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Biofilms in water industry: case study of *Salmonella enterica* serovar Typhimurium - biofilm formation and control *in vitro* and *in situ.*

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ABSTRACT

Biofilm formation is an important issue in food industries constituting an important factor of cross contamination. On the other hand,*Salmonella* is recognized as one of the most significant enteric foodborne pathogens. In recent years, the resistance of biofilms to antimicrobials has led to the search of novel antimicrobial compounds. To this direction, the aim of the present work was 1) to monitor biofilm formation in mono- and multi-species cultures of *Salmonella* Typhimurium (*S*T) with bacteria isolated from a bottling plant by fluorescence-based bioreporters and 2) evaluate the disinfection activity of *Thymbra capitata* hydrosol against *S*T biofilm cells, in comparison with the commonly used benzalkonium chloride (BC). In order toachieve this, a set of 16 samples were collected from a bottling plant. After bacterial identification, they left to form biofilm on stainless steel (SS) for 6 days at 20°C. In parallel, *S*T biofilm development with mixed communities was examined by using ten different ST bioreporters by fluorescence microscopy. Furthermore, the disinfectant efficacy of hydrosol and BC was comparatively tested against 24 h ST planktonic and biofilm cells following 6 min of treatment. Additionally, their bactericidal effect inside biofilm was evaluated by real-time visualization of the disinfection activity using time-lapse confocal laser scanning microscopy (CLSM). Regarding the obtained results, mixed bacterial communities recovered from bottling plantconsist of *Citrobacter*, *Staphylococcus*, *Pseudomonas*, *Bacillus*, *Exiguobacterium* species. The presence of indigenous bacteria neither inhibited nor enhanced biofilm formation of *S*T. Furthermore, the csrA-based bioreporter was shown to be induced in multispecies biofilms with *Citrobacter*. The role of CsrA, which is a key global regulator during multispecies biofilm formation needs to be further studied. Moreover, regarding the hydrosol resistance coefficient (Rc=1.56) was significantly lower compared to the one of BC (Rc=208.33), showing that hydrosol was almost equally active against both planktonic and biofilm cells, whereas 200 times higher concentration of BC was needed to achieve the same effect against biofilm cells, as planktonic ones. Furthermore, CLSM revealed a significant advantage of the hydrosol as its great ability to penetrate quickly the biofilm structure and act effectively.

Keywords: Salmonella, biofilms, reporters, hydrosol, confocal laser scanning microscopy

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1. INTRODUCTION

1.1 *Salmonella* species: overview

In United States of America, in 1885, Salmon and Smith detected in pigs suffering from cholera *Bacillus* cholarae-sius which is nowadays reported as *Salmonellaenterica* serovar Choleraesius. The name *Salmonella* was coined much later in 1900 by Lignieres (D' Aust and Maurer, 2007). In 1920 White and Kauffmann developed a serotyping scheme based on the discovery of flagella H antigen, the somatic O antigen and the phase-shift in the H antigen for *Salmonella* taxonomy. The Kauffmann-White method, used worldwide, is consider the gold standard for identification of *Salmonella* serotypes. Identification of *Salmonella* serotypes provides information about the severity of the disease, the source of contamination and the resistance pattern (Molbak et al., 2006).

1.1.1 General features

All species of *Salmonella* genus are gram negative, facultative anaerobic, flagellated, rod shaped bacteria that belongs to *Enterobacteriaceae* family. *Salmonelleae* are heterotrophic bacteria and have the ability to metabolize nutrients both by aerobic respiration and fermentation. The optimum temperature of growth is 37 °C. They catabolize D-glucose and other carbohydrates by producing acid and gas and they are oxidase negative and catalase positive (Adams and Moss, 2008).

Generally, species of *Salmonella* genus consists of bacteria that can adapt to extreme environmental conditions. A few *Salmonella* strains can grow in high temperatures (≤54) whereas some other demonstrate psychrotrophic properties as they can grow in foods stored at 2-4 °C. Salmonellas are heat sensitive, thusthey are readily destroyed by pasteurization temperatures. The minimum a_w for growth is around 0.93 but cells survive well in dried foods, however the survival rate increasing as a_w is reduced.Optimal growth occurs around pH 7 (Adams et Moss, 2008).

Salmonelleae are primarily inhabitants of the gastrointestinal tract. They are carried by a wide range of food animals, wild animals, rodents, pets, birds, reptiles, and insects, usually without the display of an apparent illness. They can be disseminated via faeces to soil, water, foods and feeds and thence to other animals (including humans).

1.1.2 Taxonomy

Through serotyping based on the antigenic structure of the *Salmonella* cell surface. The genus *Salmonella* consists of two species, *Salmonella enterica* and *Salmonella bongori*. According to the current White-Kauffmann-Le Minor Scheme *Salmonella enterica* is further divided into six subspecies (*S. enterica* subspecies enterica (I), *S. enterica* subsp. salamae (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. diarizonae (IIIb), *S. enterica* subsp. houtenae (IV), *S. enterica* subsp. *Indica* (VI) (Grimont et Weill, 2007). Fermentation of substances allows the differentiation into the particular subspecies. There are currently over 2,600 *Salmonella* serotypes, however 99% of these serovars are in *S. enterica* and almost 60% belong to *S. enterica* subsp. *enterica* (I). The serovar discussed in this study is *S. enterica* subspecies *enterica* serovar Typhimurium.

1.1.3 Pathogenesis and clinical features

Salmonelleae are responsible for a number of different clinical syndromes grouped here as enteritis and systemic disease.

Enteritis. Gastrointestinal infections are predominantly associated with those serotypes which occur widely in animals and humans. They can range in severity from asymptomatic carriage to severe diarrhoea and are the most common type of salmonellosis.

The incubation period for *S.* Enteritis is typically between 6 and 48 h. The principal symptoms of mild fever, nausea and vomiting, abdominal pain and diarrhea last for a few days but, in some cases, can persist for a week or more. The illness is usually self-limiting but can be more severe in particularly susceptible groups such as the very young, the very old and those already ill.

Ingested organisms, which survive passage through the stomach acid, adhere to the epithelial cells of the ileum via mannose-resistant fimbriae. They are then engulfed by the cells in a process known as receptor mediated endocytosis. The ability of salmonellas to enter non-phago-cytic cells is a property essential to their pathogenicity. Our understanding of the molecular basis of this process has increased considerably with the discovery that it is largely encoded on a 35–40 kb region of the chromosome, described as a pathogenicity island (SPI) (Marcus et al., 2000). This region of the DNA encodes a complex secretion system for the proteins required in

the signalling events which subvert the host cell and ultimately lead to bacterial uptake. Known as a type III or, in some cases, a contact dependent secretion system, such systems are also present in a number of other enteropathogens such as *Shigella*, *Yersinia*, enteropathogenic and enterohaemorraghic *Escherichia coli*. Phylogenetic analysis and their base composition suggest that these regions of DNA may have been acquired from another micro-organism as a block; an event which clearly marks an important evolutionary step towards pathogenicity. Endocytosed salmonellas pass through the epithelial cells within a membrane-bound vacuole, where they multiply and are then released into the lamina propria via the basal cell membrane. This prompts an influx of inflammatory cells leading to the release of prostaglandins which activate adenylate cyclase producing fluid secretion into the intestinal lumen (Adams et Moss., 2008). The picture is a little more complex than this since there are at least four other pathogenicity islands also contributing to the overall pathogenicity of the organism (Marcus et al., 2000).

As a general rule, the infectious dose of *Salmonella* is high, of the order of 10^6 cells, but this will vary with a number of factors such as the virulence of the serotype, the susceptibility of the individual and the food vehicle involved (Teunis et al., 2010). A number of outbreaks has occurred where epidemiological evidence points to an infective dose as low as 10–100 cells. This appears to be particularly associated with more susceptible individuals such as children and the elderly, and with fatty foods such as cheese, salami and chocolate. After symptoms have subsided, carriage of the organismand itspassage in high numbers in the stools may occur for a few weeks, or occasionally months.

Systemic Disease. Host-adapted serotypes are more invasive and tend to cause systemic disease in their hosts; a feature which is linked to their resistance to phagocytic killing. In humans, this applies to the typhoid and paratyphoid bacilli, *S.* Typhi, and *S.* Paratyphi A, B, and C, which cause the septicaemic diseases, enteric fever (Wain et al., 2015). Typhoid fever has an incubation period from 3 to 56 days, though it is usually between 10 and 20 days. Invasive *Salmonella* penetrate the intestinal epithelium and is then carried by the lymphatics to the mesenteric lymph nodes. After multiplication in the macrophages, they are released to drain into the blood stream and are then disseminated around the body. They are removed from the blood by macrophages but continue to multiply within them. This eventually kills the

macrophages which then release large numbers of bacteria into the blood stream causing a septicaemia. In this, the first phase of the illness, the organism may be cultured from the blood. There is a slow onset of symptoms including fever, headache, abdominal tenderness and constipation and the appearance on the body of rose red spots which fade on pressure (Crump et Mintz, 2010).

During the second stage of the illness, the organism reaches the gall bladder where it multiplies in the bile. The flow of infected bile reinfects the small intestine causing inflammation and ulceration. The fever persists but with the onset ofdiarrhoea in which large numbers of the bacteria are excreted with the characteristic 'pea soup' stools and, to a lesser extent, with the urine. In more serious cases, haemorrhage of the ulcers may occur and perforation of the intestine leading to peritonitis. In milder cases, the ulcers heal and fever falls with recovery after 4–5 weeks. Unlike the more localized enteric infections, typhoid is usefully treated with antibiotics such as chloramphenicol, ampicillin and amoxicillin (Adams et Moss, 2008).

