acids were very low, suggesting that the metabolic
392 activity was also very low.

393 One can emphasize that the contribution of PLs to the global spectral profile is low and the
394 loss of the latter is negligible since no cell debris showing bacterial lysis has been observed
395 either in AFM or in epifluorescence microscopy.

396 When E2146 was exposed to D-Ctl at the MIC, the general features of the spectra were
397 similar at every time of exposure to D-Ctl, looking as those of bacteria from the control
398 experiment (**Figure 9b**). In addition, the whole intensity of the spectrum after 1 hour of the D-
399 Ctl exposure was similar to the control experiment after 1 hour of culture. The whole
400 intensities of the spectra did not change drastically suggesting that bacteria did not grow upon
401 the exposure to D-Ctl. However, bands assigned mainly to nucleic acids around 1230 and
402 1086 cm^{-1} , were present. They suggested that the metabolic activity was maintained at least
403 partially for 14 hours at the MIC. The features of the spectra from the bacteria after contact
404 with D-Ctl at $5 \times$ and $10 \times$ MIC were different (**Figures 9c, d**). Indeed, bands at ~ 1621 and
405 $\sim 1526\text{ cm}^{-1}$ newly appeared in the region of amide bands from proteins and peptides. These
406 bands are assigned to β -sheets. It has been shown that D-Ctl, which is unstructured in
407 solution, forms antiparallel β -sheets that aggregate at the surface of negatively charged
408 bacterial mimetic membranes.⁹ Therefore, it can be suggested that D-Ctl accumulated on

409 or/and in the bacteria forming such aggregates on or/and in the bacteria. The spectral region
410 between 1250 and 1000 cm^{-1} was very different with respect to the control spectra. The
411 general shape of these bands, mainly assigned to PO_2 stretchings of nucleic acids, were less
412 resolved as compared to the control spectra and their intensities relative to amide bands were
413 lower (**Figures 9c, d**). The structure and the amount of nucleic acids were affected by the
414 presence of D-Ctl. This suggested an intracellular action of D-Ctl when its concentration was
415 very high. Altogether, spectroscopic analyses suggested that D-Ctl activity led to a dramatic
416 decrease in metabolism of both bacterial strains until a total break depending on AMP
417 concentration. Such interruption in bacterial metabolism seemed to be enhanced when
418 bacterial cell-wall are decorated with type-1 *fimbriae*.

419

420 **3.5. Impact of parietal structures on the D-Ctl mode of action**

421 As regarding the results obtained from both microbiological and physico-chemical
422 investigations, one can notice the occurrence of two different behaviors in D-Ctl activity as a
423 function of the D-Ctl concentration (at MIC and ≥ 5 MIC). D-Ctl antibacterial activity is not
424 related to the initial bacterial concentration considered for both strains, *i.e.* the concentrations
425 are very similar whatever the strain for a given OD_{600} . Besides, this activity is very different
426 between the nude cell-wall and the Type 1 *fimbriae* decorated bacteria.

427 In details, when both bacterial strains are exposed to D-Ctl at MIC, almost all membranes are
428 damaged after 4 and 14 hours without any pore formation upon AFM observations. However,
429 the membranes are more impacted for the nude cell-wall bacteria than those with the Type 1
430 *fimbriae*. Besides, *BacLight*TM assays and ATR-FTIR measurements supported that their
431 metabolism is not totally interrupted but disturbed. Indeed, E2146 bacteria appeared
432 filamentous; the presence of D-Ctl disrupted the cell division. On the contrary very few cells
433 of E2152 appeared filamentous. Both strains bacteria appeared inflated with higher diameter

434 size and continued to elongate but failed to septate. This can be subsequent to the blocking of
435 DNA replication, interruption in chromosome segregation, or inhibition of cell division.⁴⁶⁻⁴⁷
436 One can notice that there is no significant evolution of bacterial stiffness at MIC
437 concentration as evidenced in **Figure 7d** while important modifications occurred at higher D-
438 Ctl concentrations as reported in **Figure 7a, b**. These observations suggested that the D-Ctl
439 action on bacterial stiffness strongly depends on the occurrence of parietal structures (*i.e.* here
440 type-I *fimbriae*). This assumption is supported by the progressive bacterial stiffness drop of
441 E2146 when D-Ctl concentration was increased from MIC to $5 \times \text{MIC}$ (**Figure 7d**). One can
442 notice that a slight evolution in bacterial stiffness has been evidenced for E2152 strain (data
443 not shown) in accordance with the one observed for high D-Ctl concentrations reported in
444 **Figure 7a, b**.

