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## Comparative assessment of the disinfection effectiveness of thymol and benzalkonium chloride against adapted and non-adapted to thymol biofilm cells of a *Salmonella* Typhimurium epidemic phage type DT193 strain

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1 ORIGINAL RESEARCH ARTICLE

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3 **Comparative assessment of the disinfection effectiveness of thymol and**  
4 **benzalkonium chloride against adapted and non-adapted to thymol biofilm**  
5 **cells of a *Salmonella* Typhimurium epidemic phage type DT193 strain**

6

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18

19 **Highlights**

20

21 • Thymol presents better anti-biofilm potential compared to benzalkonium chloride.

22 • Thymol presence at ½ its MIC significantly decreases biofilm formation.

23 • The application of thymol at just four times its MBC eradicates *Salmonella* biofilm.

24 • Thymol adapted biofilm cells present increased resistance to thymol and BAC.

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## 36 A B S T R A C T

37        Nowadays there is a need to develop cost-effective, safe, and preferably eco-friendly methods to combat  
38 the pathogenic and other detrimental robust biofilms. In this work, the disinfection actions of thymol  
39 (THY), a phytochemical exhibiting wide antimicrobial action, and benzalkonium chloride (BAC), a  
40 synthetic biocide commonly used as surface disinfectant in the food industry and elsewhere, were  
41 comparatively evaluated against adapted and non-adapted to THY biofilm cells of a *Salmonella enterica*  
42 ser. Typhimurium epidemic phage type DT193 strain. Initially, the minimum inhibitory and bactericidal  
43 concentrations (MICs, MBCs) of each compound were determined against planktonic bacteria, together  
44 with their minimum biofilm inhibitory and eradication concentrations (MBICs, MBECs) against biofilms  
45 formed on polystyrene (PS). Bacteria were subsequently left to form biofilms on model stainless steel (SS)  
46 surfaces incubated in 1/10 diluted tryptone soy broth (dTSB) containing or not a sub-inhibitory THY  
47 concentration. Those sessile populations were finally submitted to disinfection (for 15 min) with THY or  
48 BAC and the viable biofilm bacteria were quantified by using in parallel agar plating and selective  
49 fluorescence staining. The results showed that when the terpenoid was applied at sub-MIC during biofilm  
50 formation, it was able to cause significant reductions of the final sessile populations on both surfaces (PS  
51 and SS). Disinfection results revealed the significant anti-biofilm action of THY on the non-adapted  
52 (control) biofilms (5 log reductions at MBC) and its superiority to that of BAC (< 2 log reductions at 2 x  
53 MBC). However, the disinfection treatments applied on the THY-adapted biofilms were quite less efficient  
54 (achieving 2.6 and 1.3 log reductions, for THY and BAC, respectively). This demonstrates adaptation of  
55 *Salmonella* to THY, conferring co- and cross-disinfection resistance, as for many other antibacterial  
56 chemicals. Overall, the results demonstrate that natural compounds extracted from plants may be promising  
57 agents in helping to combat biofilms, multicellular microbial structures well-known for their remarkable  
58 hardiness against many stresses and antimicrobials.

59

60 *Keywords: Salmonella enterica, biofilm, disinfection, stress adaptation, thymol, benzalkonium chloride*

61

## 62 **1. Introduction**

63 *Salmonella enterica* is a Gram-negative enteric bacterium and major foodborne pathogen (Jajere, 2019).  
64 Based on the most recent data, salmonellosis is the second most reported gastrointestinal infection in  
65 humans after campylobacteriosis in Europe (EFSA & ECDC, 2019). Thus, in 2018, *Salmonella* caused the  
66 30.7% of the 5,146 food- and waterborne outbreaks, with a total of 91,857 confirmed human cases and 119  
67 deaths (0.19% case-fatality rate). As in previous years, the most frequently reported serovars were  
68 Enteritidis and Typhimurium (including its monophasic variant *S. Typhimurium* 1,4,[5],12:i:-),  
69 representing together 71.0% of all reported serovars in confirmed human salmonellosis cases. Like many  
70 other microorganisms, *Salmonella* is capable to create robust biofilms on various (either abiotic or biotic)  
71 surfaces, a phenotypic trait which is linked to its resistance to various stresses and antimicrobials,  
72 persistence in the environment and its ability to colonize the host (Lamas et al., 2018; MacKenzie et al.,  
73 2017).

74 Disinfection aims to kill microorganisms on inanimate surfaces and is usually achieved by applying a  
75 variety of liquid chemicals (disinfectants), such as quaternary ammonium compounds (QACs).  
76 Benzalkonium chloride (BAC) belongs to QACs which are membrane-active agents and among the most  
77 used disinfectants, due to their broad antimicrobial spectrum (Gerba, 2015; Merchel Piovesan Pereira &  
78 Tagkopoulos, 2019). BAC is usually applied at concentrations ranging from 0.02% to 0.1% (v/v) (Gaulin  
79 et al., 2011). However, long-term sublethal bacterial exposure to that compound can promote increased  
80 resistance towards that compound itself (Capita et al., 2017; Mangalappalli-Illathu & Korber, 2006;  
81 Mangalappalli-Illathu et al., 2008), as well as cross-resistance to other distinct chemicals, such as clinically  
82 important antibiotics (Kampf, 2018; Kim et al., 2018; Tandukar et al., 2013). Alarmingly, easily

83 transferable genes (e.g., plasmid and/or integron encoded) conferring efflux-mediated resistance to QACs  
84 and other drugs have been identified in *Salmonella* (Long et al., 2016; Hoffmann et al., 2017). Not  
85 surprisingly, some countries have already prohibited the use of BAC for some applications due to safety  
86 and antimicrobial resistance concerns (Ferk et al, 2007; Merchel Piovesan Pereira & Tagkopoulos, 2019).  
87 Recent studies also showed that biofilms of several *S. enterica* serotypes increased their biovolume and  
88 surface coverage after a 10-min exposure to 0.1% (v/v) of BAC (Capita et al., 2019), while *S. Enteritidis*  
89 biofilm cells surviving BAC have been additionally found to over-express some critical virulence genes  
90 (Romeu et al., 2020).