1.1.3 Association with foods

Salmonellosis is described as a zoonotic infection since the major source of human illness is the infected animals. Transmission is by the faecal–oral route whereby intestinal contents from an infected animal are ingested with food or water. A period of temperature abuse which allows the salmonellae to grow in the food and an inadequate or absent final heat treatment are common factors contributing to outbreaks (Litrup et al., 2010).

Meat, milk, poultry, and eggs are primary vehicles; they may be undercooked, allowing the salmonellas to survive, or they may cross contaminate other foods that are consumed without further cooking. Cross-contamination can occur through direct contact or indirectly via contaminated kitchen equipment and utensils. Human carriers are generally less important than animals in the transmission of salmonellosis. (Jay M. J., 2000).

In 2013, a total of 82,694 confirmed salmonellosis cases were reported by 27 EU MS, resulting in an EU notification rate of 20.4 cases per 100,000 population. This represented a 7.9 % decrease in the EU notification rate compared with 2012, and there was a declining trend of salmonellosis in the EU/European Economic Area (EEA) I the five-year period of 2009-2013. Fifty-nine fatal cases were reported by 9

MS among the 14 MS that provided data on the outcome of their cases. This gives an EU case-fatality rate of 0.14 % among the 40,976 confirmed cases for which this information was available (EFSA, 2015).

Similar to the previous years, the two most commonly reported *Salmonella* serovars in 2013 were *S.* Enteritidis and *S.* Typhimurium, representing 39.5 % and 20.2 %, of all reported serovars in confirmed human cases respectively.

Salmonella was most frequently detected in poultry meat, and less often in pig or bovine meat. The highest proportions of *Salmonella*-positive single samples were reported for fresh turkey meat at an average level of 5.4 %, followed by fresh broiler, pig and bovine meat. *Salmonella* was rarely found in table eggs, at levels of 0.03 % (single samples) or 0.5 % (batch samples) (EFSA, 2015).

Salmonella remained the most frequently detected causative agent in the food-borne outbreaks (22.5 % of total outbreaks). The most important sources of food-borne *Salmonella* outbreaks wereeggs and egg products. However, *Salmonella* was also detected in other foods at low to very low levels (EFSA, 2015).

1.1.4 Association with food processing environments

As previously mentioned, the native habitat of *Salmonella* is considered to be the intestinal tract of taxonomically diverse group of vertebrates. Interestingly, this genus has been shown to survive for extended periods of time in nonenteric habitats (White et al., 2006). It is strongly believed that its survival and persistence in non-host environments and its transmission to new hosts should be attributed to the ability of *Salmonella* to form biofilms on inanimate surfaces.

It has been observed that the resistance of biofilm cells to antimicrobials is significantly increased compared with what is normally seen with the same cells being planktonic. Thus, it is believed that biofilm formation enhances the capacity of pathogenic *Salmonella* bacteria to survive stresses that are commonly encountered within food processing (Giaouris et al., 2012).

In food industry, biofilms may create a persistent source of product contamination, leading to serious hygienic problems and also economic losses due to food spoilage (Brooks & Flint, 2008, Lindsay & von Holy, 2006). Improperly cleaned surfaces promote soil build-up, and, in the presence of water, contribute to the

development of bacterial biofilms which may contain pathogenic microorganisms, such as *Salmonella*. Cross contamination occurs when cells detach from biofilm structure once food passes over contaminated surfaces or through aerosols originating from contaminated equipment. Till now, there is only limited information on the presence of *Salmonella* in biofilms in real food processing environments. However, numerous studies have shown that *Salmonella* can easily attach to various food-contact surfaces (such as stainless steel, plastic and cement) and form biofilms under laboratory conditions (Chia et al., 2009,Giaouris & Nychas, 2006, Marin et al., 2009, Oliveira et al., 2006, Rodrigues et al., 2011). Modern food processing supports and selects for biofilm forming bacteria on food-contact surfaces due to mass production of products, lengthy production cycles and vast surface areas for biofilm development (Lindsay & von Holy, 2006).

1.2 Bacterial biofilms

Biofilms are the predominant mode of bacterial growth, reflected in the observation that approximately 80% of all bacterial infections are related to biofilms (Hall-Stoodley & Stoodley, 2009). The defiition of biofim has evolved significantly since its discovery and researchers are still debating a common definition. However, the definition of Donlan and Costerton (2002) remains the most appreciated. These authors defied the biofilm as a structured community of microbial cells, enclosed in a self-produced polymeric matrix, and adherent to a surface, to interface, and to each other (Donlan and Costerton 2002).

1.2.1 Biofilm formation

Biofilm formation has four common stages. The first stage begins with the bacterial adhesion to surface (1), followed by the formation of microcolonies (2), and biofilm maturation (3). The final stage of the biofilm lifecycle is known as dispersion, in which the cells leave the biofilm structure in order to contaminate other surfaces (4) (Abdalah et al., 2014).

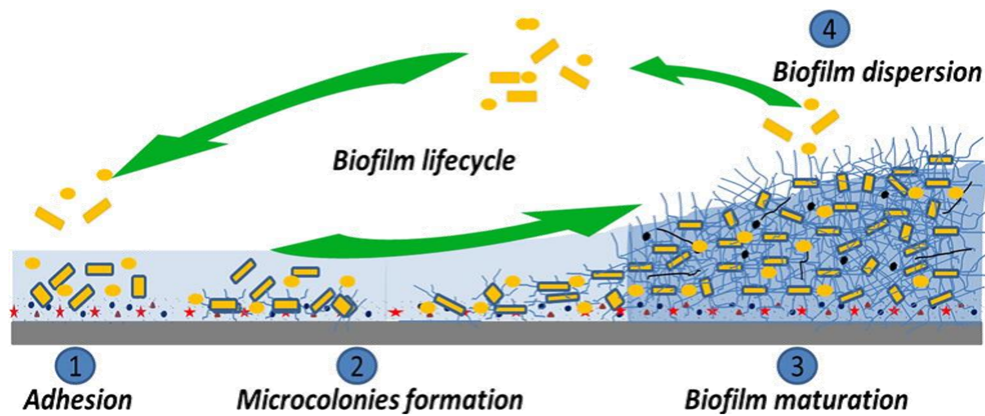

Figure 1.1: Different steps of biofilm formation.

Bacterial adhesion to the surface constitutes the fist and essential step of the biofilm formation (Fig. 1.1). This step is considered reversible and seems to be facilitated by many physical, chemical, and biological interactions (Renner and Weibel 2011). As the bacterial cell approaches a surface of interest, the entire cell will be exposed to nonspecific physiochemical forces such as Lifshitz-van der waals, Lewis acid–base, and electrostatic interactions (Bos et al. 1999). The resultant force will allow a reversible bacterial adhesion to the surface. There is increasing evidence that bacteria may sense the substratum, which allows them to conform to a biofilm condition. The bacterial appendages may constitute operative structures, which sense the abiotic surface and facilitate bacterial adhesion. For example, it has been reported that motile bacteria sense the drag on its flagella motor caused by its interaction with the surface. This phenomenon triggers a signal, which induces the expression of genes involved in biofilm formation, and repress flagellum synthesis (inhibition of motility) (Karatan and Watnick 2009). The adhesion to abiotic surfaces is also influenced by the environment surrounding the bacterial cells, such as temperature, organic matter, and pH. These factors may change bacterial and substrata surface properties and therefore the ability of bacteria to adhere to abiotic surfaces.

After attachment, the cells start to replicate into microcolonies. Reversible adhesion becomes irreversible mainly through the secretion of exopolymeric substances (ePS) that form the biofilm matrix. The extracellular matrix consists of a mixture of polymeric compounds such as polysaccharides, proteins, nucleic acids, and lipids (Flemming and Wingender 2010). These substances allow bacteria tostick to

surfaces and to each other. At this stage, the process of biofilm maturation begins in order to create a mature biofilm in which the cells are encased in an extracellular matrix complete with a complex architecture with water channels. Such a matrix acts as a scaffold for the stabilization of the three-dimensional biofilm structure. It is now increasingly clear that the formation of a biofilm is under the control of several environmental signals such as temperature, nutrient availability. Bacteria may sense these environmental signals and trigger regulatory networks in order to modulate biofilm formation (Abdalah et al., 2014). The cell-to-cell communication mechanism or the quorum sensing (QS) also regulates the formation of biofilms.

The last phase of the biofim lifecycle is dispersion, which represents an option for the sessile cells to leave,to contaminate other surfaces, and then to repeat the cycle. The biofilm dispersion is the result of several environmental events, such as alterations in nutrient availability, oxygen depletion, and other stress conditions, which promote the expression of genes involved in dispersion (McDougald et al. 2011).

1.2.2 Structural components of *Salmonella* biofilms

The extracellular matrix components of *Salmonella* biofilms vary considerably with the used biofilm set-up and the applied environmental conditions (Table 1.1). The rdar morphotype is the best studied form of *Salmonella* multicellular behaviour with respect to regulation and exopolysaccharide (EPS) composition. However, one should be cautious when generalizing themes about biofilm regulation and/or EPS composition between different test systems (Steenackers et al., 2012).

Table 1.1: Most important and already experimentally validated structural determinants important for *Salmonella* biofilm formation on particular surfaces (Steenackers et al., 2012).

