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Hydrosol of *Thymbra capitata* is a highly efficient biocide against biofilms of *Salmonella* Typhimurium: real-time visualization of bacterial inactivation by CLSM

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RUNNING TITLE: *T. capitata* hydrosol is an efficient antibiofilm agent

Abstract

Salmonella is recognized as one of the most significant enteric foodborne bacterial pathogens. In recent years, the resistance of pathogens to biocides and other environmental stresses, especially when these are embedded in biofilm structures, has led to the search and development of novel antimicrobial strategies capable of displaying both high efficiency and safety. To this direction, the aim of the present work was to evaluate the antimicrobial activity of hydrosol of the Mediterranean spice *Thymbra capitata* against both planktonic and biofilm cells of *Salmonella* Typhimurium, and to compare its action with the one of benzalkonium chloride (BC), a commonly used industrial biocide. In order to achieve this, the disinfectant activity following 6 min treatments was comparatively evaluated for both disinfectants by calculating their concentrations needed to achieve the same log-reductions against both types of cells. Their bactericidal effect against biofilm cells was also comparatively determined by *in situ* and real-time visualization of cell inactivation through the use of time-lapse confocal laser scanning microscopy (CLSM). Interestingly, results revealed that hydrosol was almost equally effective against both biofilms and planktonic cells, whereas 200 times higher concentration of BC was needed to achieve the same effect against biofilm compared to planktonic cells. Similarly, time-lapse CLSM revealed the significant advantage of the hydrosol to easily penetrate within the biofilm structure and quickly kill the cells, despite the 3D- structuration of *Salmonella* biofilm.

Importance

The results of this paper highlight the significant antimicrobial action of a natural compound that is hydrosol of *T. capitata*, against both planktonic and biofilm cells of a common foodborne pathogen. Hydrosol has numerous advantages as disinfectant of

food-contact surfaces. It is an aqueous solution which can easily be rinsed out from surfaces, it does not have the strong smell of the EO and it is a by-product of EOs distillation procedure without any industrial application. Consequently, hydrosol obviously could be of great value to combat biofilms and thus to improve product safety not only for the food industries, but probably also for many other industries which experience problems related to biofilms.

Keywords: Disinfection, hydrosol, natural antimicrobial agent, benzalkonium chloride, anti-biofilm, confocal laser scanning microscopy

1. Introduction

Salmonella is an important foodborne pathogenic bacterium that remains the most frequent causative agent of food-borne outbreaks occurring in the European Union (EU). In 2013, this accounted for 22.5 % of the total outbreaks reported (1). That year, a total of 82,694 confirmed cases of salmonellosis were reported by 27 EU member states, with an average notification rate of 20.4 cases per 100,000 people. The serovars Enteritidis and Typhimurium were the most commonly involved representing respectively 39.5 % and 20.2 %, of all reported serovars in confirmed human cases. It is also noted that while the most important sources of food-borne *Salmonella* outbreaks are eggs and egg products, *Salmonella* was also detected in many other types of foods as well (1).

Although the native habitat of *Salmonella* is considered to be the intestinal tract of diverse vertebrates, this genus has been shown to be also able to survive for extended periods of time in non-enteric habitats. It is strongly believed that its survival and

71 persistence in these non-host environments may be attributed to its great ability to form
72 biofilms on various inanimate surfaces (2, 3). Biofilms are consortia of microorganisms
73 adherent to each other and/or to a surface and embedded in a scaffold of self-produced
74 extracellular polymeric substances (EPS) (4, 5). Thus, there is extended documentation
75 on the ability of *Salmonella* to attach to various food-contact surfaces (such as stainless
76 steel, plastic and cement) and form biofilms under *in vitro* conditions (6-10), while
77 *Salmonella* cells have also been recovered from surfaces and products of various food
78 industries (11-14). During the last decades, it has become increasingly clear that
79 biofilms are the predominant mode of bacterial life in most environments (15-17).
80 Biofilms formed in food processing environments are of special importance since they
81 may act as a persistent source of product contamination which may lead to food
82 spoilage and / or transmission of diseases (2, 3, 18, 19).

83 Poor sanitation of food-contact surfaces is believed to be an essential
84 contributing factor in foodborne disease outbreaks. Disinfection procedures using either
85 physical or chemical methods have been extensively used over the years to reduce or
86 eliminate bacteria found on food-contact surfaces. However, current methods of
87 sanitation of food facilities are not always sufficiently efficient because of the great
88 resistance displayed by many foodborne bacteria, especially when these are grown into
89 biofilms (15, 20, 21). Several mechanisms have been proposed to be involved in biofilm
90 resistance to disinfectants, including: (i) limitations to the free diffusion of
91 antimicrobial agents through the biofilm matrix, (ii) variability in the physical and
92 chemical microenvironments within the biofilm (e.g., varied conditions of pH, osmotic
93 strength, or nutrients) leading to varied levels of metabolic activity and cell adaptive
94 responses, (iii) mutations and horizontal transfer of genes coding for resistance
95 mechanisms (e.g. detoxifying membrane transporters), (iv) differentiation of bacterial

cells into physiological states less susceptible to treatments (e.g. dormant, viable but not culturable, VBNC), together with the presence of extremely resistant "persister" cells and (v) bacterial protection among multi-species consortium (15, 22).

This persistence of pathogens in food environments despite continuous disinfection led to the search and development of novel antimicrobial strategies capable of displaying both high efficiency and safety. The growing negative consumer perception against artificial synthetic chemicals has shifted this research effort toward the development and application of environmental friendly disinfectants. The latter should display high lethal activity against biofilms in small concentrations, be safe and easily degraded in the environment (15, 16, 23).

Plant extracts can provide unlimited opportunities for microbial control, owing to their great chemical diversity (24-26). In recent years, several reports demonstrating the antibacterial effect of crude essential oils (EOs) and / or their active components against bacteria embedded in biofilms have been published (27-33). However, there are still strict limitations on the practical application of these compounds for the disinfection of industrial surfaces, arising from their strong hydrophobic nature, which hampers their efficient rinsing out from surfaces after a disinfection program, and their intense smell. Hydrosols, which are by-products of the EOs distillation procedure (these are situated just under the organic phase at the end of steam distillation), are aqueous solutions and may thus be easily rinsed out from surfaces. In addition, they do not present the strong smell of the EOs and they do not have any industrial application until now (34). Interestingly, the antimicrobial action of the hydrosol fraction of *Satureja thymbra* EO has been demonstrated against single- and mixed-species biofilms composed of technological, spoilage and pathogenic bacteria (35), but to the best of our knowledge, this is the only report available on the anti-biofilm action of hydrosols. In

addition, antibacterial activity of hydrosols of several aromatic plants against planktonic pathogens has been shown (36-40). Using such compounds for the disinfection of surfaces in food industrial environments could thus be an interesting way to valorize them (16).

