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Hydrosol of *Thymbra capitata* Is a Highly Efficient Biocide against *Salmonella enterica* Serovar Typhimurium Biofilms

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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 they are embedded in biofilm structures, has led to the search for and development of novel antimicrobial strategies capable of displaying both high efficiency and safety. In this direction, the aims of the present work were to evaluate the antimicrobial activity of hydrosol of the Mediterranean spice *Thymbra capitata* against both planktonic and biofilm cells of *Salmonella enterica* serovar Typhimurium and to compare its action with that 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 the 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 biofilms and planktonic cells, whereas a 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 three-dimensional (3D) structure of *Salmonella* biofilm.**

IMPORTANCE

The results of this paper highlight the significant antimicrobial action of a natural compound, hydrosol of *Thymbra capitata*, against both planktonic and biofilm cells of a common foodborne pathogen. Hydrosol has numerous advantages as a 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 essential oil (EO) and it is a byproduct of the EO distillation procedure without any industrial application until now. 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 biofilm-related problems.

Salmonella is an important food-borne pathogenic bacterium that remains the most frequent causative agent of foodborne 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 *Salmonella enterica* serovars Enteritidis and Typhimurium were the most commonly involved, representing 39.5% and 20.2%, respectively, of all reported serovars in confirmed human cases. It is also noted that, while the most important sources of foodborne *Salmonella* outbreaks were 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 tracts of diverse vertebrates, this genus has been shown to be able to survive for extended periods of time in nonenteric habitats. It is strongly believed that its survival and persistence in these nonhost environments may be attributed to its great ability to form biofilms on various inanimate surfaces (2, 3). Biofilms are consortia of microorganisms adherent to each other and usually to a surface and embedded in a scaffold of self-produced extracellular polymeric substances (EPS) (4, 5). Thus, there is extended documentation on the ability of *Salmonella* to attach to various food-contact surfaces (such as stainless steel, plastic, and cement) and form biofilms under *in vitro* conditions (6–10), while *Salmonella* cells have also been recovered from surfaces and products of various food industries (11–14). During the past decades, it has become increasingly clear that biofilms are the predominant mode of bacterial life in most environments (15–17). Biofilms formed in food-processing environments are of special importance, since they may act as a persistent source of product contamination which may lead to food spoilage and/or transmission of diseases (2, 3, 18, 19).

Poor sanitation of food-contact surfaces is believed to be an

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essential contributing factor in foodborne disease outbreaks. Disinfection procedures using either physical or chemical methods have been extensively used over the years to reduce or eliminate bacteria found on food-contact surfaces. However, current methods of sanitation of food facilities are not always sufficiently efficient because of the great resistance displayed by many foodborne bacteria, especially when these are grown into biofilms (15, 20, 21). Several mechanisms have been proposed to be involved in biofilm resistance to disinfectants, including (i) limitations to the free diffusion of antimicrobial agents through the biofilm matrix, (ii) variability in the physical and chemical microenvironments within the biofilm (e.g., varied conditions of pH, osmotic strength, or nutrients) leading to varied levels of metabolic activity and cell adaptive responses, (iii) mutations and horizontal transfer of genes coding for resistance 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) together with the presence of extremely resistant persister cells, and (v) bacterial protection among a multispecies 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 be 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 due to both their strong hydrophobic nature, which hampers their efficient rinsing out from surfaces after a disinfection program, and their intense smell. Hydrosols, which are byproducts of the EO 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 did 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 antibiofilm 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 has 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 work, the aims of the present work were to evaluate the antimicrobial activity of hydrosol of the Mediterranean spice *Thymbra capitata* against both planktonic and biofilm cells of *Salmonella enterica* serovar Typhimurium and to compare its action with that 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.

MATERIALS AND METHODS

Bacterial strain, growth conditions, and preparation of bacterial suspension. The bacterium used in this study was *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain ATCC 14028 isolated from 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 24 h and subsequently harvested by centrifugation (5,000 × *g*, 10 min, at 20°C) for disinfectant testing. For the biofilm formation, the pellets were resuspended and diluted in 10 ml of 150 mM NaCl in order to obtain a bacterial suspension adjusted to 10^6 cells/ml.

Antibacterial agents. The hydrosol of *Thymbra capitata* (pharmacy Provata, Athens, Greece) and the quaternary ammonium compound benzalkonium chloride C_{14} (BC) (molecular weight [MW], 368.04; anhydrous, 99.0%; Fluka, France) were used to carry out disinfectant tests. Different concentrations of hydrosol (25% to 75% for planktonic cells; 50% to 100% for biofilms) and BC (0.0006% to 0.002% for planktonic cells; 0.125% to 0.5% for biofilms) were prepared by diluting appropriate quantities of each antimicrobial compound in sterilized deionized water the day of the experiment.