Surface	**Structural determinants important for *Salmonella* biofilm formation on this particular surface**
Agar plates (rdar morphotype)	curli (csgDEFG–csgBAC)
	BapA (bapABCD)
	Cellulose (bcsABZC–bcsEFG)
	O-Ag-capsule (yihU–yshA and yihVW)
	Other capsular polysaccharide
	LPS
Epithelial cells	Type 1 fimbriae (fim)
	Plasmid encoded fimbriae (pef)
	Curli fimbriae (csg)
	Long polar fimbriae (lpf)
	Bovine colonization factor (bcf)
	Sth fimbriae (sth)
	Colanic acid (wca genes and wza, wzb and wzc)
	Cellulose (bcsABZC–bcsEFG)
Gallstones	Flagella
	O-Ag-capsule(yihU–yshA and yihVW)
	Type I fimbriae (fim)
Glass	**Cellulose (bcsABZC–bcsEFG)**
	LPS
	Type-three secretion apparatus (TTSS)
	Flagella
Alfalfa seeds	Curli (csg genes)
	Cellulose (bcsABZC–bcsEFG)
	O-Ag-capsule(yihU–yshA and yihVW)

Proteinaceous fraction

Curli are highly aggregative, nonbranching, amyloid-like cell-surface proteins that are important in processes such as host colonization, persistence, motility and invasion. *Salmonella* curli are important during biofilm formation because they

promote initial cell surface and subsequent cell–cell interactions. White and colleagues pointed out that in the native state, curli (csg) exist as a complex with cellulose and the O-Ag-capsule, physically linking the cells together (White et al., 2003). In addition, *S.* Typhimurium genome contains 12 other putative fimbrial operons, some of which were shown to be important in biofilm formation (Type I fimbriae). The large (386 kDa), proline-threonine-rich secreted, multidomain protein BapA of *S.* Enteritidis is a second important component of the proteinaceous fraction of the rdar morphotype which has been shown to be important for bacterial aggregation and subsequent pellicle formation.Flagella are indispensable for swarming (and swimming) but can serve different roles during *Salmonella* biofilm formation. Swarming is a multicellular process involving the generation of slimy colonies that expand rapidly via flagella mediated motility. It is similar, but to some extent inversely related to biofilm formation (Steenackers et al 2012).

Exopolysaccharide fraction

Cellulose, a β-1-4-D-glucose polymer, encoded by the bcsABZC–bcsEFG genes, is an important biofilm-associated EPS. In relation to the characteristic rdar morphotype expression pattern, cellulose supports long-range cell–cell interactions responsible for the sticky texture (Solano et al., 2002). Colanic acid is a capsular extracellular polysaccharide found to be important for *Salmonella* to create extensive three-dimensional structures on epithelial cells but itwas found not to be required for biofilm formation on abiotic surfaces, gallstones and alfa alfa seeds. Next to cellulose, the EPS fraction of *S.* Enteritidis biofilms on agar plates consists of an anionic O-antigen capsule, different from colonic acid and covalently attached to lipids. This capsule consists of more than 2300 repeating tetrasaccharide units, is highly hydrated (as are all capsules) and was proven to be involved in desiccation tolerance (Steenackers et al., 2012). In addition, de Rezende et al. purified another *Salmonella* capsule from the extracellular matrix fraction of the multiresistent *S.* Typhimurium DT104, which has a different chemical composition as compared to the above mentioned O-Ag-capsule, since it lacked rhamnose (de Rezende et al., 2005).

1.2.3 Regulators of *Salmonella* biofilms

As demonstrated in Fig. 1.2 , the synthesis of the structural components of *Salmonella* biofilms is regulated by a highly complex regulatory network. In this section, a synopsis is given on the current understanding of this network and the

interactions between its different components. For an extensive overview on this, reader is advised to refer to the recently published review of Steenackers et al. (2011).

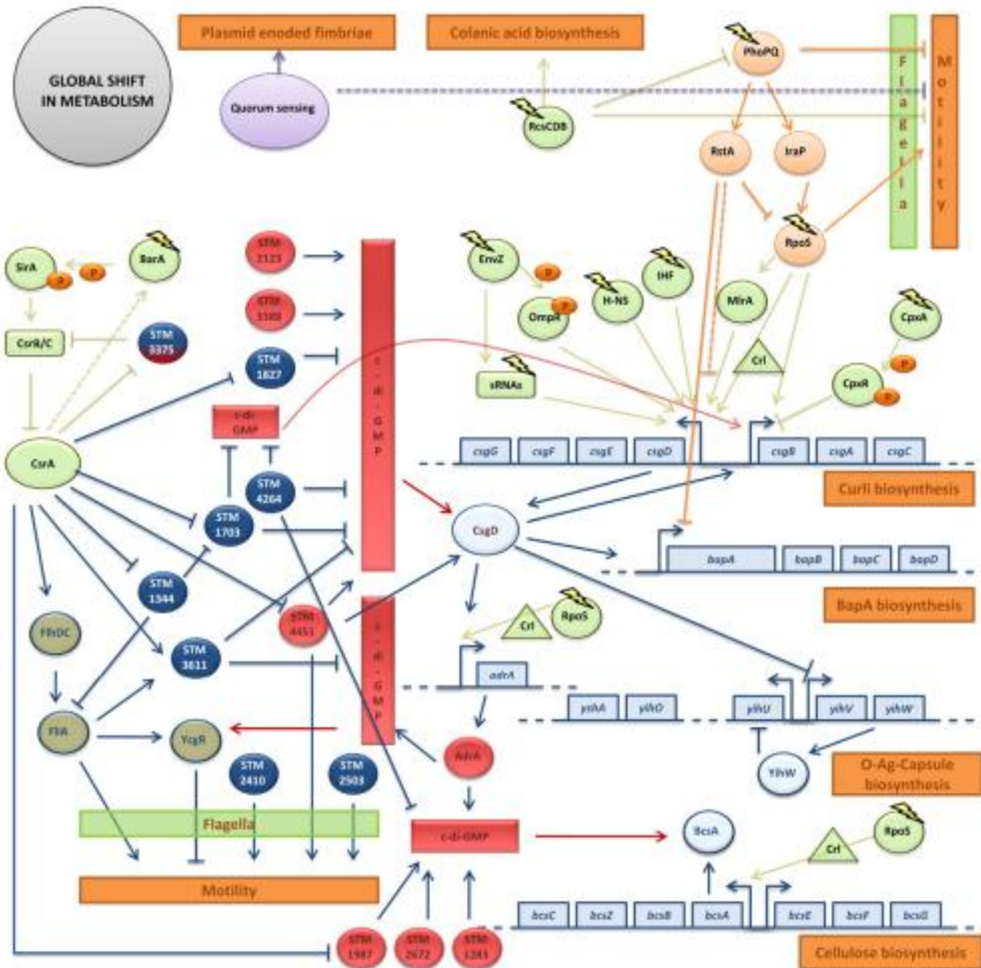

Figure 1.2: Complex regulatory network governing Salmonella biofilm formation

CsgD

CsgD is a major control and integration unit for *Salmonella* biofilm formation regulating the expression of specific biofilm associated matrix compounds (Gerstel & Römling, 2003), as can be seen on Fig. 1.2. CsgD is a transcriptional response regulator containing an Nterminal receiver domain with a conserved aspartate (D59) and a Cterminal LuxR-like helix-turn-helix (HTH) DNA-binding motif belonging to the FixJ/NarL family. In a genomic context, csgD is an integral part of the curling biosynthesis system consisting of the divergently transcribed csgBAC and csgDEFG operons.

High degree of conservation at nucleotide and protein level between the corresponding curli operons of *S.* Typhimurium and *E. coli*, together with cross-complementation ability and similar regulation patterns, suggested these genes were already present in their common ancestor (White& Surette, 2006). Using a comparative genetic analysis of the csgB–csgD intergenic region of the SARC16, they showed that, with the exception of two *S. enterica* subsp. *arizonae* isolates (belonging to *Salmonella* group IIIa), promoter functionality of the csgD and csgB genes was conserved, despite sequence differences (being the biggest for two group V *S. bongori* isolates), for six of the seven *Salmonella* subgroups. This indicates that most changes in the csgB–csgD intergenic region were the result of neutral mutations originating from genetic drift.

It is already known for a long time that CsgD regulates the transcription of the structural curli subunits encoded by csgBAC (Römling, Bian, et al., 1998). Both curli operons (csgBAC and csgDEFG) are necessary for the production of intact, highly stable curli. *Salmonella* curli assembly, following activation by CsgD, occurs via the extracellular nucleation precipitation pathway (ENP) (White et al., 2003). Using luciferase (lux) expression reporters, White et al. visualized that curli production probably initiates extracellular matrix production and as a consequence specific rdar surface patterns, since csgB expression peaks coincided with the sharp transition to these specific patterns. Similar progressive transition towards a rugose phenotype was visualized on TSA broth at ambient temperatures. This transition, however, was not unequivocally confirmed to be primarily curli mediated but importance of curli was noticed in a later study. Consistent with this, curli were shown to provide specific short-range cell–cell interactions yielding this adhesive structure (Steenackers et al., 2012).

Biosynthesis of cellulose, occurring at the inner bacterial membrane, is also positively regulated by CsgD via direct binding and subsequent transcriptional stimulation of adrA (AgfD regulated gene) in *S.* Typhimurium (Zakikhany et al., 2010). As for csgBAC activation, it was noticed that the unphosphorylated CsgD form binds specifically to the adrA promoter, although in a more complex manner. AdrA in turn regulates bcsABZC, the constitutively transcribed genes encoding the cellulose biosynthesis machinery at the post-transcriptional level, by altering the cellular levels of c-di-GMP (Robbe-Saule et al., 2006).