For the better characterization and understanding of biofilm resistance mechanisms, development of innovative microscopy techniques, such as confocal laser scanning microscopy (CLSM), together with improvements in fluorescent labeling have emerged. Direct investigation of biocide reactivity within the native structure of biofilms can now provide *in situ* important information on the dynamics of biocide action and spatial heterogeneities of bacterial susceptibility within these structurally and physiologically heterogeneous sessile consortia (41-44).

Taking into account all the previous, the aim of the present work was to evaluate the antimicrobial activity of hydrosol of the Mediterranean spice *Thymbra capitata* against both planktonic and biofilm cells of *Salmonella* Typhimurium and to compare its action with the one of benzalkonium chloride (BC), a commonly used industrial biocide. In order to achieve this, the antimicrobial activities of both disinfectants were first comparatively evaluated against 24 h planktonic and biofilm cells (on stainless steel coupons) by plate counting. Concentrations needed to achieve after 6-min treatments the same log-reductions of both type of cells were thus quantitatively determined. Afterwards, the bactericidal effect of the two tested disinfectants, when these were used at some selected concentrations (based on the previously obtained results), was comparatively evaluated by monitoring in real-time and *in-situ* inactivation of biofilm cells on polystyrene microplates through time-lapse confocal laser scanning microscopy (CLSM) analysis.

2. Materials and methods

2.1. Bacterial strain, growth conditions, and preparation of bacterial suspension

The bacterium used in this study was *Salmonella enterica* subsp. enterica serovar Typhimurim strain CDC 6516-60 (ATCC 14028) isolated from animal tissue of 4-week-old chickens (45). Before each experiment, the microorganism, stored in cryovials at -80°C, was resuscitated in two successive subcultures (7 h and 16 h) in Tryptone Soy Broth (TSB, Biomérieux, France) at 37°C. The working culture was a third subculture, incubated at 20°C for 24h and subsequently harvested by centrifugation (5000 g, 10 min, at 20 °C) for disinfectant testing. For the biofilm formation, the pellets were re-suspended and diluted in 10 ml of 150 mM NaCl in order to obtain a bacterial suspension adjusted at 10^6 cells/mL.

2.2. Antibacterial agents

The hydrosol of *Thymbra capitata* (pharmacy Provata, Athens, Greece) and the quaternary ammonium compound, benzalkonium chloride C14 (BC: MW, 368.04; puriss., anhydrous, 99.0%, Fluka, France) were used to carry out disinfectant tests. Different concentrations of hydrosol (25-75% for planktonic cells; 50-100% for biofilms) and BC (0.0006-0.002 % for planktonic cells; 0.125-0.5 % for biofilms) were prepared by diluting appropriate quantities of each antimicrobial compound in sterilized deionized water the day of the experiment.

2.3. Chemical analysis of Thymbra capitata hydrosol and its related essential oil

T. capitata hydrosol was chemically analyzed by Solid Phase Micro-Extraction followed by Gas Chromatography - Mass Spectrometry (SPME GC/MS) (46).

171 Hydrosol samples (10 mL) together with 3 g NaCl were placed into a 20 mL headspace
172 vial fitted with a Teflon-lined septum sealed with an aluminum crimp seal, through
173 which the SPME fiber 50/30 mm divinylbenzene/carbozen in poly-dimethyl-siloxane 2
174 cm (Supelco, Bellefonte, PA, USA) was introduced. 4-methyl-2-pentanol was used as
175 an internal standard at a final concentration 0.8 mg/L. The mix was equilibrated at 60
176 °C for 45 min. After head-space extraction, the fiber was transferred to the GC injection
177 port where the absorbed compounds were thermally desorbed for 3 min at 240 °C.
178 The essential oil was diluted in pure hexane (1:9), 3 g NaCl were added to 10 mL of
179 diluted oil and the mixture was then chemically analyzed by either SPME GC/MS
180 analysis as described above or by GC/MS analysis following filtration using 0.22 μm
181 filters and direct injection (1 μL). Gas chromatography-mass spectrometry (GC/MS)
182 analysis was carried out with a 6890N system (Agilent Technologies, USA) equipped
183 with an HP-5MS column (30 m, 0.25 mm inner diameter, film thickness 0.25 μm,
184 Agilent technologies) and coupled with an Agilent Technologies 5973Networked mass
185 detector. Column temperature was set at 35 °C for 6 min, increased at 60 °C at the rate
186 of 2 °C/min, kept constant for 5 min, increased again to 200°C at the rate of 5 °C/min
187 and then to 250°C at the rate of 25 °C/min and finished constant for 6 min. Helium was
188 used as the carrier gas (linear velocity of 1.8 or 1.5 mL/min for SPME or direct analysis,
189 respectively). The injector was operated in splitless mode for SPME analysis and in
190 split mode (1:50 split ratio) for direct injection analysis. Both injector and detector
191 temperatures were 240 °C. The mass spectrometer was operated in the electron impact
192 mode with the electron energy set at 70 eV and 45–400m/z scan range. Results acquired
193 were processed by ChemStation integrated software (Agilent Technologies) and
194 constituents were identified by comparing mass spectra with reference spectra from
195 NB575, stack and Wiley275 libraries, by spectra from standard compounds (in house

196 libraries) and by determining kovats' retention indexes and comparing them with those
197 reported in the literature. In SPME GC/MS analysis, the volatile compounds were semi-
198 quantified by dividing the peak areas of the compounds of interest by the peak area of
199 the IS and multiplying this ratio by the initial concentration of the IS (expressed as
200 mg/L), while in direct injection GC/MS analysis the % area of volatile compounds was
201 estimated. The peak areas were measured from the full scan chromatograph using total
202 ion current (TIC). Each experiment was carried out in duplicate and the mean data are
203 presented.
204
205 *2.4. Biofilm formation*
206 Biofilms of *S.* Typhimurium were grown on 1-cm² stainless steel (SS) AISI 204
207 coupons (Goodfellow, Cambridge Science Park, UK) and also on the wells of 96-well
208 polystyrene microtiter plates (Greiner Bio-One 655090, France) with a Clear base
209 (polystyrene; 190±5 µm thick) (47). Before use, the SS coupons were cleaned with
210 surfactant RBS 35 (Société des traitements chimiques de surface, Lambersart, France),
211 rinsed with deionized water and settled in the wells of a 24-well polystyrene microtiter
212 plate (Techno Plastic products, Switzerland) (29, 48). 1 mL or 250 µL of bacterial
213 subculture, as prepared above (~ 10^6 cells/mL), were respectively poured into the wells
214 of the 24-well (containing SS coupons) and 96-well microplates. Adhesion on SS
215 coupons or on polystyrene wells was done by sedimentation for 2 h at 20 °C.
216 Subsequently, the planktonic bacterial suspension was removed and 1 mL (or 250 µL)
217 of TSB was added in each well. Microtiter plates were incubated at 20 °C for 24 h
218 without shaking to allow biofilm development (on either SS coupons or the polystyrene
219 wells).
220

