Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Disinfectant test against monoculture and mixed-culture biofilms composed of technological, spoilage and pathogenic bacteria: bactericidal effect of essential oil and hydrosol of *Satureja thymbra* and comparison with standard acid–base sanitizers

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Keywords
antimicrobial, biofilms, disinfection, essential oil, hydrosol, inhibitors, *Satureja thymbra*.

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2007/0700: received 1 May 2007, revised and accepted 1 November 2007

doi:10.1111/j.1365-2672.2007.03694.x

Abstract

Aims: To assess the antimicrobial action of three natural-derived products (essential oil, decoction and hydrosol of *Satureja thymbra*) against biofilms, composed of useful, spoilage and pathogenic bacteria (formed as monoculture or/and mixed-culture), and to compare their efficiency with three standard acid and alkaline chemical disinfectants.

Methods and Results: Two acids (hydrochloric and lactic, pH 3), one alkali (sodium hydroxide, pH 11), the essential oil of *S. thymbra* (1% v/v) and the two by-products of the essential oil purification procedure (the decoction and the hydrosol fraction of essential oil, 100%), were tested against biofilms formed by five bacterial species, either as monospecies, or as mixed-culture of all species. The tested bacterial species were *Staphylococcus simulans* and *Lactobacillus fermentum* (useful technological bacteria), *Pseudomonas putida* (spoilage bacterium), *Salmonella enterica* and *Listeria monocytogenes* (pathogenic bacteria). Biofilms were left to be formed on stainless steel coupons for 5 days at 16°C, before the application of disinfection treatments, for 60 and 180 min. The disinfection efficiency was evaluated by detaching the remaining viable biofilm cells and enumerating them by agar plating, as well as by automated conductance measurements (using Rapid Automated Bacterial Impedance Technique). Both these methods revealed that the essential oil and the hydrosol of *S. thymbra* exhibited a strong antimicrobial action against both monospecies and mixed-culture biofilms. Surprisingly, the efficiency of the other three acid–base disinfectants was not adequate, although a long antimicrobial treatment was applied (180 min).

Conclusions: The essential oil of *S. thymbra* (1%), as well as its hydrosol fraction (100%), presents sufficient bactericidal effect on bacterial biofilms formed on stainless steel.

Significance and Impact of the Study: Use of natural antimicrobial agents could provide alternative or supplemented ways for the disinfection of microbial-contaminated industrial surfaces.

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Introduction

Biofilm formation is an extremely common phenomenon and most materials in contact with a natural fluid may rapidly become colonized by bacteria (Carpentier and Cerf 1993; Costerton *et al.* 1995; Geesey 2001). In the majority of natural environments, monospecies biofilms are relatively rare. Conversely, micro-organisms are associated with surfaces in complex multispecies communities (James *et al.* 1995; Stoodley *et al.* 2002). In the food industry of fermented products (e.g. traditional sausages), mixed bacterial biofilms may contain strains beneficial to the quality of the food product (e.g. lactobacilli and staphylococci). However, biofilms of both spoilage and pathogenic microflora are related to problems of food contamination, leading to lowered shelf-life of products and transmission of diseases (Zottola and Sasahara 1994; Hood and Zottola 1995, 1997). The risk becomes even more serious since bacteria in biofilms have been shown to express increased resistance to disinfectants compared with their planktonic counterparts (Mah and O'Toole 2001; Gilbert *et al.* 2002; Stewart *et al.* 2004).

Nowadays, it is priority for food-processing industries to apply well cleaning and sanitation programmes to minimize the risk of biofilm contamination (Gibson *et al.* 1999). However, this is not always easy to accomplish because used disinfectants, in order to be effective, should be able to penetrate the extracellular polymeric substances matrix surrounding the biofilms and kill the cells (Sutherland 2001). Research for new substances in biofilm disinfection is therefore an important area of focus. The recent negative consumer perception against artificial synthetic chemicals, however, has shifted this research effort towards the development of alternatives that consumers perceive as 'naturals' (Roller 1995; Davidson 1997). Previous studies have indicated that the essential oils and extracts of edible and medicinal plants, herbs and spices constitute a class of very potent natural antibacterial agents (Koutsoumanis *et al.* 1999; Skandamis and Nychas 2001; Nychas *et al.* 2003; Burt 2004). However, both their strong flavour and their lipophilic nature have limited, until now, their widely practical application. Substitutionally, hydrosols (by-products of essential oils purification procedure) are aqueous solutions and do not present the strong – usually undesirable for foodstuffs – smell of the essential oils. They are also documented to have antimicrobial properties (Sagdic and Ozcan 2003).

To date, limited information is available on the evaluation of essential oils as disinfectants against bacterial biofilms, while no information, to our knowledge, is available on the antimicrobial action of their hydrosol fractions against biofilm bacteria. Polytoxinol, an essential oil-based compound, has been shown to be effective against biofilms of Gram-positive coagulase-negative staphylococci (Al-Shuneigat *et al.* 2005). The antimicrobial action of carvacrol against mixed-species biofilm composed from *Staphylococcus aureus* and *Salmonella typhimurium* has also been shown (Knowles *et al.* 2005), as well as of plant essential oil components against biofilms formed by *Escherichia coli* strains (Niu and Gilbert 2004). In a recent report, Lebert *et al.* (2007) have demonstrated that the essential oil of *Satureja thymbra* was highly effective on *Pseudomonas fragi* and *E. coli*, when grown as mixed biofilm with *S. aureus* and *Listeria monocytogenes*.