Expression of bapA, part of the bapABCD operon responsible for Bap synthesis and export, is also regulated by CsgD (Latasa et al., 2005). Despite the finding that the bapA promoter region contains a similar CsgD binding sequence as the inverted repeat of the adrA promoter, Zakikhany et al. did not identify bapA as a CsgD regulated gene in *S.* Typhimurium, using a combined bioinformatics and global transcriptomic approach (Zakikhany et al., 2010).

The *S.* Enteritidis O-Ag-capsule, assembled and translocated by the divergently oriented operons, yihU-yshA and yihVW, is another compound of the EPS fraction that is regulated by CsgD (White et al., 2003).

Taken together, CsgD can be seen as the biofilm control point, regulating the expression of all major *Salmonella* biofilm constituents (under rdar conditions) and controlling the transition between planktonic and multicellular behaviour. As such and since csgD has a low basal transcription level (Gerstel & Römling, 2003), it is not surprising that the expression of csgD itself is highly regulated by different environmental stimuli (temperature, oxygen tension, nutrients and starvation, osmolarity, ethanol, iron and pH) via different transcriptional regulators (OmpR, Crl, RpoS, MlrA, CpxR, H-NS and IHF) and the secondary bacterial messenger molecule c-di-GMP, as discussed below and visualized in Figure 1.2. This complex regulation enables fine-tuning of the regulatory network and the generation of quick and well-controlled responses to changing environmental conditions.

RpoS and Crl

RpoS and Crl are two other main regulators of *Salmonella* biofilm formation, influencing this highly complex process at different points (Figure 1.2). A σ factor directs the Eσ complex (holoenzyme of *Enterobacteriaceae* RNA polymerase) to a specific set of promoters. Sigma factor (σ^5) encoded by rpoS, regulates the transcription of genes important for general stress response and stationary phase survival and it has been shown that RpoS regulon is up-regulated in biofilm cells (Hamilton et al.,2009, White et al., 2010, White et al., 2006). Transcription of csgD is depended upon RpoS (rdar morphotype) and it was shown that RpoS itself is also required at some steps in csgBAC and adrA expression (Steenackers et al., 2012).

Robbe-Saule et al. showed that a functional Crl protein, a DNA binding transcriptional regulator, is required for rdar development and that Crl exerts its

function together with RpoS. Although Crl was found to be required for maximal csgB, bcsA, csgD and adrA expression, *in vitro* experiments indicated Crl only directly activates σS-dependent, and not σ70-dependent, initiation of transcription at the latter two promoters by enhancing the rate of open complex formation and as such transcription (Steenackers et al., 2012).

c-di-GMP

Central to the mechanism of post-transcriptional regulation of biofilm formation is a tiny cyclic RNA chemical second messenger molecule, c-di-GMP. C-di-GMP is implicated in controlling various cellular functions including virulence, motility, and adhesion, although its principal role is controlling the switch from motile planktonic lifestyle to the sessile biofilm forming state. Elevated intracellular levels of c-di-GMP promote synthesis of exopolysaccharides and enhanced auto-aggregation and surface adhesion leading to biofilm formation (Martinez L. et Vadyvaloo V., 2014). Also repress virulence and motility. In contrast, reduced intracellular c-di-GMP concentrations are associated with decreased biofilm formation. Especially in *Salmonella* elevated c-di-GMP concentrations, next to activating cellulose biosynthesis by upregulating AdrA, also enhance curli production through increasing CsgD and CsgA expression transcriptionally and post-transcriptionally and negative regulate flagellar genes though repressing swimming motility (Steenackers et al 2012).

C-di-GMPis synthesized by GGDEF motif containing proteins that encode diguanylate cyclase (DGC) enzyme activity required to convert two molecules of GTP to c-di-GMP. Degradation of c-di-GMP is carried out by EAL or HD-GYP motif containingproteinsthatencodephosphodiesteraseactivityleadingtohydrolysis of c-di-GMP to pGpG. The GGDEF or EAL/HD-GYP protein encoding genes controlling c-di-GMP synthesis are present in multiple copies in bacterial genomes especially in those pathogens that infect multiple hosts, e.g., *E. coli* encodes 34, *S.* Typhimurium encodes 27 such genes with GGDEF, EAL, or HD-GYP domains in their genomes (Kulasakara et al., 2006). Thus, it stands to reason that the c-di-GMP signal transduction is tightly synchronized and regulated to avoid interference between the functionally distinct c-di-GMP responsive systems.

***BarA/SirA* system**

The BarA/SirA two component system, widely conserved within the gamma-proteobacteria, is an important global regulatory system involved in *Salmonella* virulence, motility and biofilm formation. SirA is a response regulator of the FixJ family (as is CsgD) that is phosphorylated by its cognate sensor kinase BarA or by cellular acetyl phosphate. SirA transcriptionally activates the sRNAs csrB and csrC. Both sRNAs are part of the *Salmonella* Csr system and antagonize the activity of the RNA-binding protein CsrA (Steenackers et al., 2012).

***Csr*A**

CsrA (RsmA) proteins are a family of RNA binding proteins that are widely distributed central components of the global carbon storage regulatory system (Csr) involved in the control of many cellular functions and virulence traits, like motility, quorum sensing, carbon metabolism, interaction with hosts and biofilm production. CsrA proteins control the expression of target genes at a post-transcriptional level by various methods: binding sequences overlapping the Shine–Dalgarno (SD) sequence in target mRNAs, occluding ribosome binding and translation, and enhancing mRNA degradation (Martinez et Vadyvaloo, 2014). The CsrA proteins have been shown to repress biofilm formation post transcriptionally in several different ways described below.

Generally CsrA directly and indirectly affects extracellular polysaccharide production, induces motility, down-regulates the expression of several genes encoding GGDEF/EAL proteins so negative affects ci-d-GMP levels and activates biofilm dispersal (Martinez et Vadyvaloo, 2014).

Especially in *Salmonella*, it was shown that CsrA directly and indirectly regulates the expression of at least eight genes involved in this network encoding GGDEF (STM1987 and STM4451), GGDEF/EAL (STM1703 and STM2275) as well as EAL (STM1687, STM1827, STM3611) domain proteins and the unconventional STM1344 (Simm et al., 2009). STM3611 was found to be the only positively regulated gene, while the rest was down-regulated by CsrA. Further experiments pointed at a complex regulation integrating the Csr, flagellar and c-di-GMP system to

control biofilm formation. CsrA controls the switch between sessility and motility at multiple hierarchical levels, generally activating motility and inhibiting sessility. Firstly, by direct regulation of the master regulator FlhDC (class 1 flagellar gene), resulting in a fliA (class 2 flagellar gene)-mediated upregulation of STM3611, STM1798 and class 3 flagellar genes (fliC, fljB,etc.). Secondly, CsrA directly represses STM1344. This unconventional EAL domain protein in turn transcriptionally represses STM1703 (Simm et al., 2009) and interferes with the flagella-cascade upstream of fliA, resulting in an up-regulation of STM3611, STM1798 and class 3 flagellar genes (fliC, fljB, etc.). Thirdly, the direct interaction with and stabilization of the STM3611 mRNA transcript. Fourthly, CsrA directly regulates STM3375 (csrD), a degenerative GGDEF/EAL domain protein with no apparent DGC/PDE activity, resulting in destabilization of CsrB and CsrC activity (in *E. coli*) (Jonas et al., 2008; Suzuki et al., 2006). As such, CsrA controls its own activity through an autoregulatory loop, providing a direct link between these regulatory cascades and *Salmonella* multicellular behaviour. However, a *S.* Typhimurium csrA mutant does not show increased biofilm formation (Teplitski et al., 2006), suggesting that additional components are involved in this complex network.

PhoPQ–RstA

The PhoPQ system of *Salmonella* is a two-component system consisting of the cytoplasmic response regulator PhoP and the inner membrane located sensor kinase PhoQ. Upon activation, PhoP directly and indirectly controls the expression of more than 120 genes, involved in several functions such as LPS modification, magnesium transport, invasion of epithelial cells and intramacrophage survival and it has been shown to repress biofilm formation. PhoP has also been shown to activate the expression of RstA, a protein that induces RpoS degradation independently of the ClpXP-SsrB proteolytic pathway (Steenackers et al., 2012).

Rcs system

The RcsC–RcsD–RcsB phosphorelay system consists of the sensor kinase RcsC, the intermediate phosphotransfer protein RcsD, the transcriptional regulator RcsB and the transcriptional co-regulator. RcsA Activation of the Rcs system results in a drastic induction of genes for colanic acid capsule synthesis and repression of

flagellar synthesis genes and virulence genes. These findings suggest the Rcs system to be in support of a sessile lifestyle within biofilms (Wang et al., 2007).

