ORIGINAL RESEARCH ARTICLE

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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Highlights

- Thymol presents better anti-biofilm potential compared to benzalkonium chloride.
- Thymol presence at ½ its MIC significantly decreases biofilm formation.
- The application of thymol at just four times its MBC eradicates *Salmonella* biofilm.
- Thymol adapted biofilm cells present increased resistance to thymol and BAC.

A B S T R A C T

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

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Bacterial strain and preparation of the working cell suspension

The bacterial strain used in this work was a human isolate of *S.* Typhimurium epidemic phage type DT193 (strain FMCC_B137) which was kindly provided by Professor G.-J. Nychas (Agricultural University of Athens, Greece). Before its experimental use, this was kept frozen (at −80 °C) in Tryptone Soy Broth (TSB; Lab M, Heywood, Lancashire, UK) containing 15% glycerol and was revitalized when needed by streaking a loopful of its frozen suspension on to the surface of Tryptone Soy Agar (TSA; Lab M) and incubating at 37 °C for 24 h (preculture). Working culture was prepared by inoculating a colony from the preculture into 10 mL of fresh TSB and incubating at 37 °C for 18 h. Bacteria from that final working culture were harvested by centrifugation (4000 × *g* for 10 min at room temperature), washed twice with quarter-strength Ringer's solution (Lab M), and finally suspended in the same solution, to display an absorbance at 600 nm ($A_{600\ nm}$) equal to 0.1 (*ca.* 10^8 CFU/mL).

2.2. *Biocides and preparation of their stock solutions*

Thymol (THY) was purchased from Penta Chemicals (Radiová, Prague, Czech Republic) (powder min. 99.0%, molar mass: 150.22 g/mol; product code: 27450-30100), while benzalkonium chloride (BAC) was acquired from Acros Organics (Thermo Fisher Scientific, Geel, Belgium) (liquid, alkyl distribution from C8H17 to C16H33; product code: 215411000). Two stock solutions were prepared for THY, both in absolute ethanol, at 10% w/v and 40% w/v, for subsequent application against the planktonic and biofilm cells, respectively, following appropriate dilutions (see below for further details), while the stock solution of BAC (1% v/v) was prepared in sterile distilled water. All stock solutions were maintained in airtight falcon tubes at 4 ºC for up to 2 weeks.

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

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

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

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

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

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

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

S. Typhimurium strain FMCC_B137 was left to form biofilms on rectangular SS coupons (3×1×0.1 cm, type AISI 304) as previously described (Iliadis et al., 2018). Briefly, individual (cleaned and sterilized) coupons were initially incubated for 2 h at 20 °C in saline bacterial suspension (*ca.* 10^8 CFU/mL), to allow bacteria to attach onto them, loosely attached cells were then removed by rinsing (with quarter-strength Ringer's solution), and coupons were finally incubated in dTSB containing or not a sub-inhibitory THY 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 allow biofilm growth. Following biofilm formation, each SS coupon was thoroughly washed with quarter-strength Ringer's solution (to remove the loosely attached cells) and placed for 15 min at 20 °C in each disinfectant solution. For this experiment, THY was applied at its MBC (i.e., 312.5 ppm), BAC at double its MBC (i.e., 70 ppm), while sterile distilled water (also containing 0.3% v/v ethanol when THY was tested) was used as the negative disinfection control. The applied concentration for BAC was based on preliminary experiments showing negligible killing action against biofilm cells when this chemical was applied at its MBC (i.e., 35 ppm). Following disinfection, each coupon was washed with quarter-strength Ringer's solution (to remove any disinfectant residues) and placed, for 10 min, in a 10 mL-plastic falcon tube containing 6 mL of Dey-Engley (D-E) Neutralizing broth (Lab M) and 10 glass beads (3 mm in diameter). Biofilm cells were then detached from the SS surface through its strong 2-min vortexing (at 3000 rpm using ZX3 Advanced Vortex Mixer; VELP Scientifica Srl; Usmate, Italy) and were finally enumerated

206 by counting colonies on (duplicate) spot inoculated (10 µL) 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^2 and for each compound, the logarithmic reduction ($\log_{10}$ CFU/cm^2) 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.