On open surfaces in food industry premises, a deficient cleaning and disinfection programme may leave what is called a 'resident microflora'. Hence, a crucial feature in the assessment of the effectiveness of sanitizers is the reliable determination of the remaining attached population on sanitized surfaces. This requires use of proper methods for biofilm quantification. Available methods vary significantly and the removal of attached bacteria is dependent on the efficiency of the procedure (e.g. swabbing). Conductance measurements may be a useful tool to quantify indirectly the remaining biofilm bacteria via their metabolic activity (Giaouris *et al.* 2005).

The study reported here was conducted to: (i) evaluate the disinfection efficiency of three natural-derived products (essential oil, decoction and hydrosol of *S. thymbra*) against bacterial biofilms, grown both as monospecies and as mixed-culture of all species and (ii) compare their antimicrobial action with three standard acid/base chemical sanitizers (hydrochloric acid, lactic acid and sodium hydroxide), using two approaches simultaneously (bead vortexing followed by plate counting and conductance detection times). All disinfectants were tested on five bacterial species: *Staphylococcus simulans* and *Lactobacillus fermentum* (useful technological bacteria in fermented traditional sausages), *Pseudomonas putida* (spoilage bacterium), *Salmonella enterica* and *L. monocytogenes* (pathogenic bacteria).

Materials and methods

Plant material and isolation of essential oil, hydrosol and decoction

Fresh plant materials (stem, leaves and flowers) of *S. thymbra* were collected in 2005 during the period of full flowering on the mountain of Imittos (continental Greece, 350 m altitude). A voucher specimen of the plant is also deposited in the herbarium of the Division of Pharmacognosy and Natural Products Chemistry, Department of Pharmacy, University of Athens, Greece. Plant samples were transferred at the same time of collection to

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laboratory and were chopped giving 290 g of material which was subsequently subjected to steam distillation in a Clevenger apparatus for 3 h with 3 l of H_2O (Chorianopoulos *et al.* 2006). The resulting essential oil (5·4 ml) was stored at −20°C, until utilization in biofilm disinfection experiments (see Disinfectants and biocide treatment procedure). In addition to collecting the essential oil, its hydrosol (13·6 ml) and the decoction (2·75 l) were collected and stored at −20°C. At the end of distillation, the decoction was picked up from the flask containing the plant chopped material, while the hydrosol was easily separated as the water fraction situated under the organic phase (essential oil).

Bacterial strains and preparation of inocula

Two technological, one spoilage, and two pathogenic strains were studied. The technological bacteria *S. simulans* (LQC5187) and *L. fermentum* were isolated from Greek traditional sausages. The spoilage bacterium was the reference strain *P. putida* ATCC 12633. The two pathogens tested were *L. monocytogenes* Scott A (kindly provided by Dr Eddy Smid, Agrotechnological Research Institute ATO-DLO, Wageningen, the Netherlands) and *S. enterica* serovar Enteritidis PT4 (supplied by Division of Enteric Pathogens, Central Public Health Laboratory, London, UK).

All bacteria were subcultured three times successively, with shaking (except from *L. fermentum*), in flasks containing Brain Heart (BH) broth (Merck, Darmstadt, Germany), until stationary phase, for each bacterium, was reached. Thus, *S. simulans* was incubated at 37°C for 48 h, *L. fermentum* at 30°C for 72 h, *P. putida* at 25°C for 48 h, *S. enterica* at 37°C for 18 h and *L. monocytogenes* at 30°C for 48 h. Cells from final stationary cultures were harvested by centrifugation (3000 ***g*** for 10 min at 4°C), washed twice with Ringer solution (Ringer's tablets; Merck) and finally resuspended in Ringer solution to provide working cultures of bacterial concentration of *c.* 10^8 CFU ml^{-1} for each strain. These cultures were used, after appropriate dilution, to inoculate BH broth in test tubes containing the stainless steel coupons (see Test surface and biofilm development).

Test surface and biofilm development

Stainless steel was the surface chosen for biofilm development, as it is extensively used throughout the food-processing industry (AISI-304, Halyvourgiki Inc., Athens, Greece). Before utilization for biofilm experiments, the stainless steel coupons (3 × 0·8 × 0·1 cm) were subjected to cleaning procedure according to Giaouris *et al.* (2005). To produce biofilms, quadruplicate stainless steel coupons were placed individually in test tubes containing 3·5 ml of BH broth and the test tubes with the coupons inside them were autoclaved afterwards (121°C for 15 min). Following autoclaving, aliquots of the working cultures were inoculated in the test tubes to yield initial bacterial populations of *c.* 10^7 CFU ml^{-1} for each bacterium, in the case of monospecies and mixed-culture biofilms. Test tubes were then incubated for 5 days at 16°C, under static conditions, to allow biofilm development on the coupons. The temperature for biofilm development (16°C) was chosen so as to imitate the thermal conditions encountered at Greek traditional sausages workshops (mean temperature according to European project Tradisausage QLK1-CT-2002-02240).