Chemical analysis of *T. capitata* hydrosol and its related essential oil. *T. capitata* hydrosol was chemically analyzed by solid-phase microextraction (SPME) followed by gas chromatography (GC)-mass spectrometry (MS) (46). Hydrosol samples (10 ml) together with 3 g NaCl were placed into a 20-ml headspace vial fitted with a Teflon-lined septum sealed with an aluminum crimp seal, through which the SPME fiber 50/30-mm divinylbenzene/carboxen/polydimethylsiloxane (2 cm; Supelco, Bellefonte, PA, USA) was introduced. 4-Methyl-2-pentanol was used as an internal standard at a final concentration of 0.8 mg/liter. The mix was equilibrated at 60°C for 45 min. After headspace extraction, the fiber was transferred to the GC injection port, where the absorbed compounds were thermally desorbed for 3 min at 240°C.

The essential oil was diluted in pure hexane (1:9), 3 g NaCl was added to 10 ml of diluted oil, and the mixture was then chemically analyzed by either SPME GC-MS analysis as described above or by GC-MS analysis following filtration using 0.22-μm filters and direct injection (1 μl). GC-MS analysis was carried out with a 6890N system (Agilent Technologies, USA) equipped with an HP-5MS column (5% phenyl methylpolysiloxane; 30 m; 0.25-mm inner diameter; film thickness, 0.25 μm) (Agilent Technologies) and coupled with a 5973Network mass detector (Agilent Technologies). Column temperature was set at 35°C for 6 min, increased to 60°C at the rate of 2°C/min, kept constant for 5 min, increased again to

200°C at the rate of 5°C/min, and then increased to 250°C at the rate of 25°C/min and held constant for 6 min. Helium was used as the carrier gas (linear velocity of 1.8 or 1.5 ml/min for SPME or direct analysis, respectively). The injector was operated in splitless mode for SPME analysis and in split mode (1:50 split ratio) for direct injection analysis. Both injector and detector temperatures were 240°C. The mass spectrometer was operated in the electron impact mode, with the electron energy set at 70 eV and a 45 to 400 *m/z* scan range. Results acquired were processed by ChemStation integrated software (Agilent Technologies), and constituents were identified by comparing mass spectra with spectra from the NBS75K and Wiley275 reference libraries, by spectra from standard compounds (in-house libraries) and by determining the Kovats retention indexes and comparing them with those reported in the literature. In SPME GC-MS analysis, the volatile compounds were semiquantified by dividing the peak areas of the compounds of interest by the peak area of the internal standard (IS) and multiplying this ratio by the initial concentration of the IS (expressed as mg/liter), while in direct-injection GC-MS analysis, the percentage area of volatile compounds was estimated. The peak areas were measured from the full-scan chromatograph using total ion current (TIC). Each experiment was carried out in duplicate, and the mean data are presented.

Biofilm formation. Biofilms of *S. enterica* serovar Typhimurium were grown on 1-cm^2 stainless steel (SS) AISI 204 coupons (Goodfellow, Cambridge Science Park, United Kingdom) and also on the wells of 96-well polystyrene microtiter plates (catalog no. 655090; Greiner Bio-One, France) with a clear base (polystyrene; 190 ± 5 μm thickness) (47). Before use, the SS coupons were cleaned with surfactant RBS 35 (Société des Traitements Chimiques de Surface, Lambersart, France), rinsed with deionized water, and settled in the wells of a 24-well polystyrene microtiter plate (Techno Plastic Products, Switzerland) (29, 48). Then, 1 ml or 250 μl of bacterial subculture, as prepared above (~10^6 cells/ml), was poured into the wells of the 24-well (containing SS coupons) or 96-well, respectively, microplates. Adhesion on SS coupons or on polystyrene wells was done by sedimentation for 2 h at 20°C. Subsequently, the planktonic bacterial suspension was removed, and 1 ml (or 250 μl) of TSB was added in each well. Microtiter plates were incubated at 20°C for 24 h without shaking to allow biofilm development (on either SS coupons or the polystyrene wells).