221 *2.5. Antimicrobial testing against planktonic cells*

222 Planktonic cells were challenged with the disinfectants using the EN 1040
223 standard protocol (49). Briefly, 1 ml of a 100-fold diluted solution of the working
224 culture (prepared as previously described) was centrifuged at 5000 g for 10 min.
225 Subsequently, the pellet (~10^7 cells) was resuspended in 1 mL of each antimicrobial
226 solution (at different concentrations) and left in contact for 6 min at 20°C. The
227 antimicrobial action was halted by transfering a volume (1:9) to a quenching solution
228 (3 g/L L-α-phosphatidyl cholin, 30 g/L Tween 80, 5 g/L sodium thiosulfate, 1 g/L L-
229 histidine, 30 g/L saponine) for 10 min. Serial dilutions were then prepared and survivors
230 were enumerated on Tryptic Soy Agar (TSA) using the 6 × 6 drop count method (50).
231 The control was performed in the same way with sterile deionized water instead of the
232 disinfectant. The logarithm reduction achieved was the difference between the $\log_{10}$ of
233 the survivors after the test with deionized water (control) and the $\log_{10}$ of the survivors
234 after the test with the antimicrobial agent. For each condition, three to seven replicates
235 were performed.

236

237 *2.6. Antimicrobial testing against biofilms formed on stainless steel coupons*

238 Following biofilm formation on SS coupons, the planktonic suspension was
239 removed from each well and each coupon was once rinsed with 1 ml of 150 mM NaCl.
240 Afterwards, each coupon was immediately challenged with 1 mL of each disinfectant
241 solution (at different concentrations) for 6 min at 20°C (the same time as for planktonic
242 cells). Antimicrobial action was halted by placing coupons for 10 min at 20°C in the
243 quenching solution, as previously described. The survivors were removed from the
244 surface by scratching with a plastic rake (folded pipette cone) in a standardized way
245 (horizontal/vertical/oblique) and enumerated by plate counting as previously described.

Control was performed in the same way with sterile deionized water instead of the disinfectant and log reductions were calculated as previously described. For each condition, three to height replicates were performed.

2.7. Calculation of resistance coefficients of the two biocides

The resistance coefficient (Rc) was determined for each biocide. Rc is equal to $C_{biofilm}/C_{planktonic}$, where $C_{biofilm}$ corresponds to the biocide concentration required to kill a given level of biofilm cells and $C_{planktonic}$ to the concentration needed to kill the same level of planktonic cells (15).

2.8. Antimicrobial testing against biofilms formed on polystyrene evaluated by time-lapse microscopy

This method allows the direct investigation of biocide reactivity within the native structure of biofilms (42). To achieve this, cells were initially labelled with a viability fluorescent marker and subsequently submitted to disinfection. The antimicrobial action induces cell membrane's permeabilization and subsequently loss of fluorescence that represents cell's death.

Biofilms formed on polystyrene wells were initially stained with the esterase viability marker Chemchrome V6 (AES Chemunex, Ivry-sur-Seine, France) which can penetrate passively into the cell where it is cleaved by cytoplasmic esterases, leading to the intracellular release of fluorescent residues (green fluorescence). After the 24 h biofilm growth period, 100 µL of the medium were gently removed from each well and replaced with 100 µl of V6 solution (diluted 1:100 in B16 buffer) (42). Microplate was incubated in the dark for 20 min at 37 °C in order to reach fluorescence equilibrium. Afterwards, the whole liquid part above the biofilm was gently removed from each well

271 and biofilms were once rinsed with 100 µl of 150 mM NaCl to eliminate any excess of
272 fluorescent dye. All the wells were refilled with 100 µl of 150 mM NaCl, except those
273 wells where the action of 100% hydrosol would be checked (no dilution).

274 Time lapse CLSM analysis of antimicrobial action of the two biocides against
275 *S.* Typhimurium biofilms was performed by using Leica SP2 AOBS confocal laser
276 scanning microscope at MIMA2 microscopy platform (INRA)
277 (http://www6.jouy.inra.fr/mima2_eng/). The following acquisition parameters were
278 adjusted: objective 63x oil with 1.4 numerical aperture, series of time lapse image scans
279 256 x 256 pixels, speed of scan: 400 Hz, excitation 488 nm with argon laser, emission
280 from 500 nm to 600 nm. A first xyz stack was measured (z-step 1 µm) to quantify the
281 structural parameters of the biofilm before disinfection (biovolume, thickness, density
282 etc). Subsequently a xyzt scan was done with a xyz measurement (with five sections in
283 the biofilm) every 15 s during 25 min. Appropriate quantities of either hydrosol or BC
284 were gently added to each well just after completion of the first xyz scan. Tested
285 concentrations were 100%, 75%, 50% for hydrosol and 0.5% for BC (final
286 concentrations in the wells). For each concentration, at least 8 replicates were
287 performed.

288

289 *2.9. Image analysis of fluorescence loss and estimation of inactivation kinetic*
290 *parameters*

291 The intensity of green fluorescence was quantified by the LCS Lite confocal
292 software (Leica microsystems). Intensity curves showing fluorescence loss were
293 extracted separately for four different sections within the biofilms (from the attachment
294 surface to the top of biofilm) (see results for distances), as well as for their maximum
295 projection, which is the 2D projection of the 3D biofilm structure and represents the

fluorescence in the whole biofilm. Intensity values were normalized by dividing the fluorescence intensity recorded at the different time points by the initial fluorescence intensity values obtained at the same location. Three-dimensional projections of biofilm structure were reconstructed using the Easy 3D function of the IMARIS 7.0 software (Bitplane, Switzerland). Quantitative structural parameters of the biofilms (i.e. biovolume, density and thickness), were calculated using ICY, an open community platform for bioimage informatics, created by Quantitative Image Analysis Unit at Pasteur Institute (http://www.bioimageanalysis.org/).

GinaFiT, a freeware add-in for Microsoft Excel was used to model inactivation kinetics (51). This tool enables testing of nine different types of microbial survival models, and the choice of the best fit depends on five statistical measures (i.e., sum of squared errors, mean sum of squared errors and its root, R^2, and adjusted R^2). During the present study, the "shoulder log-linear tail", "log-linear tail", or "log-linear" inactivation models were fitted to the fluorescence intensity curves obtained from the CLSM image series during biocide treatment. Two inactivation kinetic parameters were then extracted from this fitting: Sl, the shoulder length (min) that corresponded to the length of the lag phase (time period where fluorescence remains unreduced), and k_{max}, the inactivation rate (min^{-1}).

2.10. Statistical analysis

All statistical analyses (oneway ANOVA, linear regression) were performed using JMP v8.0 software (SAS, Cary, USA). Significance was defined as a P value associated with a Fisher test value lower than 0.05.