Disinfectants and biocide treatment procedure

Six disinfectants that comprised hydrochloric acid (1 mmol l^{-1}, pH 3; Merck), sodium hydroxide (1 mmol l^{-1}, pH 11; Merck), lactic acid (8 mmol l^{-1}, pH 3; Merck), essential oil of *S. thymbra* (1% v/v, pH 6·2), decoction of *S. thymbra* (100%) and hydrosol fraction of essential oil of *S. thymbra* (100%) were tested (Table 1). Each disinfectant was prepared in distilled water, except from the essential oil, which was first solubilized in ethanol and then diluted in distilled water (19% ethanol – 1% essential oil – 80% distilled water). Ringer solution (pH 6·8) and ethanol (20% v/v; Merck) were used as controls for disinfection.

For disinfection testing, coupons – carrying biofilm bacteria on them – were aseptically removed from test tubes (previously incubated for 5 days at 16°C), rinsed two times with Ringer solution and were thereafter introduced in new test tubes, containing 5 ml of each of disinfectant. Disinfection was carried out at 16°C for 60 and 180 min. After disinfection, coupons were rinsed two times with Ringer solution and were immediately: (i) either subjected to 'bead vortexing' to detach the remaining viable biofilm cells and enumerate them by plate counting (see Enumeration of biofilm bacteria) or/and

Table 1 List of applied disinfection treatments

Treatment	Composition/parameters of treatment
A	Ringer solution (control)
B	20% (v/v) ethanol, 80% (v/v) distilled water (control)
C	1 mmol l^{-1} hydrochloric acid (pH 3)
D	8 mmol l^{-1} lactic acid (pH 3)
E	1 mmol l^{-1} sodium hydroxide (pH 11)
F	1% (v/v) essential oil of *Satureja thymbra*, 19% (v/v) ethanol, 80% (v/v) distilled water
G	Decoction (100%)
H	Hydrosol (100%)

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(ii) placed into the Rapid Automated Bacterial Impedance Technique (RABIT) tubes (Don Whitley Scientific Limited, Shipley, UK) for conductance measurements, to quantify indirectly the remaining viable biofilm bacteria, via their metabolic activity (see Conductance measurements).

Enumeration of biofilm bacteria

The enumeration of biofilm cells after 5 days of incubation at 16°C, as well as the enumeration of remaining biofilm cells after disinfection, was performed using the 'bead vortexing method' described by Giaouris *et al.* (2005), with some minor modifications. Briefly, following incubation or disinfection, respectively, each coupon was carefully removed using sterile forceps, rinsed twice with Ringer solution and finally transferred to new test tube containing 6 ml of Ringer solution and 10 sterile glass beads (diameter, 3 mm). The test tube was then vortexed for 2 min, at maximum intensity, on a vortexer (Velp Scientifica, Milano, Italy) to detach the cells from the coupons. Quantification of biofilm production was performed by agar plating. For this, 1 ml of bacterial suspension was removed from each test tube after vortexing with beads, 10-fold serial dilutions were prepared in sterile Ringer solution for each sample and bacteria were enumerated on the surface of duplicate agar plates. Detection limit of the plating counts was 1·03 and 0·03 log CFU cm^{-2}, for spread and pure method, respectively.

In the case of monospecies biofilm development, Tryptone Soy Agar (LAB M; International Diagnostics Group Plc, Bury, Lancashire, UK) was used for the enumeration of micro-organisms (see below details for incubation temperatures and times). In the case of mixed-culture biofilms, the different species were enumerated using the following selective media: Baird-Parker agar for *S. simulans* (incubation at 37°C for 48 h), de Man, Rogosa, Sharpe agar (MRS; Merck) for *L. fermentum* (poor plating method and incubation at 30°C for 72 h), Cetrimide Fucidin Cephaloridine agar (Oxoid, Basingstoke, Hampshire, UK) for *P. putida* (incubation at 25°C for 48 h), Xylose Lysine Deoxycholate agar (Merck) for *S. enterica* (incubation at 37°C for 18 h) and PALCAM Listeria Selective agar (PALCAM; Merck) for *L. monocytogenes* (incubation at 30°C for 48 h).

Conductance measurements

The RABIT (Don Whitley Scientific) was also used for indirect quantification of remaining biofilm cells after disinfection (Flint *et al.* 1997; Giaouris *et al.* 2005). Conductance methods measure the production of ionic molecules in the growth medium, as an indication of the metabolic activity of growing micro-organisms. Moreover, conductance measurements can result in detection of bacterial cells that cannot be recovered by bead vortexing, providing thus a more sensitive tool.