Antimicrobial testing against planktonic cells. Planktonic cells were challenged with the disinfectants using the EN 1040 standard protocol (49). Briefly, 1 ml of a 100-fold diluted solution of the working culture (prepared as previously described) was initially centrifuged at 5,000 × *g* for 10 min. Subsequently, the pellet (~10^7 cells) was resuspended in 1 ml of each antimicrobial solution (at different concentrations) and left in contact for 6 min at 20°C. The antimicrobial action was halted by transferring a volume (1:9) to a quenching solution (3 g/liter L-α-phosphatidylcholine, 30 g/liter Tween 80, 5 g/liter sodium thiosulfate, 1 g/liter L-histidine, 30 g/liter saponine) for 10 min. Serial dilutions were then prepared, and survivors were enumerated on tryptic soy agar using the 6 × 6 drop count method (50). The control was performed in the same way with sterile deionized water instead of the disinfectant. The logarithmic reduction achieved was the difference between the $\log_{10}$ of the survivors after the test with deionized water (control) and the $\log_{10}$ of the survivors after the test with the antimicrobial agent. For each treatment, at least three replicates were performed using independently grown bacterial cultures.

Antimicrobial testing against biofilms formed on SS coupons. Following biofilm formation on SS coupons, the planktonic suspension was removed from each well and each coupon was once rinsed with 1 ml of 150 mM NaCl to remove the loosely attached cells. Afterward, each coupon was immediately challenged with 1 ml of each disinfectant solution (at different concentrations) for 6 min at 20°C (the same conditions used for the planktonic cells). Antimicrobial action was halted by placing coupons for 10 min at 20°C in the quenching solution, as previously described. The survivors were removed from the surface by scratching with a plastic rake (folded pipette cone) in a standardized way (horizontal/vertical/oblique) and enumerated by plate counting, as previously described. The 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 treatment, at least three replicates were performed using independently grown bacterial cultures.

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 $C_{planktonic}$ corresponds to the concentration needed to kill the same level of planktonic cells (15).

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 labeled with a viability fluorescent marker and subsequently submitted to disinfection. The antimicrobial action induces cell membrane permeabilization and, subsequently, loss of fluorescence that represents cell 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 was gently removed from each well and replaced with 100 μl of V6 solution (diluted 1:100 in B16 buffer) (42). The microplate was incubated in the dark for 20 min at 37°C in order to reach fluorescence equilibrium. Afterward, the whole liquid part above the biofilm was gently removed from each well, and biofilms were once rinsed with 100 μl of 150 mM NaCl to eliminate any excess of fluorescent dye. All of the wells were refilled with 100 μl of 150 mM NaCl, except those wells where the action of 100% hydrosol would be checked (no dilution).

Time-lapse CLSM analysis of antimicrobial action of the two biocides against *S. enterica* serovar Typhimurium biofilms was performed by using Leica SP2 AOBS confocal laser scanning microscope at MIMA2 microscopy platform (INRA) (http://www6.jouy.inra.fr/mima2_eng/). The following acquisition parameters were adjusted: objective, 63× oil with 1.4 numerical aperture; series of time-lapse image scans, 256 × 256 pixels; speed of scan, 400 Hz; excitation, 488 nm with argon laser; emission, from 500 nm to 600 nm. A first *xyz* stack was measured (z-step 1 μm) to quantify the structural parameters of the biofilm before disinfection (biovolume, thickness, density, etc). Subsequently, an *xyzt* scan was done for 25 min (an *xy* measurement in four different sections of the biofilm every 15 s). Appropriate quantities of either hydrosol or BC were gently added to each well just after completion of the first *xyz* scan. Tested concentrations were 100%, 75%, and 50% for hydrosol and 0.5% for BC (final concentrations in the wells). For each treatment, at least seven replicates were performed using three independently grown bacterial cultures.

Image analysis of fluorescence loss and estimation of inactivation kinetic parameters. The intensity of green fluorescence was quantified by the LCS Lite confocal software (Leica Microsystems). Intensity curves showing fluorescence loss were extracted separately from four different sections within the biofilms (from the attachment surface to the top of biofilm) (see Results for distances) as well as for their maximum projection, which is the two-dimensional projection of the three-dimensional (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. The 3D 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 the Quantitative Image Analysis Unit at Pasteur Institute (http://www.bioimageanalysis.org/).