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

214 The LIVE/DEAD® *Bac*Light™ 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® 9 and propidium iodide (PI),
219 with excitation/emission maxima at about 480/500 nm and 490/635 nm, respectively. SYTO® 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 Microsystemes SAS, France) at the MIMA2 imaging platform
227 (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

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

2.7. Statistics

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

3. Results

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

To inhibit the visible planktonic growth (in dTSB at 20 ºC for 24 h) of the *S.* Typhimurium strain FMCC_B137, THY and BAC needed to be applied at 156.3 and 20 ppm, respectively, while a double concentration was approximately required for both compounds to kill (by ≥ 99.9%) the planktonic bacteria (with MBC values equal to 312.5 and 35 ppm, respectively). Regarding the ability of each compound to inhibit biofilm formation, **Fig. 1** analytically shows the biofilm biomasses recorded on PS at each one of the eight different tested concentrations for each one, together with the planktonic populations found in each well just before their removal for biofilm quantification (both expressed as absorbances at 620 nm). THY could drastically inhibit biofilm formation (by 57.5%) compared to the positive control (no antimicrobial added) when applied at ½ of its MIC (MBIC 78.1 ppm), whereas BAC caused a 74% biofilm

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

3.2. Biofilm formation on SS surfaces and disinfection

The counts of the sessile populations found on SS coupons following their incubation, at 20 ºC for 120 h, in dTSB containing or not sub-MIC of THY (i.e., ½ MIC = 78.1 ppm) were 4.59 ± 0.56 and 6.39 ± 0.19 $\log_{10}$ CFU/cm^2, respectively. Thus, the presence of THY at its MBIC (as this was determined for biofilms formed on PS) was found to strongly reduce (*ca.* 100-fold) the *Salmonella* population encountered on the SS surface. The results on the disinfection of those communities are presented in **Fig. 2**. The application of THY at its MBC (i.e., 312.5 ppm) was always much more effective against sessile cells compared to the application of BAC at double its MBC (i.e., 70 ppm), with *ca*. a 10-1000 fold difference in efficiencies between those two compounds, depending on whether the incubation had been previously done in either the presence or absence of sub-MIC of THY. Thus, when cells had not been previously adapted, 312.5 ppm of the terpenoid were enough to provoke a *ca*. 5-log reduction of the viable sessile population (4.89 ± 0.39 $\log_{10}$ CFU/cm^2), while under such conditions the reduction did not exceeded 2 logs being limited to 1.77 ± 0.52 $\log_{10}$ CFU/cm^2 when BAC was applied at 70 ppm. On the other hand, the previous sessile growth in the presence of sub-MIC of THY (i.e., 78.1 ppm) resulted in the subsequent increased resistance of the sessile cells to disinfection with that compound, indicating thus a strong co-adaptation effect. In that case,

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

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

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

4. Discussion

Several previous studies have shown the strong bactericidal activity of THY against many microorganisms, including *S. enterica* (Nabavi et al., 2015). In the current study, the antimicrobial efficiency of this natural terpenoid was tested against biofilm cells of a *S*. Typhimurium strain of epidemic phage type DT193 (FMCC_B137), and more importantly compared to BAC, a common biocide widely used as surface disinfectant in home, healthcare, and industrial settings. Strains of phage type DT193 are commonly associated to outbreaks of human infections and are also frequently presenting multidrug resistance (Hampton et al., 1995; Wuyts et al., 2013). The MICs and MBCs of both compounds were initially determined against planktonically grown bacteria (incubated in dTSB at 20 ^{o}C for 24 h), finding rather expected values. Thus, the determined MICs were quite close to the ones previously described for *Salmonella*, that is <500-1000 ppm for THY (Boskovic et al., 2017; Čabarkapa et al., 2019; Chauhan & Kang, 2014) and <50 ppm for BAC (Kampf, 2018; Morrissey et al., 2014). The MIC found here for the synthetic biocide (i.e., 20 ppm) denotes the sensitivity of the tested *Salmonella* isolate, considering that the proposed epidemiological cut-off (ECOFF) value defining resistance (for that biocide and bacterial species) has been previously set to 128 ppm (Morrissey et al., 2014). For both compounds, MBCs were almost twice the respective MICs, which is also again something commonly described (Čabarkapa et al., 2019; Morrissey et al., 2014). The MBICs and MBECs targeted against biofilm bacteria were then determined by leaving biofilms to be formed on PS microtiter plates in dTSB at 20°C. It should be noted that both a diluted growth medium (i.e., dTSB *vs* TSB) and an incubation temperature lower than the optimum one (i.e., 15 ^{o}C *vs* 37 ^{o}C) had previously been found to favor biofilm formation by the strain tested here (Kostaki et al., 2012). This result was reconfirmed in the conditions tested here through some preliminary experiments on PS (data not shown).