Experimentally, conductance measurements were performed by placing each stainless coupon, after the disinfection and the rinsing procedure, into a RABIT tube, that contained 4·5 ml of BH broth. RABIT tubes were then incubated into the RABIT apparatus for 48 h at 30°C, while the system was adjusted to measure broth conductance every 6 min. In the case of existence of remaining viable biofilm cells on the coupons, these start to multiply, as they are in a rich growth medium. Their metabolic activity alters the conductance of the broth because of nutrients degradation and production of charged substances. Undoubtedly, during the growth of the cells, they start to detach from the coupons and become planktonic. The system gives the 'detection time' (DT) when the bacterial concentration into the RABIT tube (planktonic cells) reaches the threshold level of *c.* 10^6 CFU ml^{-1} (when default detection criteria are applied, +5 μS). It is important to differentiate between the detection threshold of the RABIT system, and its sensitivity, which is capable of detecting presence of micro-organisms at levels as low as <10 CFU ml^{-1}, providing they are viable (Anonymous 1996). For the given test protocol, DT correlates linearly with the initial concentration of bacteria on the coupons (those remaining after disinfection). Thus, shorter DTs suggest higher level of remaining biofilm bacteria on coupons, and thus, lower disinfection efficiency.

Results

Biofilm formation (monospecies and mixed-culture biofilms)

Regarding monospecies biofilms (Fig. 1), *P. putida* and *S. enterica* were the two species with the maximum number of biofilm bacteria, compared to the other three species tested (5·81 and 5·26 log CFU cm^{-2}, respectively). *Listeria monocytogenes* formed a biofilm of 4·19 log CFU cm^{-2}, while the biofilm counts for the two technological species *S. simulans* and *L. fermentum* were 3·69 and 3·32 log CFU cm^{-2}, respectively.

Regarding mixed-culture biofilm (Fig. 1), plate counting interestingly revealed that it was mainly composed of *P. putida* cells (97·8%, 5·98 log CFU cm^{-2}). *Salmonella enterica* and *L. monocytogenes* represented together only the 2·2% of it. Thus, biofilm counts for *S. enterica* were 1·5 log less than when in monoculture biofilm, while biofilm counts for *L. monocytogenes* did not differ between monospecies and mixed-culture (about 4 log CFU cm^{-2}). Besides, the strong biofilm-forming ability of *P. putida*, it

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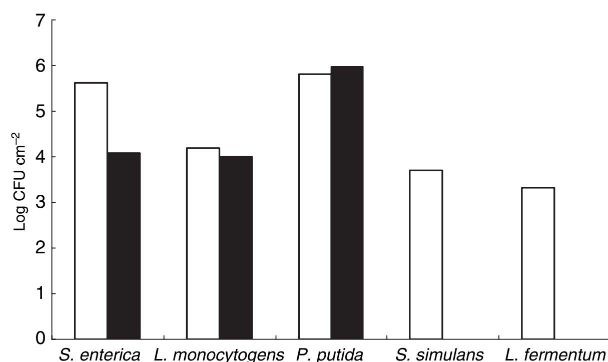

Figure 1 Formed biofilms on stainless steel coupons (log CFU cm^{-2}) before disinfection (at the fifth day of incubation at 16°C), for the five bacterial species tested, grown either as monoculture (■) or mixed-culture (□) (in mixed-culture, biofilm counts for *Staphylococcus simulans* and *Lactobacillus fermentum* were below the detection limit of plate counting method: 1·03 and 0·03 log CFU cm^{-2}, respectively).

was surprisingly found that the two technological species (*S. simulans* and *L. fermentum*) were unable to form biofilms in the presence of the other three species. Their biofilm counts were below the detection limit of plate counting method.

It should be noted that good repeatability was observed between independent biofilm formation experiments and standard deviations were always lower than 0·4 log CFU cm^{-2}.

Antimicrobial action of disinfectants against monospecies biofilms

The effect of each disinfectant on biofilms was expressed as population log-reduction of biofilm bacteria (between the initial formed and the remaining biofilm after disinfection), whereas when conductance measurements were used for the evaluation of disinfection, the 'biocide effect' was expressed as the delay of the conductance DT of the sample compared to that of controls, i.e. Ringer solution or 20% ethanol (Table 2).

The essential oil and the hydrosol of *S. thymbra* (F, H; Table 1) were the most effective treatments used for biofilm disinfection (Table 2). Both these treatments resulted in log-reductions for *L. monocytogenes*, *P. putida*, *S. simulans* and *L. fermentum* at levels below the detection limit of plate counting method, when disinfection was performed for either 60 or 180 min (Table 2). More than 4 log-reductions were also observed for *S. enterica* (Table 2). Contrary to these two natural disinfectants, the third by-product of essential oil purification procedure, i.e. the decoction (G; Table 1) was less effective, and its efficiency was similar to the other four chemical disinfectants (B, C, D, E; Tables 1 and 2). It should be noted that in all cases, no significant differences were observed between the two disinfection times (60 and 180 min). Increasing disinfection time more than 1 h, in general did not result in higher disinfection efficiencies.