GInaFiT, a freeware add-in for Microsoft Excel, was used to model

TABLE 1 Constituents of *T. capitata* hydrosol and EO, as identified by SPME GC-MS, and their (wt/vol) concentration or percentage composition

Compound	Kovats retention index	Hydrosol (mg/liter)	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
α-Pinene	917	0.1	2.7
Camphene	933		0.8
Mesitylene	957	0.2	
β-Pinene	964		0.7
1-Octen-3-ol	980	10.8	0.2
β-Myrcene	990		2.0
3-Octanol	997	0.4	
α-Phellandrene	999		0.3
3-Carene	1,005		0.1
α-Terpinene	1,013		2.4
p-Cymene	1,020	1.3	23
Eucalyptol	1,026	0.1	ND[a]
Limonene	1,028		0.8
trans-β-Ocimene	1,061		0.1
γ-Terpinene	1,063	1.2	35.3
1-Methyl-4-(1-methylethenyl)-benzene	1,105	0.1	0.1
α-Terpinolene	1,109		0.1
Linalool	1,123	0.9	0.8
Borneol	1,172	6.6	0.3
Terpinen-4-ol	1,181	9.3	0.3
α-Terpineol	1,224	2.7	<0.1
1-Isopropyl-2-methoxy-4-methylbenzene	1,250		0.7
D-Carvone	1,257		<0.1
Thymol	1,299	0.8	1.1
Carvacrol	1,332	946.3	20.4
p-Cymen-7-ol	1,323		1.2
4-Hydroxy-3-methylacetophenone	1,329		0.2
Eugenol	1,368	1.2	
Phenol, 5-methyl-2-(1-methylethyl)-, acetate	1,377		0.1
Anethole	1,381	0.2	
Caryophyllene	1,421		0.4
Ethanone	1,428	0.1	
1-Acetyl-4-methyldibenzofuran	1,451	0.2	
Phenol, 2,4-bis(1,1-dimethylethyl)-	1,517	0.1	
(−)-Spathulenol	1,581		ND
Caryophyllene oxide	1,587		0.1
α-Caryophylladienol	1,640		ND
2,3,4,6-Tetramethylphenol	2,079		ND
Hexanedioic acid, bis(2-ethylhexyl) ester	>2,200		ND

[a] ND, not determined.

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, 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: the shoulder length (SL) (min), that corresponded to the length of the lag phase (time period where fluorescence remains unreduced), and k_{max}, the inactivation rate (min^{-1}).

Statistical analyses. All statistical analyses (one-way analysis of variance, linear regression) were performed using JMP v8.0 software (SAS, Cary, NC, USA). Significance was defined as a *P* value associated with a Fisher's exact test result lower than 0.05.

RESULTS

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 byproduct 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/liter) followed by 1-octen-3-ol (10.8 mg/liter), terpinen-4-ol (9.3 mg/liter), borneol (6.6 mg/liter), α-terpineol (2.7 mg/liter), p-cymene (1.2 mg/liter), γ-terpinene (1.2 mg/

TABLE 2 Log reductions of planktonic and biofilm cells after 6-min disinfection treatments with BC or *T. capitata* hydrosol and Rc[a]

Biocide	Concn (%) (no. of replicates)		Mean ± SE log reduction		Rc
	$C_{planktonic}$	$C_{biofilm}$	Planktonic (CFU/ml)	Biofilm (CFU/cm^2)	
BC	0.0022 (3)	0.5 (10)	>7.5	>7.5	
	0.0012 (6)	0.25 (8)	6.2 ± 0.6	6.1 ± 0.6	208.3
	0.0006 (7)	0.125 (3)	2.7 ± 0.3	2.2 ± 0.1	208.3
Hydrosol	45 (3)	100 (14)	>7.5	>7.5	
	42 (6)	75 (6)	4.7 ± 0.7	4.7 ± 0.1	1.8
	37.5 (6)	50 (8)	1.2 ± 0.2	1.2 ± 0.2	1.3

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

liter), eugenol (0.9 mg/liter), linalool (0.8 mg/liter), and thymol (0.8 mg/liter). On the other hand, the main constituents of *T. capitata* EO are carvacrol (20.4%), γ-terpinene (35.3%), and p-cymene (23%) (Table 1). Other components, such as α-terpinene, α-thujene, α-pinene, E-caryophyllene, and thymol were also found in this analysis. Interestingly, among the 24 hydrosol compounds, 14 were also detected in the EO, while 10 oxygenated compounds were detected in hydrosol but not in the EO.

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. enterica* serovar 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).

Log reductions of planktonic (log CFU/ml) and biofilm cells (log CFU/cm^2) achieved after 6 min of exposure to different concentrations of BC (0.0006% to 0.5%) and *T. capitata* hydrosol (37.5% to 100%) are presented in Table 2.