3. Results

3.1. Chemical analysis of T. capitata hydrosol and essential oil

The chemical composition of *T. capitata* hydrosol was analyzed in order to identify the various antimicrobial compounds that it might contain. As hydrosol is a by-product of EO distillation procedure, it was interesting to also comparatively analyze *T. capitata* EO. Chemical composition of *T. capitata* hydrosol and EO are presented in Table 1.

This analysis revealed that *T. capitata* hydrosol is a complex mixture containing 24 constituents. Its major compound is carvacrol (946,3 mg/L) followed by 1-octen-3-ol (10,8 mg/L), terpinen-4-ol (9,3 mg/L), borneol (6,6 mg/l), α-terpineol (2,7 mg/l), p-cymene (1,2 mg/l), γ-terpinene (1,2 mg/l), eugenol (0,9 mg/l), linalool (0,8 mg/l) and thymol (0,8 mg/l). On the other hand, the main constituents of *T. capitata* EO are carvacrol (28.3%), γ- terpinene (31.2%) and p-cymene (20%). Other components such as α-terpinene, α-thujene and α-pinene, E-caryophyllene, thymol were also found in this analysis. Interestingly, among the 24 hydrosol compounds, 14 were also detected in the EO, while ten oxygenated compounds were detected in hydrosol but not in the EO.

3.2. Antimicrobial activity of hydrosol and BC against planktonic and biofilm cells on stainless steel coupons

Disinfectant efficacies of *T. capitata* hydrosol and BC were tested against 24 h *S.* Typhimurium planktonic and biofilm cells on SS coupons. Cell density in biofilms after 24 h of development reached 7.47 ± 0.46 log cfu/cm^2. To be able to make comparisons, the cell density of planktonic suspension was also adjusted to a similar level (7.96 ± 0.23 log cfu/ml).

346 Log reductions of planktonic (log cfu/ml) and biofilm cells (log cfu/cm^2)
347 achieved after 6 min exposure to different concentrations of BC (0.0006 - 0.5%) and *T.*
348 *capitata* hydrosol (37.5 - 100%) are presented in Table 2.
349 First of all, biofilm cells have been found to demonstrate significant greater
350 resistance to both biocides compared to the planktonic cells. Thus, higher
351 concentrations of each biocide were required to kill the same number of biofilm cells
352 compared to planktonic ones. More particularly, a 6 log reduction was achieved after
353 treatment of planktonic cells with 0.0012 % BC, whereas 0.25 % BC was required to
354 achieve the same log reduction of biofilm cells. An approximate 5 log reduction of
355 planktonic and biofilm cells was obtained by applying respectively 42% and 75 %
356 hydrosol solutions.
357 Rc for two different and representative log-reductions and mean Rc are
358 presented in Table 2 for the two biocides. Based on these results, BC has a significantly
359 higher Rc (208.3) than hydrosol (1.6). This actually means that BC needs to be 208 fold
360 more concentrated to exert the same effect against biofilm cells than against planktonic
361 ones, whereas hydrosol is almost equally efficient against both types of cells.
362
363 *3.3. Real time visualization of the biocide action against S. Typhimurium biofilm*
364 *cells on polystyrene by confocal laser scanning microscopy*
365 Antimicrobial efficiency of BC and *T. capitata* hydrosol against 24 h
366 *S.* Typhimurium biofilms was evaluated using real-time visualization of fluorescence
367 loss by CLSM. During control experiments (treatment with distilled water), a
368 fluorescence loss less than 10% ± 3% of initial fluorescence was observed, after 25 min
369 of treatment.

Before adding the disinfectant, the biovolume and thickness of *Salmonella* biofilm were respectively 575155±212356 μm^3 and 55.4±10.4 μm. Three illustrative experiments for treatments with BC 0.5%, hydrosol 100% and hydrosol 75% are presented in Figures 1-3. All sections into biofilms treated with the same biocide were fitted by using the same inactivation model (BC 0.5% "shoulder log-linear tail", hydrosol 100% 'log linear tail', hydrosol 75% 'log linear') demonstrating that each biocide acts with the same type of kinetics into the biofilm structure. A few images corresponding to the maximum projection of the whole biofilm as a function of time are represented under each Figure. From the Figures 1-3, it can firstly be observed that fluorescence loss is much quicker following treatment of biofilms with hydrosol 100% (and even 75 %) compared to BC 0.5%.

Inactivation parameters obtained following all repetitions conducted in this study (including the illustrative experiments described above) are shown in Table 3. Quantitative comparison of these inactivation parameters revealed some very interesting findings for the two biocides. SI for BC (10.7 ± 7, R^2=0.98 ±0.08) was significantly different and markedly higher than the close to zero SI for hydrosol 100% (0.2 ± 0.4 R^2=0.99 ±0.04). Not only SI value was high, but there was also high standard deviation. In order to explain this big variability, SI values were correlated with biofilm biovolume, and it was found a significant important linear correlation (Prob>F 0.0432* p=0.05) but Rsquare of the model was extremely low (R^2=0.26). No significant difference was recorded in SI at the different sections inside the biofilm during the application of BC 0.5 %. Nevertheless, in the 1st section, mean SI (11.34 ± 1.7) was a little higher than 2nd (10.93 ± 1.7) which was a little higher than 3rd (10.58 ± 1.7).

With regard to the inactivation rates, k_{max} for BC (0.51 ± 0.40, R^2=0.98 ±0.08) was statistically significant lower than k_{max} presented for 100% hydrosol (0.82 ± 0.36,

R^2=0.99 ±0.04). Consequently, BC 0.5 % has lower bactericidal effect on *S.* Typhimurium biofilms than hydrosol 100%, which was very efficient from the first seconds of its application leading to total biofilm eradication within 2-3 minutes. No significant difference was observed in k_{max} values at different sections with the biofilms whatever the disinfectant.

Comparing the two concentrations of hydrosol, hydrosol 75% resulted in a much lower k_{max} (0.24 ± 0.17) than hydrosol 100% and a higher SI (3.44 ± 2.79). For hydrosol 75%, no significant differences were observed in k_{max} and SI within the different sections inside the biofilm, just like for hydrosol 100%. It should be noted that hydrosol 50% was also tested in this study but it didn't exhibit sufficient bactericidal activity presenting an almost zero inactivation rate (0.03 ± 0.02) (data not shown).