Results obtained by conductance measurements corresponded well to those of plate counting method (Fig. 2). Longer DTs correlated enough properly with higher log-reductions. Thus, when disinfection treatment was performed with the essential oil and its hydrosol fraction, the observed DTs were higher compared with those given when disinfection was performed with the other disinfectants (Table 2).

Antimicrobial action of disinfectants against mixed-culture biofilms

Like in the case of monospecies biofilms, the disinfection efficiency against mixed-culture biofilms was expressed both as log-reductions and as delays at the conductance DTs (Table 3).

The essential oil and its hydrosol were again the most effective treatments for biofilm disinfection, as they resulted in log-reductions for *P. putida*, *S. enterica* and *L. monocytogenes* at levels below the detection limit of the plate counting method (1·03 log CFU cm^{-2}), when disinfection was done for either 60 or 180 min (Table 3). Increasing the duration of disinfection time from 60 to 180 min did not result in higher disinfection efficacies, like in the case of monospecies biofilms. Because of the fact that biofilm cells for *S. simulans* and *L. fermentum* were not detectable before disinfection, it had no meaning at all to evaluate the efficiency of disinfection treatments for these strains (expressed as log-reductions using the plate counting method).

Discussion

The bacterial species used in this work are typically found in complex food industrial ecosystems, such as those of sausage-processing industry. Thus, the two technological bacteria *S. simulans* and *L. fermentum* are used for sausage fermentation, while *P. putida*, *S. enterica* and *L. monocytogenes* are representatives of common spoilage and pathogenic bacteria, usually found embedded in biofilms on meat industrial equipment surfaces, when cleaning and disinfection is insufficient (Beresford *et al.* 2001; Joseph *et al.* 2001). We let biofilms to be developed for 5 days at 16°C to mimic biofilm-forming environmental conditions encountered in such environments. It is also well known that true multidynamic biofilms in meat-processing plants may take several days or even weeks to be developed (Jessen and Lammert 2003). In addition, many studies on food-borne biofilm bacteria have also

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Table 2 Log-reductions (LR, log CFU cm^{-2}) of biofilm cells, after exposure to disinfectants and conductance detection times (DT, h) of the remaining viable biofilm cells (±SD; n = 4; the bacterial species were grown in monoculture)

		Biofilm formation (log CFU cm^{-2}) of strain at fifth day of incubation at 16°C									
		S. enterica (5·62)		*L. monocytogenes* (4·19)		*P. putida* (5·81)		*S. simulans* (3·69)		*L. fermentum* (3·32)	
Treatment*	Time (min)	LR	DT	LR	DT	LR	DT	LR	DT	LR	DT
A	60	0·08 ± 0·02	5·85 ± 0·06	0·04 ± 0·03	11·90 ± 0·00	0·11 ± 0·05	6·40 ± 0·18	0·26 ± 0·09	9·40 ± 0·05	0·29 ± 0·03	12·20 ± 0·00
	180	0·11 ± 0·04	5·90 ± 0·03	0·23 ± 0·01	12·40 ± 0·12	0·23 ± 0·02	6·95 ± 0·03	0·48 ± 0·10	9·70 ± 0·03	0·82 ± 0·07	12·80 ± 0·03
B	60	0·75 ± 0·18	6·40 ± 0·12	0·18 ± 0·06	11·95 ± 0·03	1·18 ± 0·06	6·55 ± 0·01	0·66 ± 0·02	10·70 ± 0·12	1·04 ± 0·02	13·10 ± 0·18
	180	0·68 ± 0·09	6·45 ± 0·04	0·33 ± 0·12	13·65 ± 0·10	1·78 ± 0·03	6·80 ± 0·00	0·96 ± 0·08	10·15 ± 0·01	1·23 ± 0·01	13·90 ± 0·03
C	60	1·15 ± 0·01	6·05 ± 0·10	0·16 ± 0·10	12·05 ± 0·03	1·45 ± 0·01	9·40 ± 0·03	1·96 ± 0·03	10·15 ± 0·18	1·02 ± 0·05	14·20 ± 0·12
	180	1·22 ± 0·04	6·65 ± 0·01	0·54 ± 0·18	12·95 ± 0·01	1·98 ± 0·09	9·75 ± 0·06	Below DL	10·80 ± 0·03	1·14 ± 0·02	14·40 ± 0·06
D	60	1·31 ± 0·10	6·80 ± 0·18	0·47 ± 0·05	12·95 ± 0·00	1·85 ± 0·02	7·20 ± 0·12	1·18 ± 0·09	10·30 ± 0·00	0·93 ± 0·14	14·10 ± 0·03
	180	1·91 ± 0·02	7·30 ± 0·06	0·71 ± 0·02	13·05 ± 0·01	1·85 ± 0·08	8·65 ± 0·10	1·66 ± 0·06	10·30 ± 0·03	1·55 ± 0·07	14·60 ± 0·12
E	60	0·12 ± 0·09	7·05 ± 0·10	0·12 ± 0·01	12·35 ± 0·03	1·37 ± 0·10	7·35 ± 0·03	0·35 ± 0·07	9·70 ± 0·06	0·66 ± 0·02	14·50 ± 0·01
	180	1·50 ± 0·12	8·05 ± 0·12	0·24 ± 0·03	12·75 ± 0·18	2·41 ± 0·03	8·80 ± 0·06	0·48 ± 0·02	9·90 ± 0·00	0·82 ± 0·09	14·85 ± 0·03
F	60	4·23 ± 0·01	11·65 ± 0·06	Below DL†	26·20 ± 0·06	Below DL	14·50 ± 0·05	Below DL	18·05 ± 0·01	Below DL	>48
	180	4·49 ± 0·04	12·06 ± 0·09	Below DL	28·90 ± 0·03	Below DL	16·50 ± 0·03	Below DL	20·60 ± 0·03	Below DL	>48
G	60	0·99 ± 0·18	6·50 ± 0·01	0·18 ± 0·01	12·05 ± 0·00	0·49 ± 0·18	8·10 ± 0·01	0·04 ± 0·03	9·95 ± 0·01	1·06 ± 0·10	15·15 ± 0·00
	180	0·97 ± 0·02	6·70 ± 0·12	0·51 ± 0·02	12·45 ± 0·12	0·67 ± 0·02	8·30 ± 0·00	0·15 ± 0·12	10·25 ± 0·10	1·38 ± 0·18	15·80 ± 0·06
H	60	4·01 ± 0·12	14·00 ± 0·06	Below DL	22·85 ± 0·03	Below DL	13·65 ± 0·03	Below DL	17·05 ± 0·03	Below DL	>48
	180	4·41 ± 0·04	15·20 ± 0·18	Below DL	28·30 ± 0·06	Below DL	14·88 ± 0·12	Below DL	19·85 ± 0·03	Below DL	>48