It was here found that the application of THY at ½ of its MIC (i.e., 78.1 ppm) caused a significant inhibition (by 57.5%) of *Salmonella* biofilm formation (PS microtiter plates, dTSB, 20°C for 120h) (**Fig. 1A**). Similarly, a previous study with two *S.* Enteritidis strains (one expressing the *rdar* morphotype, whereas the other the *bdar* one) found that the use of THY at ½ of its MIC resulted in 50% inhibition of the biofilm formed by the *rdar* strain (PS microtiter plates, TSB, 25 ^{o}C for 48 h), while the application of the terpenoid at just the ¼ of its MIC (i.e., 39 ppm) was enough to cause the same effect against the *bdar* strain (Čabarkapa et al., 2019). In the current study, the application of THY at ½ of its MIC inhibited biofilm growth without causing any evident reduction in the number of planktonic bacteria found in the same wells indirectly expressed by the culture absorbance ($A_{620\ nm}$) (**Fig. 1A**). However, the accurate evaluation of the bacterial planktonic growth, through direct enumeration of the culturable population at different time intervals (for up to 48 h), revealed that this sub-MIC of THY was still able to significantly reduce ($P < 0.05$) the growth rate of the bacteria and in parallel their maximum (final) population densities (**Fig. S2**). This inhibitory effect of THY on planktonic proliferation could thus probably account, among other parameters, for the strong delay in sessile proliferation and thus in biofilm formation (on both PS and SS, by 57.5% and 1.8 $\log_{10}$ CFU/cm^2, respectively). It should still however be noted that the rate of planktonic growth of a given bacterium may be independent of its biofilm forming ability (Díez-García et al., 2012; Lianou & Koutsoumanis, 2012), with the latter to be significantly influenced by several other parameters, such as the auto-aggregation ability of the cells, their ability to attach them-selves to the surface, their (swarming) motility, intercellular interactions etc. (Giaouris et al., 2015; Lamas et al., 2018).

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

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

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

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

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

5. Conclusions

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

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

Conflict of Interest

The authors declare no conflict of interest.