1.2.4 Quorum sensing in *Salmonella* biofilms

It has been thoroughly suggested that bacterial cells communicate by releasing and sensing small diffusible signal molecules, in a process commonly known as quorum sensing (QS). Through cell-to-cell signaling mechanisms, bacteria modulate their own behaviour and also respond to signal produced by other species. QS involves a density-dependent recognition of signaling molecules (autoinducers, AIs), resulting in modulation of gene expression. Gram-negative bacteria primarily use a variety of Nacylhomoserine lactones (AHLs) as AI (autoinducer-1, AI-1), while Gram-positive bacteria use a variety of autoinducing polypeptides (AIPs). AHLs are synthesized and recognized by QS circuits composed of LuxI and LuxR homologues, respectively. Both AHLs and AIPs are highly specific to the species that produce them. A third QS system is proposed to be universal, allowing interspecies communication, and is based on the enzyme LuxS which is in part responsible for the production of a furanone-like compound, called autoinducer-2 (AI-2) (Giaouris et al., 2012).

Bacteria use QS communication circuits to regulate a diverse array of physiological activities, such as genetic competence, pathogenicity (virulence), motility, sporulation, bioluminescence and production of antimicrobial substances. Yet, a growing body of evidence demonstrates that QS also contributes to biofilm formation by many different species (Annous et al., 2009, Irie & Parsek, 2008, Lazar, 2011). As biofilms typically contain high concentration of cells, autoinducer (AI) activity and QS regulation of gene expression have been proposed as essential components of biofilm physiology. To date, three QS systems have been identified in *S. enterica* and are thought to be mainly implicated in the regulation of virulence (SdiA, luxS/AI-2 and AI-3/epinephrine/ norepinephrine signaling system) (Boyen et al., 2009, Walters & Sperandio, 2006).

1.2.5 *In situ* study of gene expression in *Salmonella* biofilms

Salmonella biofilm formation is an important survival strategy in non-host environments, which are fundamentally different from typical host environments, a strategy to induce chronic infections and even a possible way to colonize host

organisms. Taken together, biofilm formation can be seen as an essential and integral part of the pathogen's life cycle and a source of reappearing infections by this pathogen (Rasschaert et al.,2007). Bacterial cells residing in biofilms are not only physiologically distinct from planktonic cells (with different gene expression patterns), but also vary from each other spatially, temporally and genetically as the biofilm formation proceeds. As the ensemble of bacterial cells within a biofilm represents different physiological states, even for monospecies biofilms, gene expression patterns in these multicellular assemblages show a high degree of heterogeneity. This heterogeneity might mask differential gene expression that occurs only in subpopulations of the entire biofilm population when using methods that average expression output.

High-throughput DNA microarray and qRT-PCR studies have been conducted to study biofilm formation in many model organisms and have identified a large number of genes showing differential expression under biofilm conditions. The qRT-PCR is one of the most powerful and sensitive gene analysis techniques available at now. During qRT-PCR, the fluorescent signal is measured in real time at each amplification cycle and is directly proportional to the number of amplicons generated providing both qualitatively and quantitatively analysis. This transcriptional profiling technique, however, generate a global value for the whole biofilm population and as such, differences in gene expression patterns of subpopulations within biofilms are not taken into account (Hermans et al., 2011).

In an attempt to address this problem and to refine expression analysis in biofilm studies, the use of reporter genes in combination with advanced microscopy (fluorescence or confocal laser scanning Microscopy) and flow cytometry is emerging as a powerful tool to facilitate the *in situ* monitoring of gene expression in biofilms.

Reporter genes

In molecular biology, a reporter gene (often simply reporter) is a gene that researchers attach to a regulatory sequence of another gene of interest in bacteria, cell culture, animals or plants. Certain genes are chosen as reporters because the characteristics they confer on organisms expressing them are easily identified and measured (i.e fluorescence), or because they are selectable markers (Figure 1.3). Reporter genes are often used as an indication of whether a certain gene has been

taken up by or expressed in the cell or organism population. To introduce a reporter gene into an organism, scientists place the reporter gene and the gene of interest in the same DNA construct to be inserted into the cell or organism. For bacteria or prokaryotic cells in culture, this is usually in the form of a plasmid. It is important to use a reporter gene that is not natively expressed in the cell or organism under study, since the expression of the reporter is being used as a marker for successful uptake of the gene of interest.

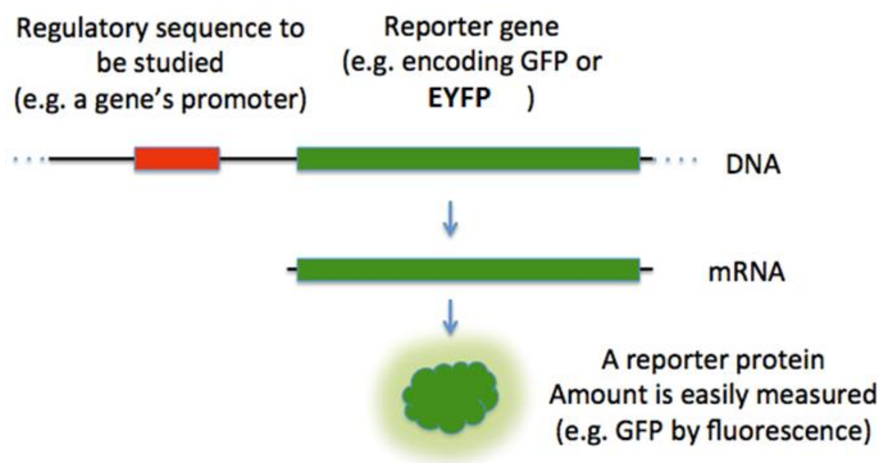

Figure 1.3: Schematic representation of reporter gene principals.

Commonly used reporter genes that induce visually identifiable characteristics usually involve fluorescent and luminescent proteins. Examples include the gene that encodes jellyfish green fluorescent protein (GFP), which causes cells that express it to glow green under blue light, the enzyme luciferase, which catalyzes a reaction with luciferin to produce light, and the red fluorescent protein from the gene dsRed (Tsien, 1998).

Reporter genes are used to assay for the expression of the gene of interest, which may produce a protein that has little obvious or immediate effect on the cell culture or organism. In these cases, the reporter is directly attached to the gene of interest to create a gene fusion. The two genes are under the same promoter elements and are transcribed into a single messenger RNA molecule. The mRNA is then translated into protein. In these cases it is important that both proteins be able to properly fold into their active conformations and interact with their substrates despite being fused (Shaner et al., 2005). In building the DNA construct, a segment of DNA

coding for a flexible polypeptide linker region is usually included so that the reporter and the gene product will only minimally interfere with one another.

1.3. Biofilm control strategies

There is good evidence indicating that biofilm mode of life leads to increased resistance to antimicrobial products (Bridier et al., 2011a). Biofilms are more resistant to antimicrobials compared to planktonic cells and this makes their elimination from food processing facilities a big challenge. Moreover, the emergence of resistant bacteria to conventional antimicrobials clearly shows that new biofilm control strategies are required. Till now, several strategies have been proposed to control biofilm formation. These strategies can be classed into two major groups. The aim of the first one is to prevent bacterial adhesion and biofilm formation with either surface property modification or antimicrobial surface coating. The second one aims to eradicate/disrupt formed biofilms using antimicrobial agents, physical forces, enzymes, phages, etc.

1.3.1 Prevention of biofilm formation

Ideally, preventing biofilm formation would be a more logical option than treating it. However, there is presently no known technique that is able to successfully prevent or control the formation of unwanted biofilms without causing adverse side effects.

The main strategy to prevent biofilm formation is to clean and disinfect regularly before bacteria attach firmly to surfaces (Simones et al., 2006). Biofilm detectors were already developed to monitor the surface colonization by bacteria and allow the control of biofilms in the early stages of development. Other preventive strategies in the past attempted to identify materials that do not promote or can even suppress biofilm, however concluding that there is hardly any material that does not allow biofilm formation. The strategies involving the modification of surface properties have emerged as an option to prevent biofilm formation. For example, the design of superhydrophobic surfaces has been found to be effective in the prevention of cell attachment and the biofilm formation of several bacteria such as *P. aeruginosa* and *S. aureus* (Lin et al. 2011, Looet al. 2012). In addition, the modifications of surface topography have also been found as promoting tool to prevent the bacterial adhesion of pathogenic bacteria (Verran and Whitehead 2005).

Surface pre-conditioning with surfactants has potential to prevent bacterial adhesion. Nonionic and anionic surfactants were evaluated in preventing the adhesion of *P. aeruginosa* to stainless steel and glass surfaces. The surfactants gave more than 90% inhibition of adhesion (Cloete and Jacobs 2001). Several studies reinforced the efficiency of surfactants and surface pre-conditioning on biofilm formation control. Splendiani et al. (2006) screened 22 surfactants for their potential to increase the cell wall charge of a Burkholderia sp. strain and reduce the ability to attach and form biofilms. The authors demonstrated that some surfactants affected the development of flagella, demonstrating significant changes in the bacteria attachment ability in the presence of surfactants.

Recently, different approaches have been proposed, which consist of antimicrobial-coated surfaces in order to reduce biofouling and associated infections. Moreover, various synthetic technologies have been extensively explored to immobilize the active agents such as antimicrobial peptides, anti-quorum sensing, essential oils, enzymes, and quaternary ammonium (QA), on abiotic surfaces. The antimicrobial coated surfaces have been found suitable for the inhibition of biofilm formation by either killing bacteria or preventing their adhesion. However, some antimicrobial coating can be toxic for humans, limiting the implementation of these methods in the food and medical fields (Abdalah et al., 2014).

As previously mentioned, there is no perfect technique to prevent the formation of biofilms. In fact, the modified surfaces may only reduce and not completely prevent bacterial adhesion. Furthermore, bacteria may use different mechanisms of attachment in response to these surface modifications. The overuse of antimicrobial-coated surfaces may allow bacteria to develop resistance against the antimicrobial of interest and increase microbiological risks. Thus, there is a demand for curative agents across industries, such as food processing and healthcare, in order to maintain a high level of hygiene and to fight against biofilm formation in these fields.