445 At $5 \times \text{MIC}$ and $10 \times \text{MIC}$, both bacterial strains did not grow anymore. After 14 hours of
446 exposure with D-Ctl, the ATR-FTIR spectroscopic investigations revealed that the amount of
447 nucleic acids for E2152 is also low with respect to the protein amount, suggesting the stop of
448 the metabolic activity. Concerning E2146, the cells have mostly damaged membranes.
449 However, the staining was not homogenous inside the bacteria, bright red-orange dots
450 occurred within the cells. It is concomitant with aggregation of D-Ctl with the formation of β -
451 sheets confirmed by ATR-FTIR investigations. As PI is a nucleic acids dye, one can suggest
452 that the bacterial nucleic acids aggregated upon the binding of D-Ctl. Previous studies have
453 shown that cationic AMP with antiparallel β -sheets and/or rich in arginine can bind nucleic
454 acids.⁴⁸⁻⁵⁰ The membrane is not the only target of the peptide as it is shown here for E2146.
455 Conversely, E2152 bacteria staining is homogeneous whatever the exposure time suggesting
456 that D-Ctl remained at the bacterial cell-wall. The constitutive expression of Type 1 *fimbriae*
457 with respect to the nude cell-wall strain can weaken the outer membrane and thus can
458 facilitate the D-Ctl translocation. Therefore, D-Ctl activity could be characterized by the

459 damaging of the outer membrane *via* electrostatic interactions followed by the peptide
460 translocation. Further intracellular translocation results in the decrease or the inhibition of the
461 acid nucleic synthesis. It is evidenced by the bacterial filamentation at the MIC mainly for
462 E2146 and by the aggregation of nucleic acids at the highest concentrations of D-Ctl.

463 The small differences observed in the bacterial growth for increasing concentration of D-Ctl,
464 are supported by macroscopic effects such as modifications of the bacterial suspension
465 turbidity. Indeed, **Figure 10** showed drastic effects of D-Ctl increasing concentration on
466 suspension turbidity and bacterial aggregation. For E2152 bacteria, the increase of D-Ctl
467 concentration led to an important bacterial aggregation at the bottom of the spectrophotometer
468 cuvettes delimited by the white dashed lines. For E2146 bacteria, the effect of increasing D-
469 Ctl concentration is lower on bacterial aggregation and the bacterial suspension became less
470 and less cloudy suggesting a more important bacteriolytic activity. These results are in
471 agreement with the difference in bacterial surface charge density of the two strains. The
472 surface of E2152 is almost 6 times more negatively charged than E2146 according to previous
473 studies that highlighted surface charge density of about -170 mV and -30 mV for E2152 and
474 E2146, respectively.²⁶ Such differences should lead to larger interactions and accumulation of
475 D-Ctl onto E2152 bacterial cell wall than the latter onto E2146. Further analyses combining
476 zeta potential and dynamic light scattering could be more relevant to decipher the molecular
477 mechanism behind this macroscopic phenomenon. Besides, overexpression of Type I *fimbriae*
478 could significantly weaken the bacterial outer-membrane and facilitate its permeabilization by
479 the antimicrobial peptides. This phenomenon has been previously reported for LPS
480 modifications/expression modulation that impact on the bacterial resistance/tolerance to
481 antimicrobial peptides and antibiotics.⁵¹⁻⁵²

482

483 **4. Conclusion**

484 In the present study, we report a systematic analysis of nanomechanical and spectrochemical
485 properties related to the antibacterial mechanism of action on two *E. coli* strains expressing or
486 not Type 1 *fimbriae* exposed to different D-Ctl doses and during several exposure periods. It
487 is found that depending of D-Ctl concentration, bacterial stiffness is dramatically modified
488 concomitantly to major membrane damages and important changes in the spectral fingerprint
489 of the bacteria. However, no pore formation has been observed while all bacterial membranes
490 were permeabilized under high D-Ctl concentrations. In case of the occurrence of fimbrial
491 structures, an intracellular mode of action was shown also. Our results evidenced that D-Ctl
492 activity is highly impacted by the cell-wall external structures and surface properties such as
493 Type 1 *fimbriae* leading or not to macroscopic aggregation. In this way, as it was reported on
494 the LPS modification, the modulation of parietal structures expressions could have a strong
495 impact on the bacterial resistance/tolerance to antimicrobial peptides and therefore can be a
496 strategy used by bacteria to bypass the immune defenses.