91 Such issues have led to the search for the development of novel antimicrobial methods to combat the  
92 robust detrimental microbial biofilms, which will be efficient (at killing the cells with the lowest possibility  
93 for resistance development), cost-effective, safe, and preferably eco-friendly. To this direction, plant  
94 extracts and purified plant secondary metabolites have been successfully evaluated (Borges et al., 2016).  
95 Phytochemicals may belong to different classes, such as terpenoids, and usually present minimum  
96 inhibitory concentrations (MICs) against free growing (planktonic) cells ranging from 100 to 1000 ppm  
97 (Sakarikou et al., 2020). Thymol (THY) is such a natural monoterpenoid phenol, derivative of cymene and  
98 isomer of carvacrol (CAR), which is found in high quantities in the essential oils (EOs) extracted from  
99 plants of the genus *Thymus*, comprising many native to the Mediterranean region species, such as *Thymus*  
100 *vulgaris* L. (thyme) (Nabavi et al., 2015). THY is known to exert its antimicrobial action mainly through  
101 its damaging effects on cells' membranes, provoking loss of their integrity, disruption of the proton motive  
102 force and ability to produce energy, harm to cellular homeostasis, and escape of valuable intracellular  
103 material including ATP (Ben Arfa et al., 2006). Besides the strong antimicrobial action (mainly verified  
104 against numerous planktonic microorganisms), THY has been efficiently applied, either alone or in  
105 combination with other agents, against biofilms formed by *Salmonella* and/or other species (Čabarkapa et  
106 al., 2019; Engel et al., 2017; Liu et al., 2015; Miladi et al., 2017).

107 To develop any novel anti-biofilm method and before its further widespread application, it is crucial to  
108 test and compare its efficiency against the classical applied methods for such purpose. This latter could  
109 demonstrate its superiority (if any) compared to the already established (insufficient/problematic) methods,  
110 together with any other issue possibly requiring further attention (e.g., microbial adaptation, resistance,  
111 toxicity). However, although several studies have been last years published related to the anti-biofilm  
112 actions of THY and/or EOs or some other plant extracts containing that compound against various  
113 microorganisms, including *Salmonella* (Sakarikou et al., 2020), very few of them have in parallel compared  
114 those actions with the ones of standard biocides (Chorianopoulos et al., 2008; Karampoula et al., 2016;  
115 Kostoglou et al., 2020; Lebert et al., 2007). Considering all those, the aim of the present study was to  
116 comparatively evaluate the disinfection actions of THY and of BAC against biofilm cells of *S.*  
117 *Typhimurium* epidemic phage type DT193 strain which were first either adapted or non-adapted to THY.

118

## 119 **2. Materials and methods**

120

### 121 *2.1. Bacterial strain and preparation of the working cell suspension*

122 The bacterial strain used in this work was a human isolate of *S. Typhimurium* epidemic phage type  
123 DT193 (strain FMCC\_B137) which was kindly provided by Professor G.-J. Nychas (Agricultural  
124 University of Athens, Greece). Before its experimental use, this was kept frozen (at  $-80\text{ }^{\circ}\text{C}$ ) in Tryptone  
125 Soy Broth (TSB; Lab M, Heywood, Lancashire, UK) containing 15% glycerol and was revitalized when  
126 needed by streaking a loopful of its frozen suspension on to the surface of Tryptone Soy Agar (TSA; Lab  
127 M) and incubating at  $37\text{ }^{\circ}\text{C}$  for 24 h (preculture). Working culture was prepared by inoculating a colony  
128 from the preculture into 10 mL of fresh TSB and incubating at  $37\text{ }^{\circ}\text{C}$  for 18 h. Bacteria from that final  
129 working culture were harvested by centrifugation ( $4000 \times g$  for 10 min at room temperature), washed twice  
130 with quarter-strength Ringer's solution (Lab M), and finally suspended in the same solution, to display an  
131 absorbance at 600 nm ( $A_{600\text{ nm}}$ ) equal to 0.1 (*ca.*  $10^8$  CFU/mL).

132

133 2.2. *Biocides and preparation of their stock solutions*

134 Thymol (THY) was purchased from Penta Chemicals (Radiová, Prague, Czech Republic) (powder min.  
135 99.0%, molar mass: 150.22 g/mol; product code: 27450-30100), while benzalkonium chloride (BAC) was  
136 acquired from Acros Organics (Thermo Fisher Scientific, Geel, Belgium) (liquid, alkyl distribution from  
137 C<sub>8</sub>H<sub>17</sub> to C<sub>16</sub>H<sub>33</sub>; product code: 215411000). Two stock solutions were prepared for THY, both in  
138 absolute ethanol, at 10% w/v and 40% w/v, for subsequent application against the planktonic and biofilm  
139 cells, respectively, following appropriate dilutions (see below for further details), while the stock solution  
140 of BAC (1% v/v) was prepared in sterile distilled water. All stock solutions were maintained in airtight  
141 falcon tubes at 4 °C for up to 2 weeks.

142

143 2.3. *Determination of minimum inhibitory and bactericidal concentrations (MICs, MBCs) of each*  
144 *compound against planktonic bacteria*

145 The MIC of each compound against the planktonic cells of the *S. Typhimurium* strain was determined  
146 using the broth microdilution method, as previously described (Vetas et al., 2017), by incubating bacteria  
147 (*ca.* 10<sup>5</sup> CFU/mL) in 1/10 diluted TSB (dTSB) at 20 °C for 24 h. That medium and incubation temperature  
148 were chosen since were the ones later used for the biofilm formation experiments (see section 2.4), given  
149 their favorable impact on biofilm formation by the tested bacterial strain (as shown in preliminary  
150 experiments; data not shown). Ten different concentrations were tested for each compound ranging from  
151 10000 to 19.5 ppm (two-fold dilutions) and from 50 to 5 ppm (in 5 ppm increments) for THY and BAC,  
152 respectively. The MIC of each compound was considered as its lowest concentration resulting in no visible  
153 bacterial growth (no increase in broth's absorbance), while the MBC was defined as its lowest concentration  
154 reducing the initial inoculum by at least 3 logs ( $\geq 99.9\%$ ).

155



156 2.4. *Determination of minimum biofilm inhibitory and eradication concentrations (MBICs, MBECs) of*  
157 *each compound against biofilm bacteria*

158 The MBIC of each compound was determined by leaving bacteria to form biofilms on 96-well  
159 polystyrene (PS) microtiter plates (transparent, hydrophobic, Ref. F-82.1581.100; Sarstedt AG & Co. KG;  
160 Nümbrecht, Germany), as previously described (Vetas et al., 2017), in dTSB containing different  
161 concentrations for each compound, at 20 °C for 120 h (with medium renewal at 48 h). Eight different  
162 concentrations were tested for each compound, ranging from 312.5 to 2.4 ppm and from 80 to 0.625 ppm  
163 (always two-fold dilutions) for THY and BAC, respectively. Those were selected based on the previous  
164 determination of the MICs. The MBIC of each compound was considered as its lowest concentration that  
165 drastically reduced biofilm formation, as this was expressed by measuring crystal violet's absorbance at  
166 620 nm (related to biomass accumulation) and compared to the positive biofilm control (no antimicrobial  
167 added). That later control also contained 468.8 ppm ethanol when THY was tested given that this ethanol  
168 concentration was the maximum one existing in the highest tested concentration for that biocide (i.e., 312.5  
169 ppm).