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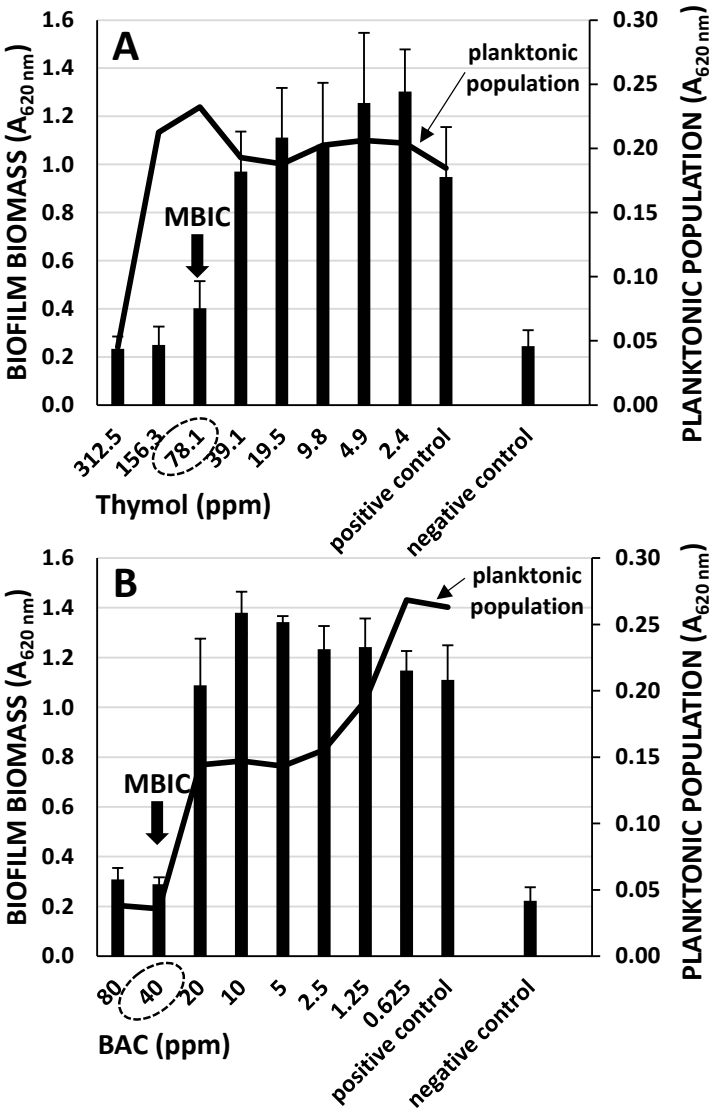
566

Table 1. Percentages (%) of surface fluorescence with total (green) and permeabilized (red) *S.* Typhimurium bacteria, both before and following the 15-min treatments (i.e., rinsing with water as negative disinfection control, disinfection with 312.5 ppm THY, disinfection with 70 ppm BAC) of 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 (i.e., 78.1 ppm = ½ MIC). Percentages of fluorescence are mean values ± standard errors (n = 40). The percentages of viable cells were extracted 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_2O (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_2O (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



574

575 **Fig 1.** Biofilm formation ($A_{620\ nm}$) 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 ± 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 ($A_{620\ nm}$)
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.