4. Discussion

Thymbra capitata is a species of aromatic plants native to the Mediterranean region of southern Europe, North Africa, and the Middle East. It belongs to the family of Lamiaceae, which includes many widely used culinary herbs (such as basil, mint, rosemary, sage, savory, marjoram, oregano, and thyme). The anti-biofilm activity of its EO has already been described (52, 53), but not the one of its hydrosol. It was first important to characterize hydrosol composition by SPME GC-MS in comparison with the one of the original essential oil (EO). The main constituents of *T. capitata* EO were oxygenated phenolic monoterpene carvacrol, monocyclic monoterpene γ-terpinene and the alkylbenzene p-cymene, as found in previous studies (54-56). However, carvacrol content in this study was much lower (20%) than previous reported concentrations (60-70%). In addition, other components detected here, such as monocyclic monoterpene α-terpinene, bicyclic monoterpenes, as α-thujene and α-pinene, acyclic monoterpenes

myrcene or linalool and the sesquiterpene E-caryophyllene, were also described as important compounds (55, 57, 58). The chemical composition of hydrosols of aromatic herbs produced in the same extraction process of EOs is not commonly studied. Their chemical analysis is the subject of only a limited number of publications (34, 59-62). However, to the best of our knowledge, the chemical composition of *T. capitata* hydrosol has not been reported until now. In this study, the chemical analysis revealed that this is a complex mixture containing part of the EO components. Thus, among 24 constituents that were detected, 14 were in common with the EO. However, some components, such as α-terpinene, myrcene, camphene and limonene, were missing in the hydrosol. Most of these missing compounds are completely insoluble in water, while some others probably disappear quickly from the acidic hydrosol with diverse degradation mechanisms (61). Some of the components that were found only in hydrosol and not in the EO could derive from chemical reactions occurring during hydro-distillation or extraction procedure before the GC analysis (59).

In this study, *T. capita* hydrosol was found to have a high bactericidal activity against planktonic cells, as 1.2±0.2, 2.8±0.5 and 4.7±0.7 log reductions were achieved by applying 37.5, 40 and 42 % (v/v) respectively. Information on the evaluation of different hydrosols as disinfectants is available (36-40). However, to the best of our knowledge, the antimicrobial action of *T. capita* hydrosol fraction has not been recorded yet.

Based on our results, *T. capitata* hydrosol presented a significant anti-biofilm action (complete eradication with 100% (v/v), approximate 5 log reduction with 75%) (Table 2). Antibacterial action of *Satureja thymbra* hydrosol against 5-days mono- and multi-species biofilms of *Pseudomonas putida*, *Salmonella enterica* and *Listeria monocytogenes* has been previously shown (35), but it is the only one reporting anti-

445 biofilm action of hydrosols. *T. capitata* and *S. thymbra* are similar species which belong
446 to the same family of Lamiaceae (also called Labiatae). Although it is difficult and often
447 misleading to compare results obtained in different studies, lower exposure time was
448 actually required in the current study for the complete eradication of biofilm (6 min
449 instead of 60 min) and a lower dose (75% instead of 100%) was also sufficient (35).

450 The Rc observed for hydrosol was very low (1.6) showing that this is almost
451 equally active against both planktonic and biofilm cells (Table 2). In the literature, the
452 Rc values of various biocides could range from 1 to 1000 depending on the considered
453 species and antimicrobial agent (15). Among all biocides reported, only hydrogen
454 peroxide and phenol exhibited the minimum Rc=1. Biocides with the second lowest Rc
455 (=4) were of plant origin (oregano, carvacrol, thymol and eucalyptus oil) (15). It is
456 noteworthy that hydrosol had a lower Rc than the ones of its main components carvacrol
457 or thymol alone (however tested against biofilms of different bacterial species),
458 revealing a very promising anti-biofilm agent.

459 BC (Rc 208.3) was shown much less efficient compared to hydrosol against
460 *Salmonella* biofilms. This thus needed to be more than 200 times more concentrated to
461 exert the same effect on biofilm cells than on planktonic ones. In the literature, the Rc
462 values for BC range from 10 to 1000, but in most cases these are above 50 (15). Typical
463 sanitizing concentrations of quaternary ammonium compounds, such as BC, range from
464 0.02 to 0.1% (v/v), but these are more generally applied at 0.02% (v/v) (63). According
465 to some authors, the recommended user concentration of BC can be a little bit higher at
466 0.07% (v/v) (64). However, in the present study it was demonstrated that BC at 0.125%
467 has an inadequate bactericidal effect against *S.* Typhimurium biofilm cells, resulting
468 only in a 2 log reduction, whereas a sufficient 6 log reduction was only achieved with
469 BC 0.25% (Table 2). Similarly, 0.02% BC failed to eradicate 48 h *S.* Typhimurium

biofilm on concrete (only 0.22 log-reduction) (65). It was also reported that BC 0.75 % was required to eradicate 3 days biofilms of *S.* Typhimurium (64). All these findings point out that BC, a nevertheless very common industrial disinfectant, is not effective against *S.* Typhimurium biofilms when this is applied at the recommended or generally used concentrations. This constitutes a common phenomenon for many industrial sanitizers, since the standards for testing disinfectant efficiency, such as European EN 1040 method (49), widely utilize planktonic cultures and results do not reflect the efficacy against bacteria in a biofilm state. However, it should be noted that the use of BC at high concentrations required for biofilm eradication may be not easily applied in food industry because of the risk of leaving residues in food. BC maximum residue level (MRL) for food and feed, laid down in Regulation (EC) No 396/2005, is 0.5 mg/kg (June 2014). Moreover, 0.1% is the maximum concentration of BC that does not produce primary irritation on intact skin or act as a sensitizer (66).

In the present study, spatial and temporal dynamics of the biocide actions were evaluated towards biofilms in real time and *in situ* by confocal laser scanning microscopy (CLSM). The spatial information obtained from CLSM analysis is of great importance because biofilms are known to present structural and physiological heterogeneity (67-69). The obtained temporal information can provide clues about the protective mechanisms of cells into the biofilm. For example, when a tolerant subpopulation is present, the shape of the inactivation curve should be concave up, and when a reaction-diffusion interaction limits the rate of access of the antimicrobial agent into the biofilm the shape of the inactivation curve should be concave down (70, 71). We have examined here the spatial action of both biocides in *S.* Typhimurium biofilms (Fig. 1-3, Table 3). BC 0.5% exhibited high SI values which means that there is an important initial time period at which it is totally ineffective against biofilm cells. Such

patterns of inactivation indicate the existence of transport limitations which is in accordance with previous references presuming that the restricted penetration of BC into biofilms might be one of the key processes explaining the resistance of biofilms to this biocide (42). However, contrarily to *P. aeruginosa* ATCC 15442 biofilm (42), no significant difference in inactivation kinetics at different depths of the biofilm were obtained, even if there was approximately 40 sec more delay in the biocide action of BC 0.5% at each section situated deeper into the biofilm. This is probably due to differences in biofilm thickness and in composition of matrix for the two bacterial species.