170 To calculate the MBEC of each compound, following biofilm formation (again in dTSB at 20 °C for  
171 120 h with medium renewal at 48 h), the planktonic suspensions were removed, and each well was twice  
172 washed with quarter-strength Ringer's solution (to remove the loosely attached cells). Subsequently, 200  
173 µL of a range of antimicrobial solutions were added to each well and left in contact for 24 h at 20 °C  
174 (disinfection/eradication step). Each compound was tested in nine different concentrations, ranging from 1  
175 to 256 x MIC (two-fold dilutions), which were all prepared in sterile distilled water, starting from each  
176 stock solution (i.e., 40% w/v and 1% v/v for THY and BAC, respectively). Sterile distilled water (also  
177 containing 6% v/v ethanol when THY was tested) was used as negative disinfection control. Following  
178 disinfection, the antimicrobial solutions were carefully removed from each well, which was then washed  
179 with quarter-strength Ringer's solution (to remove any disinfectant residues). To remove and quantify  
180 biofilm bacteria, 200 µL of quarter-strength Ringer's solution were added to each well and its PS surface

181 was thoroughly scratched with a plastic pipette tip. Recovered biomasses were vortexed (to disrupt any  
182 cellular aggregate), serially diluted, and bacteria were finally enumerated by counting colonies on  
183 (duplicate) spot inoculated (10  $\mu$ L) TSA plates, following their incubation at 37 °C for 24 h. The MBEC of  
184 each compound was determined as its lowest concentration totally eradicating biofilm bacteria (i.e., no  
185 appearance of colonies).

186

## 187 2.5. *Biofilm formation on SS surfaces in either the presence or absence of sub-MIC of THY and* 188 *disinfection*

189 *S. Typhimurium* strain FMCC\_B137 was left to form biofilms on rectangular SS coupons (3×1×0.1 cm,  
190 type AISI 304) as previously described (Iliadis et al., 2018). Briefly, individual (cleaned and sterilized)  
191 coupons were initially incubated for 2 h at 20 °C in saline bacterial suspension (*ca.* 10<sup>8</sup> CFU/mL), to allow  
192 bacteria to attach onto them, loosely attached cells were then removed by rinsing (with quarter-strength  
193 Ringer's solution), and coupons were finally incubated in dTSB containing or not a sub-inhibitory THY  
194 concentration (equal to ½ of its MIC; i.e., 78.1 ppm), at 20 °C for 120 h (with medium renewal at 48 h) to  
195 allow biofilm growth. Following biofilm formation, each SS coupon was thoroughly washed with quarter-  
196 strength Ringer's solution (to remove the loosely attached cells) and placed for 15 min at 20 °C in each  
197 disinfectant solution. For this experiment, THY was applied at its MBC (i.e., 312.5 ppm), BAC at double  
198 its MBC (i.e., 70 ppm), while sterile distilled water (also containing 0.3% v/v ethanol when THY was  
199 tested) was used as the negative disinfection control. The applied concentration for BAC was based on  
200 preliminary experiments showing negligible killing action against biofilm cells when this chemical was  
201 applied at its MBC (i.e., 35 ppm). Following disinfection, each coupon was washed with quarter-strength  
202 Ringer's solution (to remove any disinfectant residues) and placed, for 10 min, in a 10 mL-plastic falcon  
203 tube containing 6 mL of Dey-Engley (D-E) Neutralizing broth (Lab M) and 10 glass beads (3 mm in  
204 diameter). Biofilm cells were then detached from the SS surface through its strong 2-min vortexing (at 3000  
205 rpm using ZX3 Advanced Vortex Mixer; VELP Scientifica Srl; Usmate, Italy) and were finally enumerated

206 by counting colonies on (duplicate) spot inoculated (10  $\mu$ L) TSA plates, following ten-fold serial dilutions  
207 in quarter-strength Ringer's solution, plating, and incubation at 37 °C for 24 h. Plate counts were converted  
208 to  $\log_{10}$  CFU/cm<sup>2</sup> and for each compound, the logarithmic reduction ( $\log_{10}$  CFU/cm<sup>2</sup>) of cells following  
209 disinfection was calculated by subtracting the  $\log_{10}$  of the survivors from that counted following the  
210 negative disinfection control. Biofilm populations onto the coupons were also quantified before disinfection  
211 (at the end of 120 h of incubation) using the same protocol.

212

## 213 2.6. *LIVE/DEAD<sup>®</sup> fluorescence staining of sessile bacteria on SS before and following disinfection*

214 The LIVE/DEAD<sup>®</sup> BacLight™ Bacterial Viability Kit (Ref. L7012; Molecular Probes, Invitrogen)  
215 was used to quantify the surface coverage (% surface fluorescence) of the sessile communities and evaluate  
216 the percentages of viable and permeabilized (dead) cells in those populations, both before and following  
217 the 15-min disinfection treatments. This kit is based on a mixture of two fluorescent dyes that differ in their  
218 spectral characteristics and ability to penetrate bacterial cells, i.e., SYTO<sup>®</sup> 9 and propidium iodide (PI),  
219 with excitation/emission maxima at about 480/500 nm and 490/635 nm, respectively. SYTO<sup>®</sup> 9 penetrates  
220 all cells in a population and results in their green staining, while PI penetrates only those cells with damaged  
221 membranes resulting in their red staining. SS coupons were stained just after their 120 h of incubation in  
222 dTSB (containing or not sub-MIC of THY equal to ½ of its MIC; i.e., 78.1 ppm), and also after the two  
223 different 15-min disinfection treatments (i.e., THY, BAC), plus after the treatment with water as the  
224 negative disinfection control (see section 2.5), following the recommended user guidelines. Stained  
225 coupons were subsequently observed under a Leica SP8 AOBS confocal laser scanning microscope  
226 (CLSM; Leica Microsystems SAS, France) at the MIMA2 imaging platform  
227 ([https://www6.jouy.inrae.fr/mima2\\_eng](https://www6.jouy.inrae.fr/mima2_eng)). An inverted water 40X objective was used to acquire 20 images  
228 for green and red channels per coupon, while two coupons were analyzed for each treatment. Raw images  
229 (40/treatment) were binarized using the MaxEntropy function in an automatic macro executed in the open-  
230 access ImageJ software (National Institutes of Health, Bethesda, Md., USA) and the percentage of surface

231 fluorescence for each image was calculated for the total (green fluorescence) and permeabilized cells (red  
232 fluorescence).