*See Table 1 for the composition of each disinfectant.

†DL, detection limit of the plate counting method for *Salmonella enterica*, *Listeria monocytogenes*, *Pseudomonas putida* and *Staphylococcus simulans*: 1·03 log CFU cm^{-2}, for *Lactobacillus fermentum*: 0·03 log CFU cm^{-2}.

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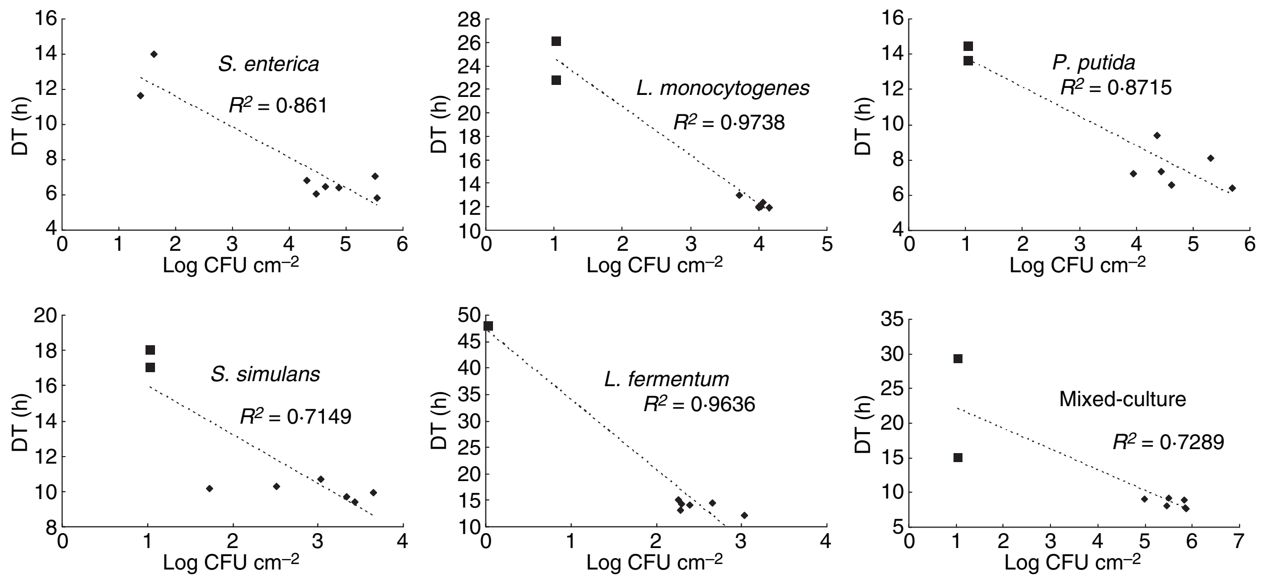

Figure 2 Relationships between biofilm counts (log CFU cm^{-2}) after disinfection (60 min, treatments: A–H) and conductance detection times (h). Rectangular points correspond to detection limit of plate counting.