1.3.2 Eradication of biofilms with disinfectant agents

In health facilities and food sector, biocides are widely used to decontaminate surfaces, instruments, and equipment that come into contact with the human body or with the food product. In addition, there are a variety of commercialized disinfectants,

commonly used within these fields, such as alcohol based products, hypochloric solutions including sodium hypochlorite, aldehydes, peracetic acid, hydrogen peroxide, ozone, chlorhexidine digluconate, polyhexamethylene biguanides (PHMB), and QA compounds (Buckingham-Meyer et al. 2007, Belessi et al. 2011). The same agent can be used by different sectors with the main difference being the concentration at which it is employed. Bacteria vary in their susceptibility to biocides, with bacterial spores being the most resistant, followed by mycobacteria, gram-negative, gram-positive, and fungal microorganisms (Maillard 2005). However, it is not possible to predict which microorganisms will be present on surfaces. Thus, disinfectant products must be adapted whatever the lifestyle and the kind of harmful microorganisms.

Unlike antibiotics, which affect a specific physiological process, the disinfectant molecules in general have more than one target site. Biocides, such as quaternary ammonium group (QA), phenols, biguanides, and alcohols, have the cytoplasmic membrane as a main target. These active agents may promote the precipitation of cellular material. PHMB, which are poly-cationic disinfectants, bind to the negatively charged phosphate head groups of phospholipids and do not integrate into the bilayer. The PHMB bridges between pairs of adjacent phospholipid head groups causing its aggregation and disruption in the cell walls. The antimicrobial action of alcohols, which are also dehydrating agents, is related to the denaturation of surface and intracellular proteins. Disinfectants based on alkylating agents, hydrogen peroxide, ozone, peracetic acid penetrate inside cells and interact with the cells constituents, such as proteins, ribosome, nucleic acid, and enzymes, causing the cells death (McDonnell and Russell, 1999).

QAs: Benzalkonium chloride

Benzalkonium chloride, also known as BZK, BKC, BAC, alkyldimethylbenzylammonium chloride and ADBAC, is a cationic surface-acting agent belonging to QAs. It has three main categories of use: as a biocide, a cationic surfactant, and phase transfer agent in the chemical industry. The chemical is a heterogeneous mixture of alkylbenzyldimethylammonium chlorides of various even-numbered alkyl chain lengths. Benzalkonium chloride is commonly used in the foodservice industry as sanitizing agents. It has been reported that the QAs interact with the bacterial membrane causing its disruption and the leakage of intracellular

components. The QA contains one quaternary nitrogen, which is associated with at least one major hydrophobic substituent and an anion such as Cl or Br. The mode of action of these cationic biocides seems to involve both hydrophilic and hydrophobic moieties (Palermo et al. 2011). The hydrophilic moiety of QAs is thought to adsorb to the relatively anionic bacterial cell walls, while the hydrophobic tail integrates into the lipid bilayer causing its disruption. In contrast to phenolics, quaternary ammonium compounds are not very effective in the presence of organic compounds. In table X the acute toxicity data in RTECS (Registry of Toxic Effects of Chemical Substances) are listed.

Table X. Acute toxicity data for BC in RTECS

Organism	Route of Exposure	Dose (LD50)
Rat	Intravenous	13.9 mg/kg
Rat	Oral	240 mg/kg
Rat	Intraperitoneal	14.5 mg/kg
Rat	Subcutaneous	400 mg/kg
Mouse	Subcutaneous	64 mg/kg

Benzalkonium chloride is a human skin and severe eye irritant. It is a suspected respiratory toxicant, immunotoxicant, gastrointestinal toxicant and neurotoxicant (Lewis, 2004). Benzalkonium chloride formulations for consumer use are dilute solutions. Concentrated solutions are toxic to humans, causing corrosion/irritation to the skin and mucosa, and death if taken internally in sufficient volumes. The maximum concentration of benzalkonium chloride that does not produce primary irritation on intact skin or act as a sensitizer is 0.1%..

Disinfectants are more effective in the absence of organic material (fat, carbohydrates, and protein based materials). Interfering organic substances, pH, temperature, water hardness, chemical inhibitors, concentration and contact time generally control the disinfectants efficacy. The disinfectants must be effective, safe and easy to use, and easily rinsed off from surfaces, leaving no toxic residues that could affect the health properties and sensory values of the final products.

Nevertheless, the literature demonstrates that there is no one strategy with absolute biofilm control efficiency.

1.3.3 Factors affecting the disinfectant efficacy against the biofilm

The activity of biocides against biofilms depends upon a number of factors. The principal physical and chemical factors that influence the efficiency of these agents are the concentration, contact time, temperature, and pH of treatment. The efficacy of disinfectants against biofilms usually increases with the increase in both biocide concentration and time of treatment (Belessi et al. 2011). However, there needs to be a balance between efficacy and toxicity. The increase in temperature and pH of the treatment may increase the efficiency of disinfectant agents. In addition to the effect of the treatment conditions, other factors related to the environmental conditions of biofilm growth may affect the efficiency of disinfectant products. For example, it has been reported that the increase in the biofilm growth temperature increases biofilm resistance to disinfectant agents. Otherwise, the increase of the duration in incubation time of biofilms often results in an increase in biofilm resistance to antimicrobial agents. The surface type may also influence the efficacy of biocides in killing and removing biofilms from the abiotic surfaces. The involvement of the surface type could be related to the effect of surface properties on the biofilm shield and architecture, or on the effectiveness of cleaning and sanitizing. In fact, the surface defects/ roughness often makes the cleaning process more difficult (Abdallah et al., 2014).

1.3.3.1 The biofilm resistance to disinfectant agents.

The most clinical guidelines for the use of biocides have been developed for planktonic microorganisms (Cerf et al. 2010). However, most of the microorganisms live as surface-adherent communities. In addition, considerable works have already shown that the cells living under a biofilm state can be up to 1,000-fold more resistant to disinfectant products than their planktonic counterparts (Belessi et al. 2011, Bridier et al. 2011a). Thus, the commercialized disinfectants may have a confirmed efficiency on the planktonic cells and often be unable to eradicate biofilm cells. This high tolerance of sessile cells to biocides may increase the risk of further disinfection failure leading to severe health problems and economic losses. Therein, the current researches are focused on the mechanisms of the biofilm resistance to disinfectant agents in order to understand them and to improve biofilm control strategies. In fact,

several mechanisms have been proposed to explain the apparent increased resistance of biofim cells (Figure 1.3). The biofim resistance is thought to be linked to the: (1) restricted penetration of biocides into the biofilm, (2) the biofilm phenotype and adaptation of cells to the biofilm environment, and (3) presence of disinfectant-adapted and persister cells (Abdallah et al., 2014).

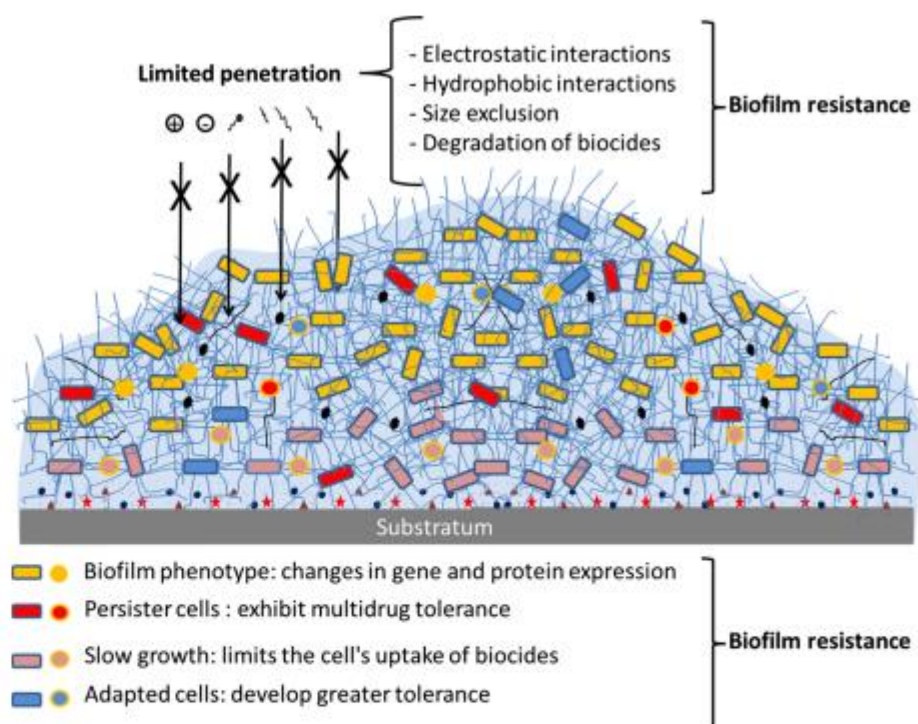

Figure 1.4: Mechanisms of biofilm resistance to antimicrobial agents (Abdallah et al., 2014)

1.3.3.2 The restricted penetration of biocides inside the biofilm.

The formation and maintenance of mature biofilms are intimately linked to the production of an extracellular matrix. The multiple layers of cells and EPS may constitute a complex and compact structure within which biocides find it difficult to penetrate and reach internal layers, thus hampering their efficacy. This hypothesis is supported by several experimental studies indicating that the biofilm matrix may hinder the penetration of numerous disinfectant molecules into biofilms. Using time-lapse confocal laser imaging Davison et al., (2010) have found that the penetration of biocides inside the biofilm of *S. epidermidis* was retarded by the factor of 600 and 60, respectively, for chlorine and QA. Using the same technique, Bridier et al. (2011c)

also observed that diffusion–reaction limitations are involved in the resistance of *P. aeruginosa* biofilms to benzalkonium chloride.