233

## 234 2.7. *Statistics*

235 Each experiment included at least two technical replicates (e.g., PS wells, SS coupons) and was repeated  
236 three times using independent bacterial cultures. Plate counts were transformed to logarithms before means  
237 and standard deviations were computed. All the biofilm forming and disinfection data concerning SS  
238 surfaces were analyzed by factorial analysis of variance (ANOVA) to check for any significant effects of  
239 THY's presence (at ½ MIC) and biocide's type (THY, BAC) on biofilm formation ( $\log_{10}$  CFU/cm<sup>2</sup>) and  
240 disinfection efficiency (expressed as log reduction), using the statistical software STATISTICA® (StatSoft  
241 Inc.; Tulsa, OK 74104, USA). All differences are reported at a significance level of 0.05.

242

## 243 3. Results

244

### 245 3.1. *Determination of MIC, MBC, MBIC and MBEC of each compound*

246 To inhibit the visible planktonic growth (in dTSB at 20 °C for 24 h) of the *S. Typhimurium* strain  
247 FMCC\_B137, THY and BAC needed to be applied at 156.3 and 20 ppm, respectively, while a double  
248 concentration was approximately required for both compounds to kill (by  $\geq 99.9\%$ ) the planktonic bacteria  
249 (with MBC values equal to 312.5 and 35 ppm, respectively). Regarding the ability of each compound to  
250 inhibit biofilm formation, **Fig. 1** analytically shows the biofilm biomasses recorded on PS at each one of  
251 the eight different tested concentrations for each one, together with the planktonic populations found in  
252 each well just before their removal for biofilm quantification (both expressed as absorbances at 620 nm).  
253 THY could drastically inhibit biofilm formation (by 57.5%) compared to the positive control (no  
254 antimicrobial added) when applied at ½ of its MIC (MBIC 78.1 ppm), whereas BAC caused a 74% biofilm

255 reduction when applied at double its MIC (MBIC 40 ppm). On the other hand, the application of BAC at  
256 its MIC (i.e., 20 ppm) was unable to cause any significant reduction at the quantity of sessile biomass, while  
257 the application of THY at its MIC (i.e., 156.3 ppm) reduced that biomass by 73.6% resulting in recorded  
258 absorbance that did not significantly differ to that of the biofilm negative control (no bacteria added). To  
259 determine MBEC values, the formed sessile communities ( $7.20 \pm 0.40 \log_{10}$  CFU/cm<sup>2</sup>; data not shown)  
260 were disinfected through 24 h exposure to nine different concentrations (up to 256 x MIC) of each  
261 antimicrobial. The concentrations needed to totally eradicate the biofilm bacteria (> 6 logs reduction) were  
262 quite similar for both compounds (i.e., 1250 and 1280 ppm for THY and BAC, respectively). Nevertheless,  
263 those values correspond at just four times its MBC for THY, whereas it is 36.6 x MBC for BAC.

264

### 265 3.2. *Biofilm formation on SS surfaces and disinfection*

266 The counts of the sessile populations found on SS coupons following their incubation, at 20 °C for 120  
267 h, in dTSB containing or not sub-MIC of THY (i.e.,  $\frac{1}{2}$  MIC = 78.1 ppm) were  $4.59 \pm 0.56$  and  $6.39 \pm 0.19$   
268  $\log_{10}$  CFU/cm<sup>2</sup>, respectively. Thus, the presence of THY at its MBIC (as this was determined for biofilms  
269 formed on PS) was found to strongly reduce (*ca.* 100-fold) the *Salmonella* population encountered on the  
270 SS surface. The results on the disinfection of those communities are presented in **Fig. 2**. The application of  
271 THY at its MBC (i.e., 312.5 ppm) was always much more effective against sessile cells compared to the  
272 application of BAC at double its MBC (i.e., 70 ppm), with *ca.* a 10-1000 fold difference in efficiencies  
273 between those two compounds, depending on whether the incubation had been previously done in either  
274 the presence or absence of sub-MIC of THY. Thus, when cells had not been previously adapted, 312.5 ppm  
275 of the terpenoid were enough to provoke a *ca.* 5-log reduction of the viable sessile population ( $4.89 \pm 0.39$   
276  $\log_{10}$  CFU/cm<sup>2</sup>), while under such conditions the reduction did not exceeded 2 logs being limited to  $1.77 \pm$   
277  $0.52 \log_{10}$  CFU/cm<sup>2</sup> when BAC was applied at 70 ppm. On the other hand, the previous sessile growth in  
278 the presence of sub-MIC of THY (i.e., 78.1 ppm) resulted in the subsequent increased resistance of the  
279 sessile cells to disinfection with that compound, indicating thus a strong co-adaptation effect. In that case,

280 the difference in log reductions between the two compounds was limited to  $1.33 \log_{10}$  CFU/cm<sup>2</sup>, with  
281 recorded log reductions following the 15-min disinfection being equal to  $2.60 \pm 0.57$  and  $1.27 \pm 0.29 \log_{10}$   
282 CFU/cm<sup>2</sup>, following application of THY and BAC, respectively. It also seems that BAC was equally  
283 effective against either adapted or non-adapted biofilm cells since the recorded log reductions did not  
284 significantly differ between these two treatments.

285 Percentages of surface fluorescence for total (green) and permeabilized (red) bacterial populations found  
286 on SS surfaces both before and following disinfection are given in **Table 1**. Before disinfection, the  
287 percentage of green fluorescence was higher for biofilm grown without THY ( $3.22 \pm 0.16\%$  vs  $2.35 \pm 0.27\%$   
288 with THY). Among those sessile cells, 99.4% were viable when bacteria were grown without THY, whereas  
289 only 55.7% remained viable when prior growth was done in the presence of sub-MIC of THY. This latter  
290 low percentage of viable cells is probably due to the permeabilizing effect of THY on bacteria during their  
291 120-h incubation with that compound. The percentages of viable cells were similar before and after  
292 treatment with water (99.4% and 91.9%, respectively for non-adapted cells and 55.7% and 63.3%  
293 respectively for THY-adapted cells). Thus, the treatment with water (used here as the negative disinfection  
294 control) seems to have no significant effect on cell viability. Nevertheless, this rinsing step provoked the  
295 detachment of cells from the surfaces since the percentage of green (total) surface fluorescence dropped  
296 from  $3.22 \pm 0.16\%$  to  $1.36 \pm 0.11\%$  for non-adapted cells and from  $2.35 \pm 0.27\%$  to  $0.90 \pm 0.39\%$  for THY-  
297 adapted cells, before and after rinsing, respectively. Concerning disinfection, the treatment with THY at  
298 312.5 ppm induced an important loss of viable cells from 99.4% to 20.8% of total sessile cells when biofilms  
299 had been previously grown without THY. However, the growth in the presence of THY induced a  
300 subsequent increased resistance of the sessile bacteria to disinfection with that compound, with percentages  
301 of viable cells only falling from 55.7% to 43.2%. Similar (cross)adaptation effects were observed with BAC  
302 treatment where the recorded percentages of remaining viable cells were found equal to 25.0 and 30.4% for  
303 not adapted and THY-adapted bacteria, respectively. **Fig. S1** presents some characteristic fluorescence

304 photos of the sessile communities encountered on the SS surfaces following each treatment for cells grown  
305 either in the absence or presence of sub-MIC of THY.