Biofilm cells have been found to demonstrate significant greater resistance to both biocides compared to the planktonic cells. Thus, higher concentrations of each biocide were required to kill the same number of biofilm cells compared to planktonic ones. More particularly, a 6-log reduction was achieved after treatment of planktonic cells with 0.0012% BC, whereas 0.25% BC was required to achieve the same log reduction of biofilm cells. An approximate 5-log reduction of planktonic and biofilm cells was obtained by applying, respectively, 42% and 75% hydrosol solutions.

The Rc values for two representative log reductions are presented in Table 2 for the two biocides. Based on these results, BC had a significantly higher Rc (208.3) than hydrosol (1.3 and 1.8, respectively; mean of 1.6). This actually meant that BC needed to be 208-fold more concentrated to exert the same effect against biofilm cells than against planktonic ones, whereas hydrosol was almost equally efficient against both types of cells.

Real-time visualization of the biocide action against *S. enterica* serovar Typhimurium biofilm cells on polystyrene by confocal laser scanning microscopy. Antimicrobial efficiency of BC and *T. capitata* hydrosol against 24 h *S. enterica* serovar Typhimurium biofilms was evaluated using real-time visualization of fluorescence loss by CLSM. During control experiments (treatment with distilled water), a fluorescence loss of less than 10% ± 3% of initial fluorescence was observed after 25 min of treatment.

Before adding the disinfectant, the biovolume and thickness of *Salmonella* biofilm were, respectively, 5.75 × 10^5 ± 2.72 × 10^4 μm^3 and 55.36 ± 1.34 μm. Three illustrative experiments for treatments with 0.5% BC (BC 0.5%), 100% hydrosol (hydrosol 100%), and 75% hydrosol (hydrosol 75%) are presented in Fig. 1, 2, and 3, respectively. All sections in biofilms receiving the same treatment 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 in a given concentration 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 Fig. 1 to 3, it can 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. The SL for BC (10.7 ± 7 min; R^2 =

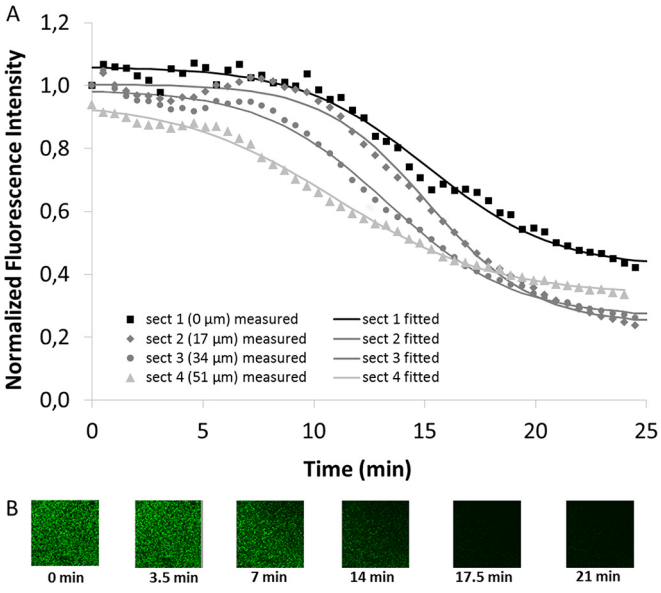

FIG 1 Quantification of fluorescence intensity during 0.5% (vol/vol) BC treatment (A) and series of images of *S. enterica* serovar Typhimurium biofilms during treatment (B). The values represent the loss of fluorescence at four sections—sect 1 (bottom), sect 2, sect 3, and sect 4—into the biofilm. Shoulder length and inactivation rate were obtained after GInaFiT modeling.