On the contrary, hydrosol 100% caused a uniform and direct linear loss of fluorescence in biofilm cell clusters of *S.* Typhimurium, suggesting that the slightly greater resistance of the biofilm compared to planktonic cells observed in this study for this biocide could not be due to limitations affecting its penetration into the biofilm. In addition, hydrosol 100% displayed a relatively high inactivation rate resulted in a rapid and total cells permeabilization throughout the biofilm within a few minutes (Fig. 2, Table 3).

The composition of hydrosol can explain its tremendous efficiency. *T. capitata* hydrosol's main constituent is carvacrol with its concentration to be 943.6 mg/L (0.0943%). Several studies have demonstrated that carvacrol has both bacteriostatic and bactericidal activity against foodborne microorganisms including *Salmonella enterica* (27, 72-74). The effectiveness of carvacrol as a natural antimicrobial compound is well established and its mechanism of action is believed to be associated with structural and functional damage to cellular membranes (25, 28, 74-76). It has also been previously demonstrated as an anti-biofilm compound. Biofilm eradication concentration (BEC, 5 log reduction) of carvacrol against *L. monocytogenes* biofilms on SS coupons and

520 microplates was obtained at 5 mM (0,0750 mg/L) (77). Similarly, it was reported that
521 carvacrol BEC against *S. aureus* and *S. epidermidis* biofilms on microplates ranged
522 from 0.125 to 0.5 % (v/v) (78). Low levels (2 and 3 log CFU/g) of *S.* Enteritidis and *E.*
523 *coli* O157:H7 were inactivated when radish seeds were treated with 4000 ppm and 8000
524 ppm (0.4-0.5%) of carvacrol for 60 min (79). The markedly effective antimicrobial and
525 anti-biofilm action of the *T. capitata* hydrosol against *S.* Typhimurium could thus be
526 partly attributed to its high carvacrol content. However, other constituents of this
527 hydrosol, such as terpinen-4-ol, thymol and eugenol, have also previously shown
528 antibacterial properties (25, 72, 78, 80, 81). Their concentrations in the hydrosol were
529 much lower compared to carvacrol's concentration, but synergistic action can occur
530 between all these compounds and can explain the overall high efficiency of the
531 hydrosol. In previous studies, combinations of different compounds from EOs, in
532 particular carvacrol, thymol and eugenol, showed synergistic activity (29, 72, 82, 83).
533 The high efficiency of hydrosol is also probably due to its high water solubility. We
534 can thus assume that active molecules of the hydrosol can better diffuse in aqueous
535 medium around planktonic bacteria or biofilm cells and their efficiency get improved
536 compared to the EOs that need initial solubilization in an organic solvent (ethanol,
537 DMSO) before introduction into an aqueous medium.
538
539 **5. Conclusions**
540 It is of great importance to take into consideration that bacteria can manage to
541 survive in many harsh environments, like those encountered in food processing, mainly
542 being enclosed into biofilms, sessile consortia which present higher resistance against
543 many environmental stresses (e.g. application of antimicrobial agents) compared to
544 their planktonic counterparts. Although the study of biofilms has come into sharp focus

545 in recent years, more research is required to further understand the intricate mechanisms
546 accounting for biofilm recalcitrance and to develop efficient methods to control them.
547 Both methods used in this study led to the same conclusion for both biocides. BC, which
548 is a common industrial disinfectant, proved to have insufficient anti-biofilm activity at
549 user's recommended concentration. On the contrary, *T. capita* hydrosol was clearly
550 demonstrated as a highly efficient anti-biofilm agent. The findings of this study i.e. the
551 efficiency to kill biofilm cells through a hydrosol fraction, an aqueous solution, which
552 is easily rinsed out from surfaces, and does not have the strong smell of the EO, may
553 be of great value not only for the food industries, but probably also for many other
554 industries which experience problems related to biofilms. This obviously could be an
555 interesting alternative and / or supplementary way to combat biofilms, being one
556 innovative, natural and environmentally safe antimicrobial agent. Indeed as hydrosols
557 are by-products of EOs distillation procedure, without any industrial application until
558 now, it could be useful and smart to apply such natural compounds for the disinfection
559 of surfaces in these environments. However, before its extensive use as disinfectant in
560 the food industry and other environments facing hygiene problems, more safety studies
561 based on toxicology data should be carried out.
562
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List of tables:

Table 1. Constituents of *T. capitata* hydrosol and EO, as identified by SPME GC/MS, and their (w/v) concentration or percentage composition

Compounds	KI	Hydrosol mg/L	Essential oil %area SPME
Toluene	<800	0.2	
2-methyl-Butanoic acid, methyl ester	<800	0.3	
N-ethyl-1,3-dithioisoindoline	821	0.6	
(Z)-3-Hexen-1-ol	847	0.7	
α-Thujene	912	0.1	2.5
.alpha.-Pinene	917	0.1	2.7
Camphene	933		0.8
Mesitylene	957	0.2	
.beta.-Pinene	964		0.7
1-Octen-3-ol	980	10.8	0.2
.beta.-Myrcene	990		2.0
3-octanol	997	0.4	
.alpha.-Phellandrene	999		0.3
3-Carene	1005		0.1
α-Terpinene	1013		2.4
p-Cymene	1020	1.3	2.3
Eucalyptol	1026	0.1	nd
Limonene	1028		0.8
trans-β-Ocimene	1061		0.1
γ-Terpinene	1063	1.2	35.3
1-methyl-4-(1-methylethenyl)-Benzene	1105	0.1	0.1
α-Terpinolene	1109		0.1
Linalool	1123	0.9	0.8
Borneol	1172	6.6	0.3
Terpinen-4-ol	1181	9.3	0.3
α-Terpineol	1224	2.7	<0.1
1-Isopropyl-2-methoxy-4-methylbenze	1250		0.7
D-Carvone	1257		<0.1
Thymol	1299	0.8	1.1
Carvacrol	1332	946.3	20.4
p-Cymen-7-ol	1323		1.2
4-Hydroxy-3-methylacetophenone	1329		0.2
Eugénol	1368	1.2	
Phenol, 5-methyl-2-(1-methylethyl)-, acetate	1377		0.1
Anethole	1381	0.2	
Caryophyllene	1421		0.4
Ethanone	1428	0.1	
1-Acetyl-4-methyldibenzofuran	1451	0.2	
Phenol, 2,4-bis(1,1-dimethylethyl)	1517	0.1	
(-)-Spathulenol	1581		nd
Caryophyllene oxide	1587		0.1
(α-Caryophylladienol)	1640		nd
2,3,4,6-Tetramethylphenol	2079		nd
Hexanedioic acid, bis(2-ethylhexyl) ester	>2200		nd

Table 2. Log reductions of planktonic (log cfu/ml) and biofilm cells (log cfu/cm^2) after 6 min disinfection treatments with BC and *T. capitata* hydrosol and resistance coefficients Rc

Biocide	Log reduction		Concentration (%)		Rc*	Mean Rc
	Planktonic (cfu/ml)	Biofilm (cfu/cm^2)	$C_{planktonic}$	$C_{biofilm}$		
BC	>7.5	>7.5	0.0022	0.5		
	6.2 ±0.6	6.1 ±0.6	0.0012	0.25	208.3	
	2.7 ±0.3	2.2 ±0.1	0.0006	0.125	208.3	
						208.3
Hydrosol	>7.5	>7.5	45	100		
	4.7 ±0.7	4.7 ±0.1	42	75	1.8	
	1.2 ±0.2	1.2 ±0.2	37.5	50	1.3	
						1.6

Rc= $C_{biofilm}/C_{planktonic}$, where $C_{biofilm}$ corresponds to the biocide concentration required to kill a given level of biofilm cells and $C_{planktonic}$ to the concentration needed to kill the same level of planktonic cells.