Table 3 Log-reductions (LR; log CFU cm^{-2}) of biofilm cells, after exposure to disinfectants and conductance detection times (DT; h) of the remaining viable biofilm cells (±SD; n = 4; the bacterial species were grown in mixed-culture‡)

		Biofilm counts (log CFU cm^{-2}) of each strain in the mixed-biofilm‡			
		Salmonella enterica (4·07) 1·2%	*Listeria monocytogenes* (4·00) 1%	*Pseudomonas putida* (5·98) 97·8%	
Treatment*	Time (min)	LR	LR	LR	DT
A	60	0·05 ± 0·07	0·09 ± 0·18	0·12 ± 0·05	7·64 ± 0·18
	180	0·18 ± 0·08	0·24 ± 0·03	0·08 ± 0·08	7·84 ± 0·03
B	60	0·43 ± 0·02	0·12 ± 0·08	0·14 ± 0·02	7·78 ± 0·00
	180	0·68 ± 0·14	0·07 ± 0·10	0·29 ± 0·14	7·54 ± 0·10
C	60	0·46 ± 0·18	0·28 ± 0·02	0·53 ± 0·07	8·06 ± 0·03
	180	0·87 ± 0·07	0·55 ± 0·05	0·67 ± 0·02	8·48 ± 0·12
D	60	1·05 ± 0·02	0·52 ± 0·08	1·02 ± 0·12	9·12 ± 0·18
	180	1·14 ± 0·09	0·67 ± 0·03	1·34 ± 0·03	9·42 ± 0·03
E	60	0·12 ± 0·12	0·49 ± 0·09	0·16 ± 0·06	8·94 ± 0·06
	180	0·36 ± 0·05	0·44 ± 0·14	0·09 ± 0·03	9·54 ± 0·01
F	60	Below DL†	Below DL	Below DL	15·12 ± 0·00
	180	Below DL	Below DL	Below DL	16·30 ± 0·12
G	60	0·87 ± 0·10	0·32 ± 0·06	0·49 ± 0·02	9·24 ± 0·06
	180	0·97 ± 0·02	0·41 ± 0·06	0·88 ± 0·08	9·06 ± 0·03
H	60	Below DL	Below DL	Below DL	29·42 ± 0·01
	180	Below DL	Below DL	Below DL	30·18 ± 0·00

Percentages of each species in mixed biofilm before disinfection are also indicated.
*See Table 1 for the composition of each disinfectant.
†DL, detection limit of the plate counting method (1·03 log CFU cm^{-2}).
‡Each strain was cultured in the presence of other four strains (*Staphylococcus simulans* and *Lactobacillus fermentum* did not form biofilm).

indicated that their resistance against various disinfectants increases with biofilm age (Frank and Koffi 1990; Wirtanen and Mattila-Sandholm 1992; Sommer *et al.* 1999). We also left biofilms to be developed in mixed-culture of all strains because, in most ecosystems, biofilms are complex communities consisting of more than one microbial

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species (Wimpenny *et al.* 2000; Stoodley *et al.* 2002). The interactions between the different species have been shown to influence the biofilm forming capacity of individual strains, as well as their resistance to antimicrobial treatments (Leriche *et al.* 2003; Carpentier and Chassaing 2004, Molin *et al.* 2004; Burmolle *et al.* 2006).

Interestingly, under given experimental conditions, mixed-culture biofilm was mainly composed of *P. putida* (97·8%), while *S. simulans* and *L. fermentum* were not detectable. Species composition of mixed-culture biofilm should be the final 'result' of competition between micro-organisms, both for available nutrients and for free surface to colonize (Leriche *et al.* 1999; Leriche and Carpentier 2000). Microscale structural heterogeneity, production of metabolites and differing rates of attachment and detachment between the five species could also be responsible for the final coexistence observed in our system (Zhao *et al.* 2004). The finding, concerning the reduced biofilm-forming ability of technological bacteria, is in contrast with the study of Ammor *et al.* (2004), where they reported that the technological bacteria *Staphylococcus carnosus* and a strain of *Lactobacillus* when cultivated in multispecies biofilms, with the spoilage bacteria *Enterococcus faecium*, *P. fluorescens, P. putida, Hafnia alvei* and the pathogen *L. monocytogenes*, were able to produce biofilms on extra-thick fibreglass discs, which, moreover, were resistant to the antimicrobial action of monolaurin (0·075 w/v) and acetic acid. However, the dynamic and environmental conditions for biofilm formation used in that study (bacterial strains, support material, biofilm incubation temperature and growth medium), were quite different from the biofilm forming conditions employed in this work.

Regarding disinfection treatments, because it is the undissociated acid molecule that mainly accounts for an acid's antibacterial activity (Doores 1993), we decided to choose lactic acid (pK_a: 3·85) – already used for decontamination in meat environments (Gordon Greer and Dilts 1995) – as well as the strong hydrochloric acid (pK_a: −8), in the same pH value of 3 for both, to see whether there would be any effect on disinfection. Sodium hydroxide, used here at pH 11, consists of the reactive compound of many alkaline disinfectants (Taormina and Beuchat 2002). Essential oil from *S. thymbra* has already been shown to suppress the growth of pathogenic bacteria and yeasts (Goren *et al.* 2004; Chorianopoulos *et al.* 2006).