306

#### 307 **4. Discussion**

308 Several previous studies have shown the strong bactericidal activity of THY against many  
309 microorganisms, including *S. enterica* (Nabavi et al., 2015). In the current study, the antimicrobial  
310 efficiency of this natural terpenoid was tested against biofilm cells of a *S. Typhimurium* strain of epidemic  
311 phage type DT193 (FMCC\_B137), and more importantly compared to BAC, a common biocide widely  
312 used as surface disinfectant in home, healthcare, and industrial settings. Strains of phage type DT193 are  
313 commonly associated to outbreaks of human infections and are also frequently presenting multidrug  
314 resistance (Hampton et al., 1995; Wuyts et al., 2013). The MICs and MBCs of both compounds were  
315 initially determined against planktonically grown bacteria (incubated in dTSB at 20 °C for 24 h), finding  
316 rather expected values. Thus, the determined MICs were quite close to the ones previously described for  
317 *Salmonella*, that is <500-1000 ppm for THY (Boskovic et al., 2017; Čabarkapa et al., 2019; Chauhan &  
318 Kang, 2014) and <50 ppm for BAC (Kampf, 2018; Morrissey et al., 2014). The MIC found here for the  
319 synthetic biocide (i.e., 20 ppm) denotes the sensitivity of the tested *Salmonella* isolate, considering that the  
320 proposed epidemiological cut-off (ECOFF) value defining resistance (for that biocide and bacterial species)  
321 has been previously set to 128 ppm (Morrissey et al., 2014). For both compounds, MBCs were almost twice  
322 the respective MICs, which is also again something commonly described (Čabarkapa et al., 2019; Morrissey  
323 et al., 2014). The MBICs and MBECs targeted against biofilm bacteria were then determined by leaving  
324 biofilms to be formed on PS microtiter plates in dTSB at 20°C. It should be noted that both a diluted growth  
325 medium (i.e., dTSB vs TSB) and an incubation temperature lower than the optimum one (i.e., 15 °C vs 37  
326 °C) had previously been found to favor biofilm formation by the strain tested here (Kostaki et al., 2012).  
327 This result was reconfirmed in the conditions tested here through some preliminary experiments on PS (data  
328 not shown).

329 It was here found that the application of THY at  $\frac{1}{2}$  of its MIC (i.e., 78.1 ppm) caused a significant  
330 inhibition (by 57.5%) of *Salmonella* biofilm formation (PS microtiter plates, dTSB, 20°C for 120h) (**Fig.**  
331 **1A**). Similarly, a previous study with two *S. Enteritidis* strains (one expressing the *rdar* morphotype,  
332 whereas the other the *bdar* one) found that the use of THY at  $\frac{1}{2}$  of its MIC resulted in 50% inhibition of  
333 the biofilm formed by the *rdar* strain (PS microtiter plates, TSB, 25 °C for 48 h), while the application of  
334 the terpenoid at just the  $\frac{1}{4}$  of its MIC (i.e., 39 ppm) was enough to cause the same effect against the *bdar*  
335 strain (Čabarkapa et al., 2019). In the current study, the application of THY at  $\frac{1}{2}$  of its MIC inhibited biofilm  
336 growth without causing any evident reduction in the number of planktonic bacteria found in the same wells  
337 indirectly expressed by the culture absorbance ( $A_{620\text{ nm}}$ ) (**Fig. 1A**). However, the accurate evaluation of the  
338 bacterial planktonic growth, through direct enumeration of the culturable population at different time  
339 intervals (for up to 48 h), revealed that this sub-MIC of THY was still able to significantly reduce ( $P <$   
340 0.05) the growth rate of the bacteria and in parallel their maximum (final) population densities (**Fig. S2**).  
341 This inhibitory effect of THY on planktonic proliferation could thus probably account, among other  
342 parameters, for the strong delay in sessile proliferation and thus in biofilm formation (on both PS and SS,  
343 by 57.5% and 1.8  $\log_{10}$  CFU/cm<sup>2</sup>, respectively). It should still however be noted that the rate of planktonic  
344 growth of a given bacterium may be independent of its biofilm forming ability (Díez-García et al., 2012;  
345 Lianou & Koutsoumanis, 2012), with the latter to be significantly influenced by several other parameters,  
346 such as the auto-aggregation ability of the cells, their ability to attach them-selves to the surface, their  
347 (swarming) motility, intercellular interactions etc. (Giaouris et al., 2015; Lamas et al., 2018).

348 The MBIC of BAC (40 ppm) that was here determined corresponds to a double concentration of its  
349 MIC. Although several studies have been till now published related to the antimicrobial action of BAC  
350 against *Salmonella* biofilms, all of those were related to already established communities (Corcoran et al.,  
351 2014; Karampoula et al., 2016; Mangalappalli-Illathu & Korber, 2006; Mangalappalli-Illathu et al., 2008;  
352 Wong et al., 2010). To the best of our knowledge, no other data exist on its minimum concentration able to  
353 inhibit *Salmonella* sessile growth. Considering that this MBIC of BAC can drastically inhibit the



354 proliferation of (or even probably kill) the planktonic bacteria, it is quite likely that the observed inhibition  
355 on biofilm growth was just due to the inhibition of cellular proliferation. Nevertheless, although BAC was  
356 also still able to delay the planktonic proliferation of the bacteria found in the same wells upon applied at  
357 its MIC and below (i.e., from 20 to 1.25 ppm), at the same time biofilm development did not seem to be  
358 affected (Fig. 1B), something clearly denoting the different physiology and resistance of the two growth  
359 modes. The doubling of the minimum required concentration of BAC to inhibit the planktonic growth of  
360 *Salmonella*, from 20 to 40 ppm (**Fig. 1B**), when growth was done for either 24 or 120 h, respectively, could  
361 probably be due to the progressive adaptation with time of the bacteria to that biocide. That adaptation of  
362 *Salmonella* to BAC has indeed been previously shown (Capita et al., 2017; Mangalappalli-Illathu & Korber,  
363 2006; Mangalappalli-Illathu et al., 2008).