497

498

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505

506

507 **Supporting Information**

508 Structures and physicochemical properties of D-cateslytin and scheme of bacterial strains cell
509 wall (**Figure S1**), AFM imaging and height profiles of reference bacteria E2152 in absence of
510 D-Ctl and performed in air after CO₂ critical point drying (**Figure S2**), AFM imaging and
511 height profiles of reference bacteria E2152 in presence of D-Ctl at MIC concentration and
512 performed in air after CO₂ critical point drying (**Figure S3**), AFM imaging and height profiles
513 of reference bacteria E2152 in presence of D-Ctl at 5 × MIC concentration and performed in
514 air after CO₂ critical point drying (**Figure S4**), AFM imaging and height profiles of reference
515 bacteria E2152 in presence of D-Ctl at 10 × MIC concentration and performed in air after CO₂
516 critical point drying (**Figure S5**), AFM imaging and height profiles of E2146 bacteria in
517 absence of D-Ctl and performed in air after CO₂ critical point drying (**Figure S6**), AFM
518 imaging and height profiles of E2146 bacteria in presence of D-Ctl at MIC concentration and
519 performed in air after CO₂ critical point drying (**Figure S7**), AFM imaging and height profiles
520 of E2146 bacteria in presence of D-Ctl at 5 × MIC concentration and performed in air after
521 CO₂ critical point drying (**Figure S8**), AFM imaging and height profiles of E2146 bacteria in
522 presence of D-Ctl at 10 × MIC concentration and performed in air after CO₂ critical point
523 drying (**Figure S9** Evolution with time of stiffness ratio for E2152 and E2146 in presence of
524 D-Ctl at 40 μg/mL (**Figure S10**), *In-situ* monitoring of D-Ctl activity on E2146 bacteria (Fast
525 scanning of trapped single bacterium) (**Figure S11**), AFM imaging of E2146 bacteria in
526 absence and in presence of D-Ctl at 5 × MIC concentration and performed in air after gentle
527 nitrogen drying (**Figure S12**), and AFM imaging of E2152 bacteria in absence and in
528 presence of D-Ctl at 5 × MIC concentration and performed in air after gentle nitrogen drying
529 (**Figure S13**). The Supporting Information is available free of charge on the ACS Publications
530 website.

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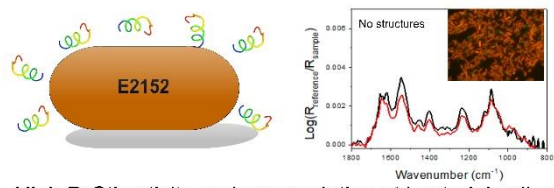
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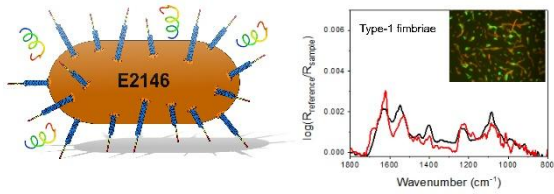
676

677 **Graphical Abstract**

678



High D-Ctl activity and accumulation at bacterial cell wall



Low D-Ctl activity and accumulation at bacterial cell wall

680

681 **FIGURE AND TABLE CAPTIONS.**

682

683 **TABLES**

684 **Table 1.** Average length (μm) of bacteria in presence or not to antimicrobial peptide D-Ctl
685 (red: average length for red bacteria in the images; green: average length for green bacteria in
686 the images).

687

688 **Figure 1:** Growth curves of *E. coli* MDR measured at 23°C by the recording of the optical
689 density at 600 nm (DO_{600}) for different concentrations of D-Ctl. (a), (b), (c): E2152; (d), (e),
690 (f): E2146; (a), (d) initial $\text{DO}_{600} = 0.001$; (b), (e): initial $\text{DO}_{600} = 0.01$; (c), (f) initial $\text{DO}_{600} =$
691 0.5.

692

693 **Figure 2.** Representative epifluorescence images of *E. coli* E2152 submitted or not to the
694 presence of D-Ctl at different concentrations and several times of exposure (*BacLight*TM
695 staining). (a), (e), (i): control experiment (0 x MIC); (b), (f), (j): D-Ctl at MIC (8 $\mu\text{g}/\text{mL}$); (c),
696 (g), (k): D-Ctl at 5 x MIC (40 $\mu\text{g}/\text{mL}$); (d), (h), (l): D-Ctl at 10 x MIC (80 $\mu\text{g}/\text{mL}$) (a), (b), (c),
697 (d): after 1 hour; (e), (f), (g), (h): after 4 hours; (i), (j), (k), (l): after 14 hours. Bars: 20 μm
698 (white), 1 μm (yellow).