Those findings showed that biofilm resistance seems to be related to the involvement of the biofilm matrix in the retention of biocides. The restricted diffusion of molecules within the range 3–900 kDa in biofilms due to size exclusion has already been reported. But because biocides are often highly chemically reactive molecules, the presence of organic matter such as proteins, nucleic acids or carbohydrates can profoundly impair their efficacy and potential interactions between antimicrobials and biofilm components seem more likely to explain the limitations of penetration into the biofilm (Bridier et al., 2011a).

During the past 10 years, the emergence of innovative optical microscopy techniques such as confocal laser scanning microscopy (CLSM), and improvements in fluorescent labeling, have provided an opportunity for the direct investigation of biocide reactivity within the native structure of bio films (Bridier et al. 2011b). This can provide information on the dynamics of biocide action in the biofilm and the spatial heterogeneity of bacteria related susceptibilities that are crucial to a better understanding of biofilm resistance mechanisms.

1.3.3.3 The phenotype of biofilm cells

Different approaches have been proposed to explain the biofilm resistance to biocides, since the inhibition of diffusion inside biofilms cannot always explain the resistance of sessile cells to an antimicrobial compound. Other factors such as decreased growth rate, membrane permeability changes and the adaptation of cells to biofilm environments could be involved in the resistance of sessile cells to biocide agents.

The reduced growth rate of bacterial cells under the biofilm state is among the hypothesis proposed for the biofilm resistance to antimicrobials (Schulte et al. 2005). It is now established that the bacteria grown in the stationary phase present an enhanced resistance to disinfectant agents than those in the exponential phase (Cherchi and Gu 2011). Moreover, cells grown under a biofilm state were found to resemble stationary phase rather than the planktonic stage. Thus, it is easy to imagine that bacterial growth in the deeper layer of biofilm is slowed or arrested, owing to substrate and oxygen limitation, and may diminish the uptake of antimicrobials.

The sessile cells have been found to be phenotypically different from their planktonic counterparts. This phenotype, also called "biofilm phenotype," was proposed to explain the resistance of sessile cells to antimicrobial agents. The upregulation of exopolysaccharide production is considered a phenotypic characteristic of surface attached bacteria. In fact, the transition from floating to sessile state increased the expression of genes involved in the biosynthesis of EPS. The transition to the sessile phenotype can induce changes in the membrane fatty acids profile, which maintain the membrane fluidity of bacterial cells. For example, the transition from planktonic to sessile state was found to decrease the membrane fluidity of *L. monocytogenes* (Gianotti et al. 2008) and *P. aeruginosa* sessile cells (Benamara et al. 2011). Such an increase in membrane rigidity may hinder the penetration of biocide into the lipid bilayer and enhance the resistance of biofilm cells to disinfectants agents at the cellular level. The transition to the sessile phenotype was found to induce changes in the expression of membrane and cytosolic proteins. *S.* Enteritidis PT4 growing under the biofilm state also changed the expression of 61 proteins (Giaouris et al. 2013). Moreover, this study showed that the sessile cells differed from planktonic ones by the expression of a group of proteins involved in the stress response, nutrient transport, and DnA metabolism (Giaouris et al. 2013). Thus, the protein expression changes may also be a part of biofilm resistance to biocides since several up-regulated proteins have been associated with the resistance to disinfectant agents (Tabata et al. 2003).

The sessile cells are subject to several stresses such as starvation, osmotic, and oxidative stress. It is known that these cells, in response to stress conditions, induce an adaptive stress response such as the expression of the stress sigma factor RpoS (σS). The σS is the master regulator of the general stress response and was found to be upregulated in the gram-negative biofilm cells such as *P. aeruginosa* and *E. coli*. Moreover, this factor (σS) was found to positively control the expression of more than 240 genes encoding stress management proteins, metabolic enzymes, membrane proteins, and regulatory proteins. The alternative sigma factor SigB (σB), controlling the cellular stress responses of gram-positive bacteria, has been found to be upregulated under the biofilm state. Thus, it can be expected that these factors may affect biocide resistance, by the regulation of biofilm formation and the regulation of genes involved in the resistance to biocides (Abdallah et al., 2014). Moreover, the

deletion of sigma factor has been found to increase the sensitivity of both planktonic and sessile *L. monocytogenes* cells to the benzalkonium chloride and peracetic acid, the *P. aeruginosa* sessile cells to hydrogen peroxide, and the sessile *S. aureus* to different house cleaners. Furthermore, it has been reported that the increased antioxidative capacities in the biofilm, in response to oxidative stresses, may also increase the resistance of sessile cells to oxidative agents such as sodium hypochlorite and hydrogen peroxide. Otherwise, the biofilm formation is under the control of several factors which in turn are regulated by the QS molecules. Although the role of QS in resistance of sessile cells to disinfectant is not yet clear, the deletion of lasI and rhlI of *P. aeruginosa* was found to increase its sensitivity to disinfectant agents (Hassett et al. 1999).

1.3.3.4 The presence of disinfectant-adapted and persister cells

The increased use of disinfectants at lower concentrations than that recommended by the manufacturer has raised some concerns about their overall efficacy, but also about the emergence of microbial resistance to biocides. In fact, the bacterial adaptation to disinfectant products has been reported for several bacteria. In addition, the food and the medical environments constitute a reservoir of bacteria presenting high tolerance to disinfectant products, which is due to misuse of these agents. Moreover, disinfectant-adapted bacteria may exhibit cross-resistance to other disinfectant agents. Thus, food and medical equipment is constantly confronted with the formation of biofilms harboring already disinfectant-resistant bacteria, which increase the chance of biofilm cells survival in the biocide treatment. Furthermore, the exposure of bacteria to a sublethal biocide concentration engendered adapted phenotype changes, and this was predominantly due to the contribution of efflx pump activity (Mc Cay et al. 2010). The efflux proteins, also known as multidrug resistance (MDR), remove toxic substances, including antimicrobial agents from the cell. However, the involvement of these structures in the resistance of the biofilm is still not fully understood, since their expression it is not induced under the biofilm state (Folsom et al. 2010).

Recently, the involvement of persister cells in biofilm has been proposed as a hypothesis of the biofilm resistance to biocides. The persister cells describe a bacterial phenotype, which is highly tolerant to antimicrobial treatments (Simones et al. 2011). This population has been estimated to reach about 0.1–10 % of total biofim cells.

However, the exact cause leading to the formation of this protected subpopulation, “persister cells,” remains not fully understood.

1.3.3.5 Pathogen protection in multispecies biofilms

In their natural environments, it is clear that biofilms are complex mixtures of different species rather than the model single species biostructures studied by the majority of laboratories (Simoes et al. 2008, Zijnge et al. 2010). In these complex consortia, species interactions can lead to the emergence of specific biofilm phenotypes.

Indeed, numerous studies have demonstrated that multi-species biofilms are generally more resistant to disinfection than mono-species biofilms (Luppens et al. 2008; Simoes et al. 2009, 2010; Van der Veen and Abee 2010). Unfortunately, the mechanisms involved in this phenomenon remain unclear. The specific nature and composition of a multi-species biofilm matrix is one of the explanations proposed. It has been suggested that chemical interactions between the polymers produced by each species may lead to a more viscous matrix and thus reduce the permeation of biocides. Another explanation is that due to the specific spatial arrangement of certain bacterial species within a biofilm, some strains may be protected from a biocide by their aggregation with others within the three-dimensional structure (Bridier et al., 2011a)

1.3.4 The green strategy for biofilms control

In an effort to stem the increase in biofilm resistance to the conventional control strategies with chemical-based disinfectants, new approaches have been introduced in order to overcome and to control the biofim-related problems such as the use of mechanical forces, green strategies, and the phage

1.3.4.1 Mechanical and enzymatic treatments

Mechanical cleaning is one of the most effective ways to fight against biofilms (Donlan and Costerton 2002). The high shear forces affect the mechanical stability of biofilms and facilitate its removal from abiotic surfaces or the accessibility of antimicrobial compounds. For example, the combination of high flow rates with detergents showed an important efficiency of biofilm removal in the endoscopes. The use of ultrasonication also seems to be a useful option for improving disinfectant efficiency and biofilm removal (Shen et al. 2010).

It is now established that the exopolymeric matrix is a part of biofilm resistance to disinfectant products. In addition, even if disinfectant agents can reduce completely the viable count of sessile cells, most biocides leave the matrix undisturbed. Thus, the dispersion of the biofilm matrix is among the approaches proposed to remove biofilms and to disrupt their structure. The uses of enzymes, promoting the degradation of exopolymers such as proteases, amylase proteases, and DNAase, have been found as a suitable option to facilitate the breakdown of the biofilm matrix. This approach has the advantage of reducing the excessive use of toxic antimicrobial agents. In addition, the combination of enzymatic treatment and other strategies such as surfactants, chelating agents, and ultrasonic may improve the enzymatic activity and biofilm removal (Abdalah et al. 2014).