364 The determined MBEC values were quite similar for THY and BAC (i.e., 1250 and 1280 ppm,  
365 respectively). However, the better anti-biofilm potential of the natural terpenoid compared to the synthetic  
366 biocide is still evident, when someone also considers their killing effectiveness against the planktonic  
367 bacteria (i.e., MBC values). Thus, THY was able to totally eradicate formed biofilm on PS upon applied at  
368 just four times its MBC, whereas BAC needed to be applied at 36.6 x MBC for the same purpose. This  
369 better anti-biofilm potential of THY was further confirmed when the two antimicrobials were applied  
370 against *Salmonella* biofilms established on SS surfaces (either in the presence or absence of sub-MIC of  
371 THY). In that case, the application of THY at its MBC (i.e., 312.5 ppm) was always much more effective  
372 against the sessile cells compared to the application of BAC at double its MBC (i.e., 70 ppm) (**Fig. 2**). In  
373 accordance with current results, this remarkable anti-biofilm potential of THY over BAC has also been  
374 recently shown against two widespread *Staphylococcus* species (i.e., *S. aureus* and *S. epidermidis*)  
375 (Kostoglou et al., 2020).

376 However, although THY was here proven to be quite efficient at eradicating *Salmonella* biofilm bacteria  
377 on both PS surfaces and SS coupons, being able to provoke a *ca.* 5-log reduction of the latter upon applied  
378 at just its MBC (i.e., 312.5 ppm = 2 x MIC), the significant increase in resistance of those cells when their

379 previous sessile growth was done in the presence of sub-MIC of THY (i.e., 78.1 ppm) is still of concern  
380 (**Fig. 2**). This strong co-adaptation effect was evident by both agar plating and the fluorescence staining  
381 results (**Table 1** and **Fig. S1**). In addition, although not so significant, this cellular adaptation to THY seems  
382 to also result in a cross-resistance effect when these THY-adapted cells were exposed to BAC. Probably in  
383 agreement with that, a positive correlation between resistance to THY and BAC has also been previously  
384 revealed for a collection of 21 antibiotic-resistant, biocide-tolerant *Salmonella* strains from hen eggshells  
385 (Márquez et al., 2018). Although the (co- and cross-) adaptation here observed was probably not so likely  
386 to happen, considering the previously described multi-target (non-specific) antimicrobial action of THY  
387 and other EOs components (Nabavi et al., 2015), some previous studies have still also revealed the adaptive  
388 response and increased survival of *Salmonella* bacteria following exposure to sublethal concentrations of  
389 THY (Di Pasqua et al., 2006, 2010; Dubois-Brissonnet et al., 2011), due to changes in membrane fatty acid  
390 composition and up-regulation of some stress and outer membrane proteins.

391

## 392 **5. Conclusions**

393 THY was proven quite effective against biofilm-enclosed bacteria of a *S. Typhimurium* phage type  
394 DT193 strain found on either PS or SS surfaces. Interestingly, this anti-biofilm action was quite evident at  
395 concentrations close to the one needed to kill the planktonic cells, that is the MBC (i.e., 312.5 ppm). Thus,  
396 THY needed to be applied at just four times its MBC (i.e., 1250 ppm) to totally eradicate a sessile population  
397 of more than  $10^7$  CFU/cm<sup>2</sup> found on PS, while its application at its MBC (i.e., 312.5 ppm) provoked *ca.* 5-  
398 log reduction of a population of more than  $10^6$  CFU/cm<sup>2</sup> found on SS. On the other hand, BAC need to be  
399 applied at 36.6 times its MBC (i.e., 1280 ppm) to eradicate that *Salmonella* population on PS, while its  
400 application at two times its MBC (i.e., 70 ppm) reduced less than two logs those pathogenic biofilm bacteria  
401 found on SS. However, the previous growth of the bacteria in the presence of sub-MIC of THY (i.e., 78.1  
402 ppm = ½ MIC), although capable to significantly reduce the formed biofilms on both surfaces (by 57.5%  
403 and 1.8 log<sub>10</sub> CFU/cm<sup>2</sup>; for PS and SS, respectively), resulted at the same time in an increased resistance of

404 the remaining viable strongly attached cells (on SS) towards the actions of either THY or BAC. Such co-  
405 and cross-adaptation effects should be surely considered when novel anti-biofilm approaches, like the ones  
406 based on natural phytochemicals, are designed and implemented to get rid of pathogenic and other  
407 detrimental microbial biofilms, within the food industry and elsewhere.

408

#### 409 **Conflict of Interest**

410 The authors declare no conflict of interest.

411

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418

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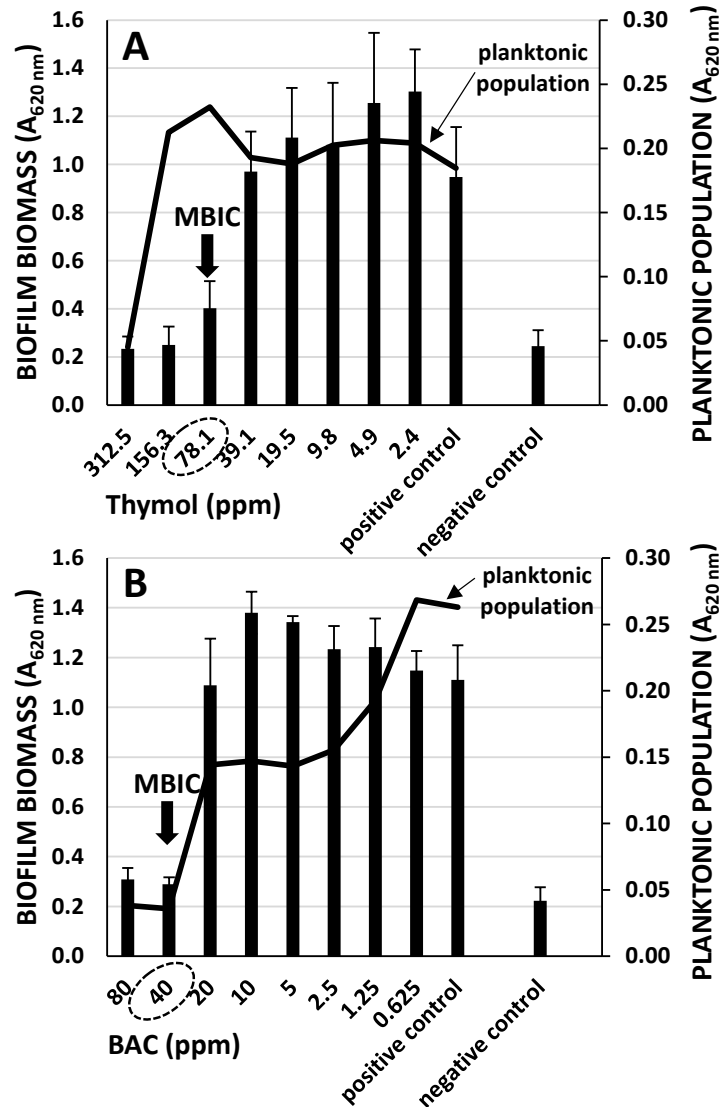
566