699

700 **Figure 3.** Representative epifluorescence images of *E. coli* E2146 submitted or not to the
701 presence of D-Ctl at different concentrations and several times of exposure (*BacLight*TM
702 staining). (a), (e), (i): control experiment (0 x MIC); (b), (f), (j): D-Ctl at MIC (8 $\mu\text{g}/\text{mL}$); (c),
703 (g), (k): D-Ctl at 5 x MIC (40 $\mu\text{g}/\text{mL}$); (d), (h), (l): D-Ctl at 10 x MIC (80 $\mu\text{g}/\text{mL}$) (a), (b), (c),
704 (d): after 1 hour; (e), (f), (g), (h): after 4 hours; (i), (j), (k), (l): after 14 hours. Bars: 20 μm
705 (white), 1 μm (yellow).

706

707 **Figure 4.** AFM imaging in air after CO₂ critical point drying of samples of *E. coli* E2152
708 submitted or not to the presence of D-Ctl at different concentrations and several times of
709 exposure. (a), (e), (i): control experiment (0 × MIC); (b), (f), (j): D-Ctl at MIC (8 µg/mL); (c),
710 (g), (k): D-Ctl at 5 × MIC (40 µg/mL); (d), (h), (l): D-Ctl at 10 × MIC (80 µg/mL) (a), (b), (c),
711 (d): after 1 hour; (e), (f), (g), (h): after 4 hours; (i), (j), (k), (l): after 14 hours. Bar: 1 µm.

712

713 **Figure 5.** AFM imaging in air after CO₂ critical point drying of samples of *E. coli* E2146
714 submitted or not to the presence of D-Ctl at different concentrations and several times of
715 exposure. (a), (e), (i): control experiment (0 × MIC); (b), (f), (j): D-Ctl at MIC (8 µg/mL); (c),
716 (g), (k): D-Ctl at 5 × MIC (40 µg/mL); (d), (h), (l): D-Ctl at 10 × MIC (80 µg/mL) (a), (b), (c),
717 (d): after 1 hour; (e), (f), (g), (h): after 4 hours; (i), (j), (k), (l): after 14 hours. Bar: 1 µm.

718

719 **Figure 6.** Morphology evolution of *E. coli* MDR exposed or not to D-Ctl at several
720 concentrations; (a): Evolution of E2152 bacterial diameter in absence and in presence of D-
721 Ctl after 1, 4 and 14 hours; (b): Evolution of E2146 bacterial diameter in absence and in
722 presence of D-Ctl after 1, 4 and 14 hours.

723

724 **Figure 7.** *In-situ* monitoring of bacterial stiffness of *E. coli* MDR exposed or not to D-Ctl at 5
725 × MIC; (a): E2152 in absence and in presence of D-Ctl (black and grey squares, respectively);
726 (b): E2146 in absence and in presence of D-Ctl (black and grey circles, respectively); (c):
727 Evolution of stiffness ratio upon D-Ctl treatment at 5 × MIC (40 µg/mL) for E2152 and
728 E2146 (black rectangles and white circles, respectively); (d): *In-situ* monitoring of E2146
729 bacterial stiffness evolution after 2 injections of D-Ctl over 3 hours (D-Ctl concentration was
730 initially set at 8 µg/mL and increased to 40 µg/mL after the second injection)..

731

732 **Figure 8:** ATR-FTIR spectra of planktonic *E. coli* E2152 submitted or not to the presence of
733 D-Ctl at different concentrations after 1 hour (black spectra), after 4 hours (red spectra) and
734 after 14 hours (blue spectra). (a): control experiment ($0 \times \text{MIC}$); (b): D-Ctl at MIC ($8 \mu\text{g/mL}$);
735 (c): D-Ctl at $5 \times \text{MIC}$ ($40 \mu\text{g/mL}$); (d): D-Ctl at $10 \times \text{MIC}$ ($80 \mu\text{g/mL}$). Key: P, proteins; NA:
736 nucleic acids; PL: phospholipids, PS: polysaccharides.

737

738 **Figure 9:** ATR-FTIR spectra of planktonic *E. coli* E2146 submitted or not to the presence of
739 D-Ctl at different concentrations after 1 hour (black spectra), after 4 hours (red spectra) and
740 after 14 hours (blue spectra). (a): control experiment ($0 \times \text{MIC}$); (b): D-Ctl at MIC ($8 \mu\text{g/mL}$);
741 (c): D-Ctl at $5 \times \text{MIC}$ ($40 \mu\text{g/mL}$); (d): D-Ctl at $10 \times \text{MIC}$ ($80 \mu\text{g/mL}$). Key: P, proteins; NA:
742 nucleic acids; PL: phospholipids, PS: polysaccharides.

743

744 **Figure 10:** Tentative mechanism of action of D-Ctl at the different concentrations on E2152
745 (top) and E2146 (bottom).

746