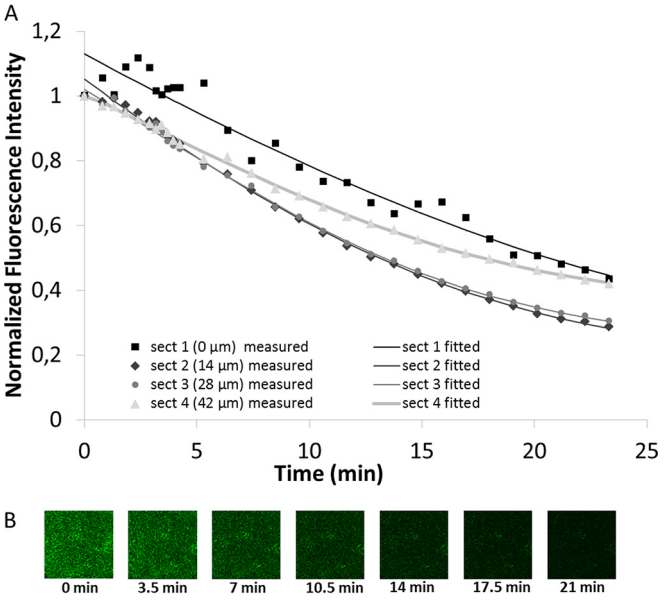

FIG 2 Quantification of fluorescence intensity during 75% (vol/vol) *T. capitata* hydrosol treatment (A) and series of images of *S. enterica* serovar Typhimurium biofilms during treatment (B). The values represent the loss of fluorescence at four sections—sect 1 (bottom), sect 2, sect 3, and sect 4—into the biofilm. Inactivation rate was obtained after GInaFiT modeling.

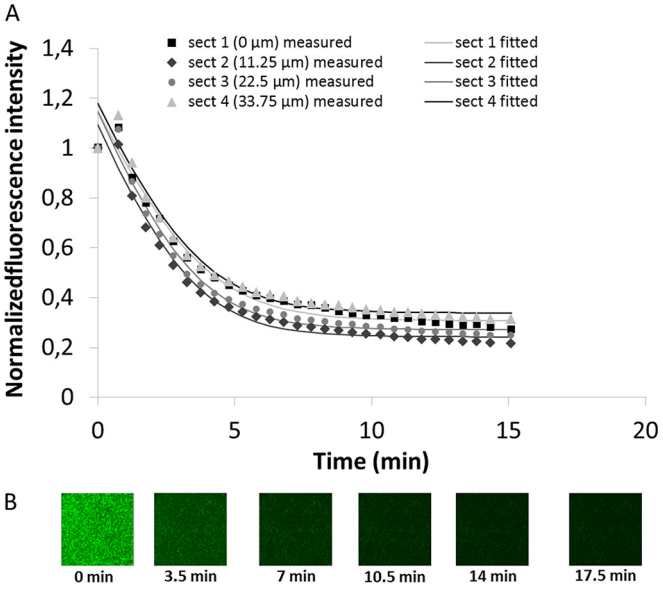

FIG 3 Quantification of fluorescence intensity during 100% (vol/vol) *T. capitata* hydrosol treatment (A) and series of images of *S. enterica* serovar Typhimurium biofilms during treatment (B). The values represent the loss of fluorescence at four sections—sect 1 (bottom), sect 2, sect 3, and sect 4—into the biofilm. Inactivation rate was obtained after GInaFiT modeling.

0.98 ± 0.08) was significantly different from and markedly higher than the close to zero SL for hydrosol 100% (0.2 ± 0.4 min; R^2 = 0.99 ± 0.04). Not only was the SL value high, but there was also a high standard deviation. In order to explain this big variability, SL values were correlated with biofilm biovolume; a linear correlation was found, but R^2 of the model was extremely low (R^2 = 0.26). No significant differences were recorded in the SL values at the different sections inside the biofilm during the application of BC 0.5%. Nevertheless, in the first section, the mean SL (11.34 ± 1.7 min) was a little higher than in the second section (10.93 ± 1.7 min), which was a little higher than in the third section (10.58 ± 1.7 min).

With regard to the inactivation rates, k_{max} for BC (0.51 ± 0.40 min^{-1}; R^2 = 0.98 ± 0.08) was statistically significantly lower than the k_{max} for 100% hydrosol (0.82 ± 0.36 min^{-1}; R^2 = 0.99 ± 0.04). Consequently, BC 0.5% has a lower bactericidal effect on *S. enterica* serovar Typhimurium biofilms than hydrosol 100%, which was very efficient from the first seconds of its application, leading to total biofilm eradication within 2 to 3 min. No significant difference was observed in k_{max} values at different sections in the biofilms whatever the disinfectant.

Comparing the two concentrations of hydrosol, hydrosol 75% resulted in a much lower k_{max} (0.24 ± 0.17 min^{-1}) than hydrosol 100%, as well as a higher SL (3.44 ± 2.79 min). For hydrosol 75%, no significant differences were observed in k_{max} and SL 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 did not exhibit sufficient bactericidal activity, presenting an almost zero inactivation rate (0.03 ± 0.02 min^{-1}) (data not shown).