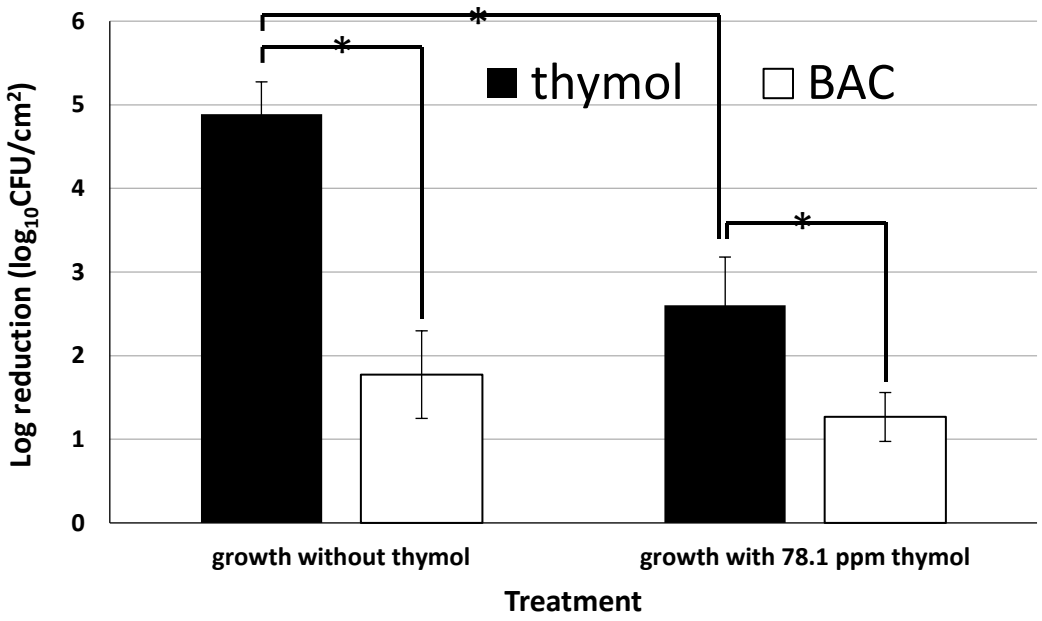


Fig 2. Log reductions (log_{10} CFU/cm^2) of sessile communities of *S*. Typhimurium strain FMCC_B137 developed on SS coupons incubated at 20 ºC for 120 h, in dTSB containing or not 78.1 ppm of THY (= ½ MIC = MBIC) following their 15-min disinfection with either 312.5 ppm THY (■) or 70 ppm BAC (□). The bars represent mean values ± standard deviations (n = 6). Asterisks denote significant differences (P < 0.05).

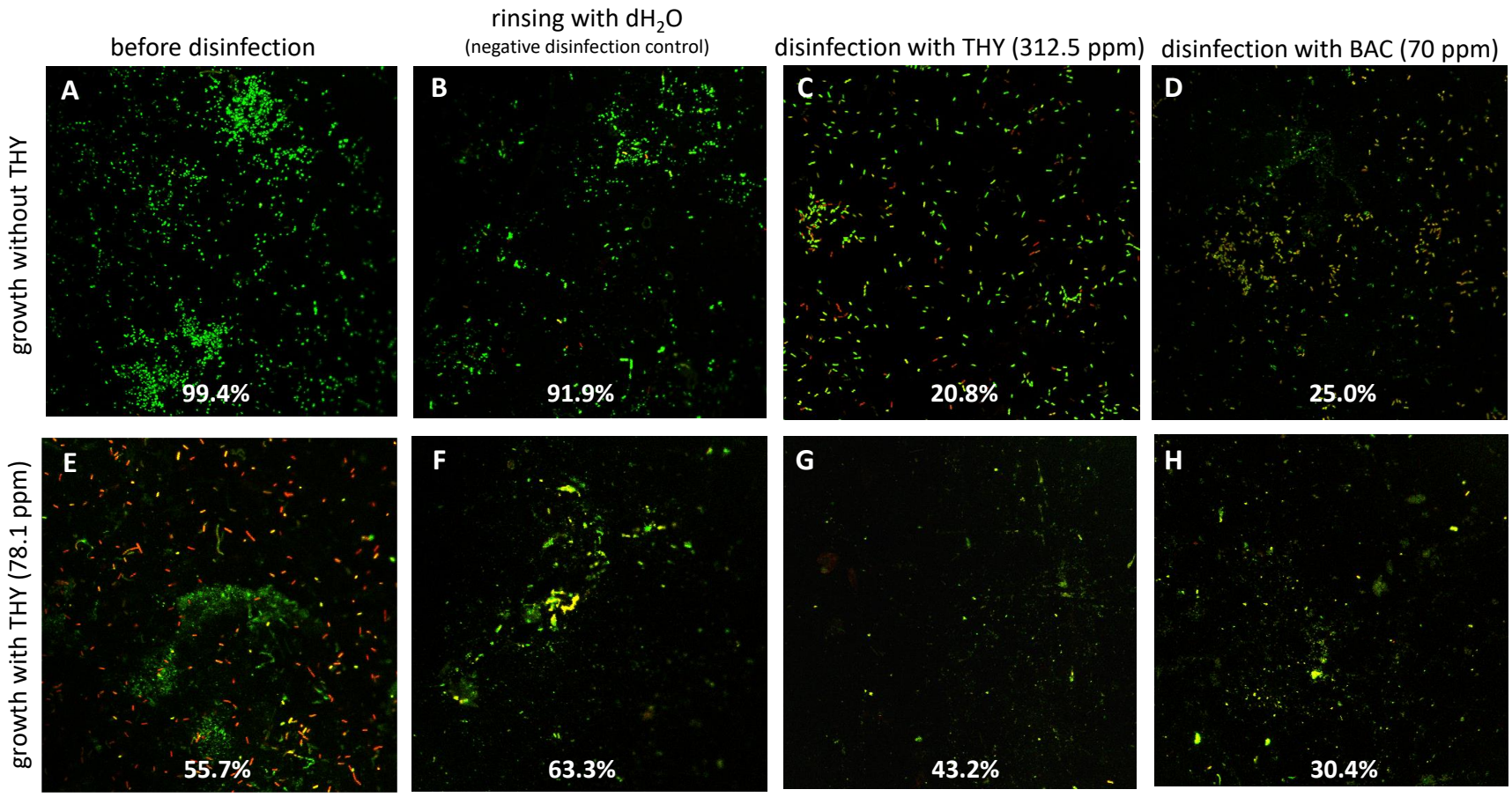
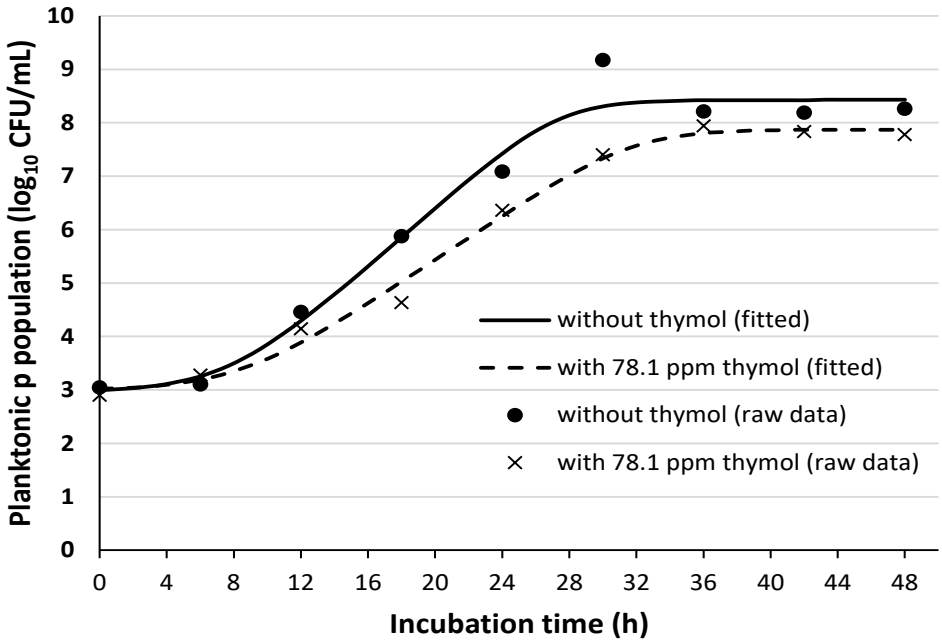