876 **Table 3**: Inactivation parameters [k_{max}, inactivation rate (min^{-1}); Sl, shoulder length
877 (min)] determined for each biocide and its respective concentrations for four different
878 sections within the biofilms, as well as for their maximum projection, which
879 represents the fluorescence in the whole biofilm. Mean values are presented ±
880 standard errors.
881

Biocide (n replicates)		k_{max}	SI
Hyd 100%	Max projection	0.82±0.09	0.2±0.13
(n=18)	Section 1	0.82±0.09	0.56±0.13
	Section 2	0.85±0.09	0.28±0.13
	Section 3	0.84±0.09	0.26±0.13
	Section 4	0.84±0.09	0.27±0.13
Hyd 75%	Max projection	0.24± 0.06	3.44±2.79
(n=7)	Section 1	0,25± 0.06	7.04±2.79
	Section 2	0.25± 0.06	3.69±2.79
	Section 3	0.24± 0.06	3.59±2.79
	Section 4	0.21± 0.06	5.05±2.79
BC 0.5%	Max projection	0.51±0.09	10.70±1.15
(n=18)	Section 1	0.64±0.09	11.34± 1.67
	Section 2	0.57±0.09	10.93±1.67
	Section 3	0.48±0.09	10.5±1.71
	Section 4	0.37±0.09	11.53±1.89

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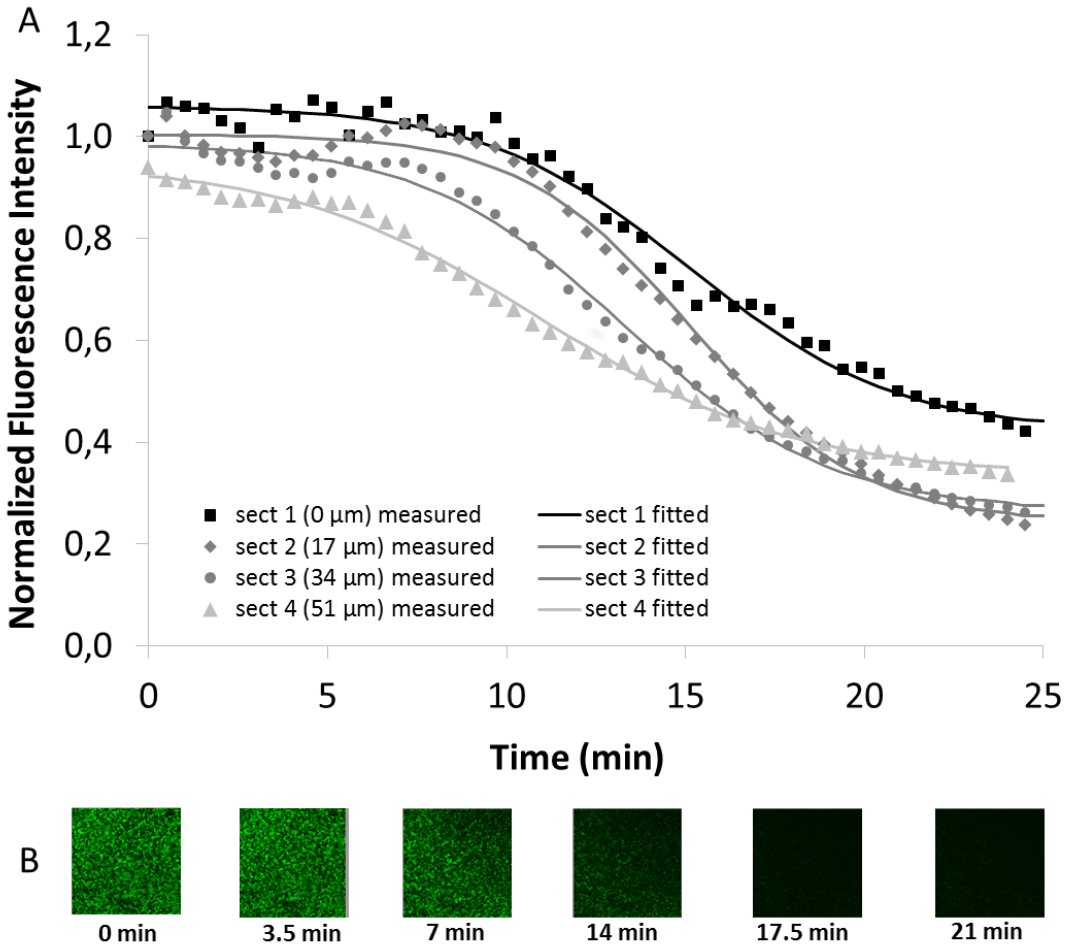


Figure 1. Quantification of fluorescence intensity during BC 0.5% (v/v) treatment (A) and series of images of Chemchrome V6 fluorescence in *S.* Typhimurium biofilms during treatment (B). The values represent the loss of fluorescence at four sections into the biofilm: **sec 1** (■) 0 µm bottom, **sec 2** (♦) 17 µm, **sec 3** (●) 34 µm and **sec 4** (▲) 51 µm. Shoulder length SI and inactivation rate kmax, were obtained after GInaFIT modeling.