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 antibiofilm activity of its EO has already been described (52, 53), but that of its hydrosol has not. It was first important to characterize hydrosol composition by SPME GC-MS in comparison with that of the original essential oil. The main constituents of the *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, the carvacrol content in this study was much lower (20%) than previous reported concentrations (60% to 70%). It should be noted that the extraction product (i.e., the EO) can vary in quality, quantity, and composition according to climate (temperature, humidity), soil composition, plant organ, age, vegetative cycle stage, and method used to extract the oil (36). 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 some 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

TABLE 3 Inactivation parameters determined for each biocide and its respective concentrations for four different sections within the biofilms, as well as for their maximum projections

Biocide (no. of replicates)	Projection or section	Mean ± SE inactivation parameters	
		k_{max} (min^{-1})	Shoulder length (min)
Hydrosol 100% (18)	Max projection[a]	0.82 ± 0.09	0.2 ± 0.13
	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
Hydrosol 75% (7)	Max projection	0.24 ± 0.06	3.44 ± 2.79
	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% (18)	Max projection	0.51 ± 0.09	10.70 ± 1.15
	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

[a] Max projection represents the fluorescence in the whole biofilm.

(61). Some of the components that were found only in hydrosol and not in the EO could derive from chemical reactions occurring during hydrodistillation or an extraction procedure before the GC analysis (59).

In this study, the *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% (vol/vol), 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 the *T. capita* hydrosol fraction has not been recorded yet.

Based on our results, *T. capitata* hydrosol presented a significant antibiofilm action (complete eradication with 100% [vol/vol], approximate 5-log reduction with 75%) (Table 2). The antibacterial action of *Satureja thymbra* hydrosol against 5-day mono- and multispecies biofilms of *Pseudomonas putida*, *Salmonella enterica*, and *Listeria monocytogenes* has been previously shown (35), but this was the only previous study reporting antibiofilm action of hydrosols. *T. capitata* and *S. thymbra* are similar species which belong to the same family of Lamiaceae (also called Labiatae). Although it is difficult and often misleading to compare results obtained in different studies, a shorter exposure time was actually required in the current study for the complete eradication of biofilm (6 min instead of 60 min), and a lower dose (75% instead of 100%) was also sufficient (35). Thus, in the previous study, biofilms were left to be formed for 5 days at 16°C under either mono- or mixed-species conditions, employing also different bacterial species and strains (35). We should also keep in mind that biofilms are microbial communities known to present great structural and physiological heterogeneity, even those formed by the same microorganism under different environmental conditions (63).

The Rc observed for hydrosol was very low (1.6), showing that this is almost equally active against both planktonic and biofilm cells (Table 2). In the literature, the Rc values of various biocides could range from 1 to 1,000 depending on the considered species and antimicrobial agent (15). Among all biocides reported, only hydrogen peroxide and phenol exhibited the minimum Rc of 1. Biocides with the second lowest Rc (Rc = 4) were of plant origin (oregano, carvacrol, thymol, and eucalyptus oil) (15). It is noteworthy that hydrosol had a lower Rc than those of its main components carvacrol or thymol alone (however tested against biofilms of different bacterial species), revealing a very promising antibiofilm agent.

BC (Rc = 208.3) was much less efficient than hydrosol against *Salmonella* biofilms. BC thus needed to be more than 200 times more concentrated to exert the same effect on biofilm cells than on planktonic ones. In the literature, the Rc values for BC range from 10 to 1,000, but in most cases these were above 50 (15). Typical sanitizing concentrations of quaternary ammonium compounds, such as BC, range from 0.02% to 0.1% (vol/vol), but these were more generally applied at 0.02% (vol/vol) (64). According to some authors, the recommended user concentration of BC can be a little bit higher at 0.07% (vol/vol) (65). However, in the present study, it was demonstrated that BC at 0.125% has an inadequate bactericidal effect against *S. enterica* serovar Typhimurium biofilm cells, resulting only in a 2-log reduction, whereas a sufficient 6-log reduction was achieved only with BC 0.25% (Table 2). Similarly, 0.02% BC failed to eradicate 48 h *S. enterica* serovar Typhimurium biofilm on concrete (only 0.22-log reduction) (66). It was also reported that BC 0.75% was required to eradicate 3-day biofilms of *S. enterica* serovar Typhimurium (65). All these findings point out that BC, a nevertheless very common industrial disinfectant, is not effective against *S. enterica* serovar 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 the 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 the high concentrations required for biofilm eradication may be not easily applied in food industry because of the risk of leaving residues in food. The BC maximum residue level for food and feed, laid down in Regulation (EC) 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 (67).