Disinfection programmes currently applied in many food-processing industries vary from 15 to 60 min. Preliminary results in our laboratory have, however, shown that 5 days biofilms of *P. putida* and *S. enterica* were very resistant against some of the tested chemical acid–base disinfectants. This was the main reason why we selected to carry out the disinfection treatments for 60 min, as well as for 180 min, to see if the increase of disinfection's duration to 3 h would result in higher bactericidal efficiencies. However, according to the results of this study, disinfection efficiencies were similar between 60 and 180 min, indicating that all tested disinfectants mainly acted against biofilm bacteria during the first hour, and after that, no more antimicrobial action was exhibited.

When bacteria attach to surfaces and form biofilms cleaning and disinfection usually become more difficult (Das *et al.* 1998; Gilbert *et al.* 2001, 2003; Gawande and Bhagwat 2002). Possible reasons for the increased resistance of biofilm cells to antimicrobial treatments include the difficulty of a disinfectant to penetrate the matrix surrounding the biofilms, the altered microenvironment, which in turn contributes to slow microbial growth and acquisition of resistance phenotypes and the existence of persistent cells (Lewis 2001; Stewart *et al.* 2004). In this work, standard chemical disinfectants, such as lactic acid, hydrochloric acid and sodium hydroxide did not efficiently disinfect the formed biofilms. The resistance of biofilm bacteria to acids and caustic soda in this study could possibly be explained by the fact that these substances may not diffuse sufficiently across the biofilm to kill cells or by an adaptive mechanism, which would allow the bacteria to maintain a relatively constant and low transmembrane pH gradient (Diez-Gonzalez and Russell 1997).

In contrast to acid–base disinfectants, the essential oil and its hydrosol (F, H; Table 1) exhibited a strong antimicrobial action, against both monoculture and mixed-culture biofilms (Tables 2 and 3). In a majority of cases, these two products caused the maximum possible log-reductions. It was also confirmed (by observing no cells after plating the run-off fluids from the coupons after disinfection) that these two compounds had killed the cells and not just removed them from the coupons into the liquid phase. Recently also, Lebert *et al.* (2007) showed that the essential oil from *S. thymbra* presented an antimicrobial action against the spoilage strains *P. fragi, E. coli*, the pathogens *S. aureus, L. monocytogenes* and the technological strain *Staphylococcus equorum*, all of them grown as multispecies biofilm on extra-thick glass fibre filters. However, in our study, conductance measurements interestingly revealed that there were still remaining metabolically active biofilm cells even after the strong disinfection treatments (Tables 2 and 3). These cells could not be recovered by the bead vortexing procedure or/and could not be detected by the plate counting (below detection limit).

In previous reports, we had showed that the essential oil of *S. thymbra* contains carvacrol and thymol at 50% of its total volume (Chorianopoulos *et al.* 2006). Also, it

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is believed that, during the steam distillation, a part of the essential oil is dissolved in hydrosol (Rajeswara Rao *et al.* 2002). The strong inhibitory effect of essential oils on micro-organisms is highly associated with the presence of these two compounds, as they are documented to cause structural and functional damages to plasma membranes, modifying thus their permeability and leading to leakage of cell contents (Sikkema *et al.* 1995; Ultee *et al.* 1999; Lambert *et al.* 2001; Falcone *et al.* 2005). Interestingly, this study showed that such compounds have also the ability to inactivate biofilm bacteria.

It must also be pointed out that carvacrol, thymol and their biosynthetic monoterpene precursors *p*-cymene and γ-terpinene – representing the bulk of the essential oil of *S. thymbra* (76%; Chorianopoulos *et al.* 2006) – have already been registered by the European Commission for use as flavourings in foodstuffs, while the United States Food and Drug Administration (FDA) has classified these substances as generally recognized as safe or as approved food additives (http://www.cfsan.fda.gov/~dms/eafus.html). However, it is recommended that more safety studies based on toxicology data be carried out before essential oil and hydrosol of *S. thymbra* are widely used as biofilm disinfectants in food industries.

Despite the obvious antimicrobial action of the essential oil of *S. thymbra* against biofilms, the practical application of this compound may still be hampered by some limitations (strong smell and difficulty to flush it efficiently from surfaces after a disinfection programme). On the other hand, its hydrosol fraction, as aqueous solution, is easily rinsed out from surfaces, and it does not have the strong smell of the essential oil. As hydrosol is a by-product of essential oil purification procedure, without any industrial application until now, it could be useful to make it productive and apply such compound for the disinfection of surfaces in food industrial environments. Hydrosols may provide valuable alternative pathways for the prevention of biofilm formation.

In conclusion, the importance of this work lies in the extension of our knowledge on the antimicrobial action of essential oils against biofilm bacteria, and moreover, new exciting data are presented on the antimicrobial activity of their hydrosol fractions. However, further research is needed (e.g. dose–response, toxicological studies) to establish these promising natural compounds as biofilm sanitizers in real complex food-processing ecosystems.

Acknowledgements

This work was supported by an EU project (ProSafeBeef) within the 6th Framework Programme (ref. Food-CT-2006-36241).

We are grateful to Michalis Kalomoiris for technical assistance.

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