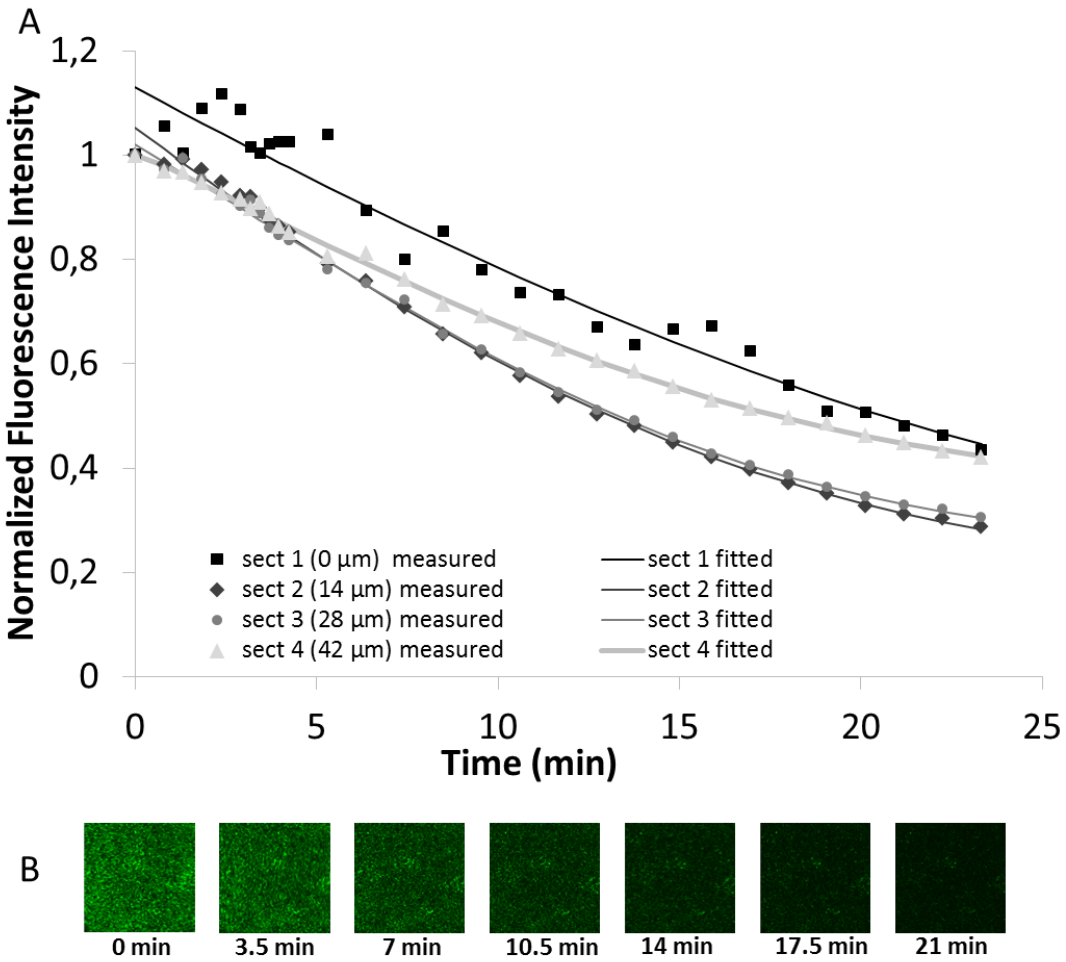
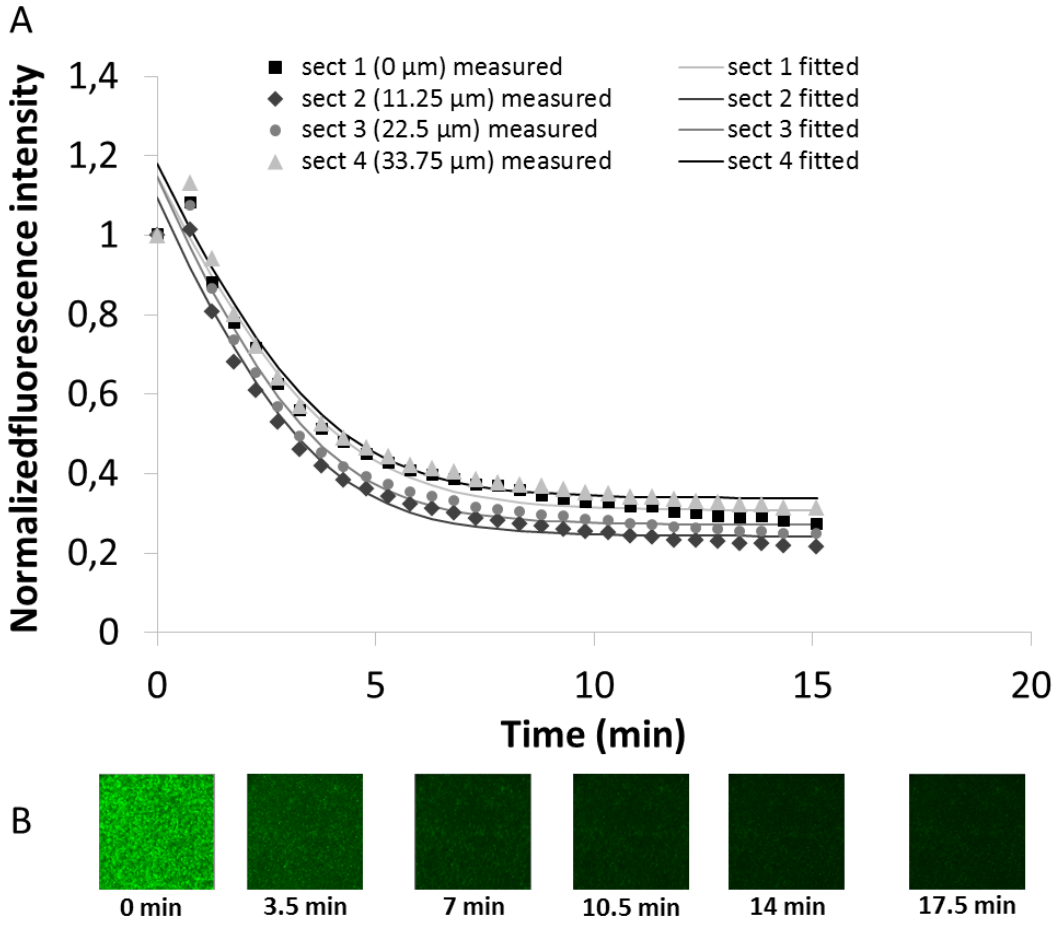


Figure 2. Quantification of fluorescence intensity during *T. capitata* hydrosol 75% (v/v) treatment (A) and series of images of Chemchrome V6 fluorescence in *S.* Typhimurium biofilms during treatment (B). The values represent the loss of fluorescence at four sections: **sec 1** (■) 0 µm, bottom, **sec 2** (♦) 14 µm, **sec 3** (●) 28 µm and **sec 4** (▲) 42 µm into the biofilm. Inactivation rate kmax, was obtained after GInaFIT modeling.



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904 **Figure 3.** Quantification of fluorescence intensity during *T. capitata* hydrosol 100%
905 (v/v) treatment (A) and series of images of Chemchrome V6 fluorescence in
906 *S.* Typhimurium biofilms during treatment (B). The values represent the loss of
907 fluorescence at four sections: **sec 1** (■) (0 µm, bottom), **sec 2** (♦) (11.25 µm), **sec 3** (●)
908 (22.5 µm) and **sec 4** (▲) (33.75 µm) into the biofilm. Inactivation rate kmax, was
909 obtained after GInaFIT modeling.

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