In the present study, spatial and temporal dynamics of the biocide actions were evaluated toward 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 (68–70). 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 (71, 72). We have examined here the spatial action of both biocides in *S. enterica* serovar Typhimurium biofilms (Table 3; Fig. 1 to 3). BC 0.5% exhibited high shoulder length 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, contrary to *Pseudomonas aeruginosa* ATCC 15442 biofilm (42), no significant difference in inactivation kinetics at different depths of the biofilm were obtained, even if there was an approximately 40-s 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 the composition of the 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. enterica* serovar 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 cell permeabilization throughout the biofilm within a few minutes (Table 3; Fig. 2).

The composition of hydrosol can explain its tremendous efficiency. The main constituent of the *T. capitata* hydrosol is carvacrol, with a concentration of 943.6 mg/liter (0.0943%). Several studies have demonstrated that carvacrol has both bacteriostatic and bactericidal activity against foodborne microorganisms, including *Salmonella enterica* (27, 73–75). 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, 75–77). It has also been previously demonstrated as an antibiofilm compound. Biofilm eradication concentration (BEC) (5-log reduction) of carvacrol against *Listeria monocytogenes* biofilms on SS coupons and microplates was obtained at 5 mM (0.0750 mg/liter) (78). Similarly, it was reported that the carvacrol BEC against *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms on microplates ranged from 0.125 to 0.5% (vol/vol) (79). Low levels (2- and 3-log CFU/g) of *Salmonella enterica* serovar Enteritidis and *Escherichia coli* O157:H7 were inactivated when radish seeds were treated with 4,000 ppm and 8,000 ppm (0.4% to 0.8%) of carvacrol for 60 min (80). The markedly effective antimicrobial and antibiofilm action of the *T. capitata* hydrosol against *S. enterica* serovar Typhimurium could thus be partly attributed to its high carvacrol content. However, other constituents of this hydrosol, such as terpinen-4-ol, thymol, and eugenol, have also previously shown antibacterial properties (25, 73, 79, 81, 82). Their concentrations in the hydrosol were much lower than the carvacrol concentration, but synergistic action can occur between all these compounds and can explain the overall high efficiency of the hydrosol. In previous studies, combinations of different compounds from EOs, in particular carvacrol, thymol, and eugenol, showed synergistic activity (29, 73, 83, 84). The high efficiency of hydrosol is also probably due to its high water solubility. We can thus assume that active molecules of the hydrosol can better diffuse in aqueous medium around planktonic bacteria or biofilm cells and that their efficiency is improved compared to the EOs that need initial solubilization in an organic solvent (ethanol, dimethyl sulfoxide [DMSO]) before introduction into an aqueous medium.

Conclusions. It is of great importance to take into consideration that bacteria can manage to survive in many harsh environments, like those encountered in food processing, mainly by being enclosed in biofilms, sessile consortia, which present higher resistance against many environmental stresses (e.g., application of antimicrobial agents) compared to their planktonic counterparts. Although the study of biofilms has come into sharp focus in recent years, more research is required to further understand the intricate mechanisms accounting for biofilm recalcitrance and to develop efficient methods to control them. Both methods used in this study led to the same conclusion for both biocides. BC, which is a common industrial disinfectant, proved to have insufficient antibiofilm activity at the recommended concentration. Conversely, the *T. capita* hydrosol was clearly demonstrated as a highly efficient antibiofilm agent. The findings of this study, i.e., the efficiency to kill biofilm cells through a hydrosol fraction, an aqueous solution which is easily rinsed out from surfaces and does not have the strong smell of the EO, may be of great value not only for the food industries but probably also for many other industries which experience problems related to biofilms. This obviously could be an interesting alternative and/or supplementary way to combat biofilms, as one innovative, natural, and environmentally safe antimicrobial agent. Indeed, as hydrosols are byproducts of the EO distillation procedure, without any industrial application until now, it could be useful and smart to apply such natural compounds for the disinfection of surfaces in these environments. However, taking into account that high concentration of hydrosols may be needed to sufficiently kill biofilm cells (i.e., 75% hydrosol achieves an approximate 5-log reduction, based on our results), high quantities of these compounds would obviously be needed for the daily disinfection of a food processing plant. Although 2 to 3 ml of hydrosol is typically received in parallel of 1 ml EO following the same distillation procedure, producing sufficient quantities may be an important practical hurdle in the wider application of such compounds. In addition, before their extensive use as disinfectant in the food industry and other environments facing hygiene problems, more safety studies based on toxicology data should be carried out.

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