Fig S1. Sessile communities of *S.* Typhimurium bacteria encountered on the SS surfaces both before (A, E) and following the 15-min treatment with water (negative disinfection control; B, F), THY (312.5 ppm; C, G), and BAC (70 ppm; D, H). SS coupons had been previously incubated at 20 °C for 120 h, in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



growth	growth rate (μ_{max})	maximum population density (log_{10} CFU/mL)	R^2 (fitting)
without THY	0.27 ± 0.04	8.45 ± 0.07	0.96 ± 0.01
with THY (78.1 ppm)	0.19 ± 0.04	7.78 ± 0.05	0.97 ± 0.01

Fig S2. Characteristic growth curves of *S.* Typhimurium strain FMCC_B137 planktonic cultures left to statically grow in dTSB, containing or not sub-MIC of THY (i.e., 78.1 ppm = ½ MIC), at 20 °C for 48 h. Both raw (●, x) and fitted data (solid and dashed lines; for growth without and with THY, respectively) are presented. The growth rates (μ_{max}; log_{10} CFU/mL/h) and maximum population densities (log_{10} CFU/mL) for each treatment are also shown. Data are mean values ± standard deviations (n = 4) and were extracted by fitting the obtained (raw) growth data with the complete Baranyi and Roberts model using the online version of DMFit available at ComBase (https://www.combase.cc/). The average regression coefficients (R^2) of the fitted regression plots are also presented.