567 **Table 1.** Percentages (%) of surface fluorescence with total (green) and permeabilized (red) *S. Typhimurium* bacteria, both before and following the  
 568 15-min treatments (i.e., rinsing with water as negative disinfection control, disinfection with 312.5 ppm THY, disinfection with 70 ppm BAC) of  
 569 the sessile communities developed on the SS surfaces following their incubation at 20 °C for 120 h, in dTSB containing or not sub-MIC of THY  
 570 (i.e., 78.1 ppm = ½ MIC). Percentages of fluorescence are mean values ± standard errors ( $n = 40$ ). The percentages of viable cells were extracted  
 571 from the mean results of surface fluorescences.

prior growth	treatment	percentages of fluorescence (%)		percentages of viable cells (not permeabilized) (%)
		total bacteria (green fluorescence)	permeabilized bacteria (red fluorescence)	
without THY	before disinfection	3.22 ± 0.16	0.02 ± 0.003	99.4
	dH <sub>2</sub> O (negative disinfection control)	1.36 ± 0.11	0.11 ± 0.02	91.9
	disinfection with THY (312.5 ppm)	2.02 ± 0.23	1.60 ± 0.23	20.8
	disinfection with BAC (70 ppm)	1.56 ± 0.09	1.17 ± 0.15	25.0
with THY (78.1 ppm)	before disinfection	2.35 ± 0.27	1.04 ± 0.10	55.7
	dH <sub>2</sub> O (negative disinfection control)	0.90 ± 0.39	0.33 ± 0.17	63.3
	disinfection with THY (312.5 ppm)	0.44 ± 0.02	0.25 ± 0.02	43.2
	disinfection with BAC (70 ppm)	1.02 ± 0.14	0.71 ± 0.19	30.4

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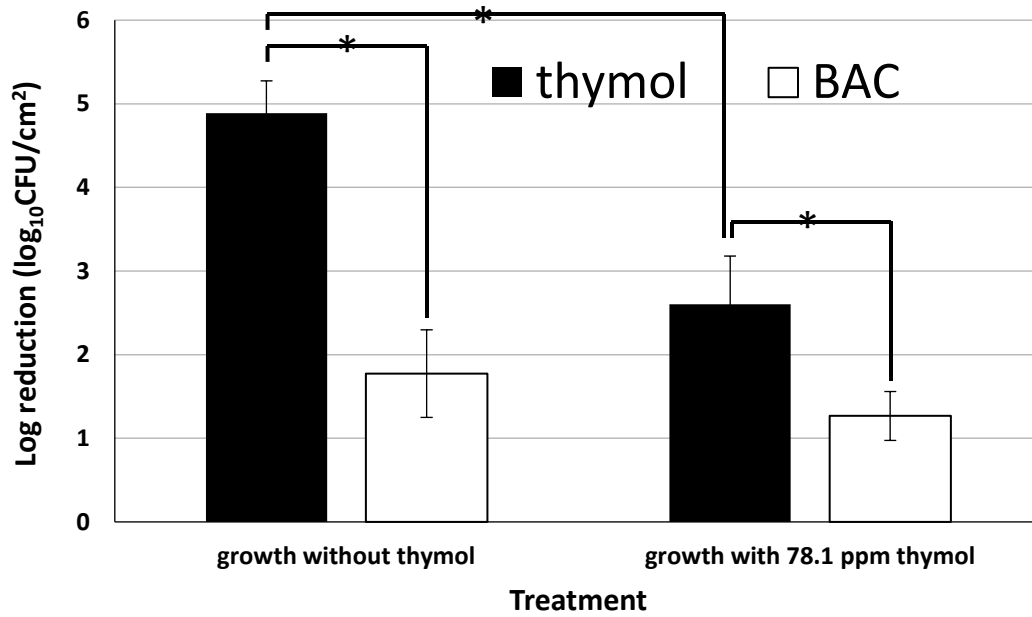
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574

575 **Fig 1.** Biofilm formation ( $A_{620\text{ nm}}$ ) by *S. Typhimurium* strain FMCC\_B137 on PS in the presence of eight  
 576 different concentrations for each compound, ranging from 312.5 to 2.4 ppm for THY (A) and from 80 to  
 577 0.625 ppm for BAC (B), respectively. The bars represent the mean values  $\pm$  standard deviations ( $n = 12$ ,  
 578 three independent experiments, each performed four times). The accumulated biofilm biomasses for  
 579 positive and negative controls are also shown (without antimicrobial and without bacteria, respectively). At  
 580 all cases, biofilms were left to be formed on PS at 20 °C for 120 h in dTSB, with medium renewal at 48 h,  
 581 and were finally quantified by the crystal violet assay. Big arrows indicate the MBIC for each compound  
 582 (resulting in drastic reduction in biofilm biomass). The absorbance of planktonic suspensions ( $A_{620\text{ nm}}$ )  
 583 found in the wells at the time of sampling (120 h) are also shown for each compound and concentration  
 584 (thick curved lines). The bars of standard deviations of the planktonic means ( $n = 12$ ) were omitted for  
 585 clarity reasons in data presentation.

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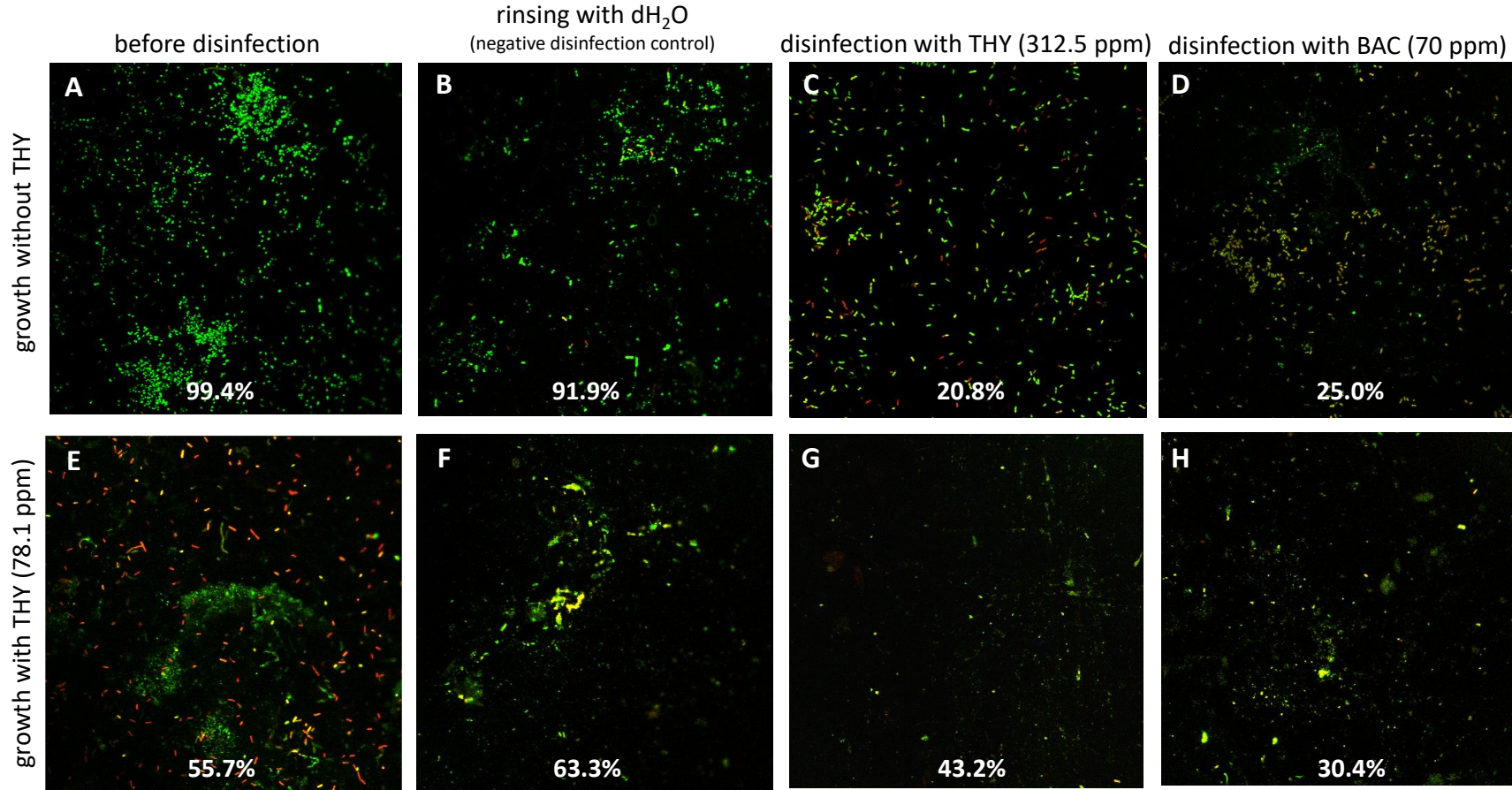
588 **Fig 2.** Log reductions (log<sub>10</sub> CFU/cm<sup>2</sup>) of sessile communities of *S. Typhimurium* strain FMCC\_B137  
589 developed on SS coupons incubated at 20 °C for 120 h, in dTSB containing or not 78.1 ppm of THY (= ½  
590 MIC = MBIC) following their 15-min disinfection with either 312.5 ppm THY (■) or 70 ppm BAC (□).  
591 The bars represent mean values ± standard deviations (*n* = 6). Asterisks denote significant differences (*P* <  
592 0.05).

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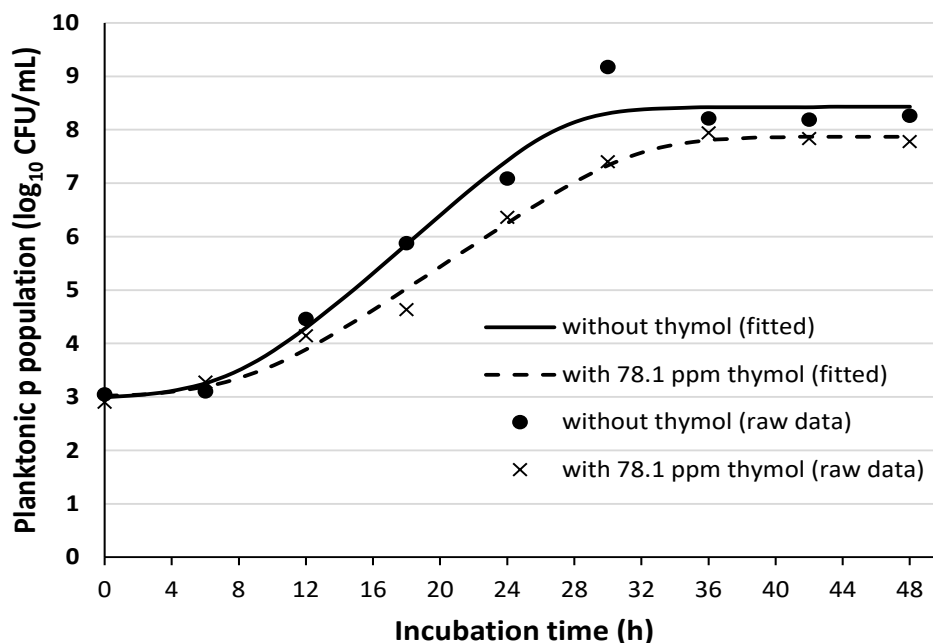


597

598 **Fig S1.** Sessile communities of *S. Typhimurium* bacteria encountered on the SS surfaces both before (A, E) and following the 15-min treatment with  
 599 water (negative disinfection control; B, F), THY (312.5 ppm; C, G), and BAC (70 ppm; D, H). SS coupons had been previously incubated at 20 °C  
 600 for 120 h, in dTSB in the absence (A, B, C, D) or presence (E, F, G, H) of sub-MIC of THY (78.1 ppm = ½ MIC). SYTO® 9 stains all bacteria in  
 601 green, whereas those permeabilized with damaged membranes are stained in red (with PI). The calculated percentage of viable cells (upon total  
 602 cells) is given on each image.

603

604



growth	growth rate ( $\mu_{\max}$ )	maximum population density (log <sub>10</sub> CFU/mL)	$R^2$ (fitting)
without THY	$0.27 \pm 0.04$	$8.45 \pm 0.07$	$0.96 \pm 0.01$
with THY (78.1 ppm)	$0.19 \pm 0.04$	$7.78 \pm 0.05$	$0.97 \pm 0.01$

605

606 **Fig S2.** Characteristic growth curves of *S. Typhimurium* strain FMCC\_B137 planktonic cultures left to  
607 statically grow in dTSB, containing or not sub-MIC of THY (i.e., 78.1 ppm = 1/2 MIC), at 20 °C for 48 h.  
608 Both raw (●, x) and fitted data (solid and dashed lines; for growth without and with THY, respectively) are  
609 presented. The growth rates ( $\mu_{\max}$ ; log<sub>10</sub> CFU/mL/h) and maximum population densities (log<sub>10</sub> CFU/mL)  
610 for each treatment are also shown. Data are mean values  $\pm$  standard deviations ( $n = 4$ ) and were extracted  
611 by fitting the obtained (raw) growth data with the complete Baranyi and Roberts model using the online  
612 version of DMFit available at ComBase (<https://www.combase.cc/>). The average regression coefficients  
613 ( $R^2$ ) of the fitted regression plots are also presented.

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