The specificity in the enzymes mode of action makes it a complex technique, increasing the difficulty of identifying enzymes that are effective against all the different types of biofilms. Formulations containing several different enzymes seem to be fundamental for a successful biofilm control strategy. Moreover, the use of enzymes in biofilm control is still limited due to the low prices of the chemicals used today compared with the costs of the enzymes. In fact, the technology and production of these enzymes and the enzyme-based detergents are mostly patent-protected. Moreover, the low commercial accessibility of different enzyme activities limits their current usage (Simoes et al., 2010).

1.3.4.2 The treatment with bacteriophages

Phages are ubiquitous in nature. Bacteriophages are viruses that infect bacteria and may provide a natural, highly specific, non-toxic, feasible approach for controlling several microorganisms involved in biofilm formation. When phages come into contact with biofilms, further interactions occur, depending on the susceptibility of the biofilm cells to the phage and to the availability of receptor sites. If the phage also possesses polysaccharide-degrading enzymes, or if considerable cell lysis is affected by the phage, the integrity of the biofilm may rapidly be destroyed (Simoes et al., 2010).

For example, the phage K has showed successful effect in the removal and prevention of *S. aureus* biofilms (Kelly et al. 2012). Similarly, T7-like lytic phages isolated from river water dispersed the biofilm of multidrug-resistant strains of *P.*

aeruginosa (Donlan 2009). The engineered enzymatic bacteriophage, producing depolymerases that hydrolyze biofilm extracellular polymers, has been found to be a promising tool of biofilm control. Moreover, biofilm removal by enzymatic bacteriophage has been found to be more efficient than the classical enzymatic treatment. The combination of phages with other antibacterial agents also showed interesting outcomes (Zhang and Hu 2013).

1.3.4.3 Treatment with natural antimicrobial agents

It is necessary for research on new antimicrobial strategies to focus on processes that display high lethal activity against pathogens, are efficient in penetrating the biofilm structure and are easily degraded in the environment. Recent years have seen the emergence of studies on the use of natural antimicrobials as antibiofilm compounds. For example, plants are a rich source of active molecules with antimicrobial properties (Lewis and Ausubel 2006). Several compounds extracted from aromatic plants, which are natural and 'generally recognized as safe', have demonstrated their antimicrobial activity on planktonic bacteria. Some are now being evaluated for their potential in eradicating biofilms. Examples include carvacrol, a natural terpene extracted from thyme or oregano (Knowles et al. 2005), casbane diterpene, isolated from the ethanolic extract of a Brazilian native plant Croton nepetaefolius (Carneiro et al. 2011), thymoquinone, an active principle of Arabian Nigella sativa seed (Chaieb et al. 2011), and a naphthalene derivative isolated from Trachyspermum ammi seeds (Khan et al. 2010) which limit the formation of biofilms of various bacterial species. More interestingly, some of these compounds have been tested for their bactericidal activity on established biofilms. The ratio of concentrations (Rc) required to achieve the same reduction in a planktonic or biofilm *Staphylococcusepidermidis* population is about 4 for oregano oil, thymol or carvacrol (Bridier et al., 2011a), which compares well with that of most chemical agents. Eucalyptus oil, tea tree oil or a-terpineol have also displayed considerable efficacy in eradicating biofilms.

However, there are strict limitations in the application of essential oil as antimicrobial agents because of their high hydrophobic nature and intense smell. A promising method for their application is to vaporize these volatile compounds so as to enhance their access to the biological targets. For example, the vaporization of allyl isothiocyanate, cinnamaldhehyde, and carvacrol has been shown to markedly

inactivate *E. coli* O157:H7 attached to the surface of lettuce leaves (Bridier et al. 2011a).

1.4 Hydrosols of aromatic plants

During essential oil extraction by distillation, the essential oil vapors/constituents remain in close contact with large volume of steam/condensate water for prolonged time. Polar, oxygenated, odor imparting, hydrophilic, volatile oil components that can form hydrogen bonds with water get disproportionately partitioned into the distillation water phase (Garneau et al., 2014). The distillation water with dissolved essential oil components is commonly referred in the literature as hydrosol. The water of hydrosol comes from the water used for generating steam and from the distilled plant biomass. Although hydrosols are produced in the same extraction process as essential oils, their analysis were the subject of a limited number of publications (Paolini et al. 2008, Inouye et al 2008, Garneau et al. 2012, Gaoming et al. 2014, Garneau et al. 2014). Hydrosols are highly dilute (water volume is many times greater than that of dissolved fragrant oil constituents), acidic (pH 3.5-6.5), mild or pleasant-scented solutions. They are complex mixtures containing variable amounts of essential oil and other volatile, water-soluble, secondary metabolites (Garneau et al., 2012, Gaoming et al., 2014, Garneau et al., 2014). Hydrosols do not have the adverse effects of essential oils such as strong scent that causes headache; skin and eye irritations on contact etc.

1.4.1 Uses and economic significance

Hydrosols with odor imparting, oxygenated, polar components of fragrant oils swept their way into global markets, especially in the west as affordable flavoring, perfumery ingredients, aromatherapy products and caught the attention of global consumers, aromatherapy practioners, beauticians and scientists. Moreover recent scientific research revealed their antimicrobial properties thus implying possible applications as disinfectants.

In Europe, Asia and Africa, floral and herbal waters are employed for food flavoring and cooking. Thyme and oregano hydrosols find application as beverages and ingredients of food products. Distillate of fresh bitter orange flowers is used in desserts, western cuisine and for masking unpleasant odors. In the Mediterranean

region cakes, pastries and beverages are flavored with orange blossom hydrosol (Paolini et al., 2008).

Rose and orange floral hydrosols are widely used in cosmetics, soaps, toiletries and perfumes throughout Europe and Asia. Undiluted, diluted, formulated hydrosols find use as bath waters, message/body lotions, steam bath, sprays, wipes, nebulizer solutions, personal/space odorants and health drinks for treating several disorders of human beings and their pets. Hydrosols find application in aromatherapy for their antibacterial, antifungal, antiseptic, astringent, analgesic, antiinfectious, antioxidant, anticoagulant, antiinflammatory, cicatrizing, aphrodisiac, digestive, healing and calming properties (Paolini et al., 2008).

In Europe, Asia and Africa, floral and herbal waters are employed for medicinal purpose (Rajeswara Rao., 2012). Hydrosol, essential oil and herbal tea of *Origanumcompactum* are used in Morocco for treating dysentery, colitis, gastrointestinal infections, gastric acidity, pulmonary, skin, mouth disorders and is used as a tonic for increasing appetite and as an aphrodisiac. Orange blossom water is used in the Mediterranean region for skin care, as carminative and for inducing sleep in babies.

Hydrosols could also safely be employed as sanitizing agents for cleaning and washing fruits and vegetables, cutlery, utensils, equipment and surfaces in food industry, hotels, homes, as insect repellents, as ingredients of room/body sprays, vaporizers, floor cleaners and in festivals and religious rituals.

1.4.2 Biological properties

The biological activities of hydrosols are attributed to the major compounds and their functional groups by some researchers (Inouye et al., 2009) while others found no correlation between major constituents and the biological activity (Moon et al., 2006). It should be noted that minor components possess biological activities and the presence or absence of the activities may be associated with the sensitivity of the organism tested, origin and composition of the hydrosols, synergistic or additive effects of the components etc.

1.4.2.1 Antibacterial Activity

With the emerging trend of employing eco-friendly, health beneficial, non-toxic, natural food preservatives, wood protectors, medicines and pesticides hydrosols were investigated for their antibacterial properties. According Chorianopoulos et al., (2008_, undiluted hydrosol of *Satureja thymbra* from Greece exhibited superior bactericidal activity against biofilm forming bacteria (*Lactobacillus fermentum*, *L. monocytogenes*, *P. putida*, *S. enterica* and *Staphylococcus simulans*) relative to chemical sanitizers. The effect was time independent (60 and 180 minutes were equally effective) and hydrosol was recommended as an effective disinfectant in food processing industry against biofilms formation by food-spoilage and pathogenic bacteria on open surfaces.

Turkish thyme and (*Thymus serpyllum*, *T. vulgaris*) and oregano (*Origanum majorana, O. onites, O. vulgare*) hydrosols were bactericidal against *E. coli* (2 strains), *Staph. aureus* and *Yersinia enterocolitica* for 4 days at concentration 50 and 75 ml/100 ml (Saðdiç, 2003). Saðdiç and Özcan, (2003) reported the antibacterial effect of 16 Turkish spice hydrosols against 15 different bacterial species demonstrating the sufficient bactericidal effect of hydrosols. Similarly, indian peppermint (*Mentha piperita cv. Kukrail*) and spearmint (*Mentha spicata cv. MSS-5*) hydrosols showed weak to moderate antibacterial activity against *E. coli*, *Enterococcus faecalis*, *Streptococcus mutans*, *Staphylococcus epidermidis* and *P. aeruginosa* (Verma et al., 2011). Washing of sliced apple and carrot pieces with hydrosols of thyme, black cumin, sage, rosemary and bay leaf for 20-60 minutes protected them from *E. coli* and *S.* Typhimurium infections. Apples and carrots were inoculated with the bacteria prior to their slicing and washing. Thyme hydrosol was most effective, hence can be employed as natural sanitizing agent for washing freshly cut fruits and vegetables (Tornuk et al., 2011).

Notwithstanding the reports of antibacterial activity of spice and aromatic plants' hydrosols, other researchers reported absence of antibacterial activity of the hydrosols of several aromatic crops (Wilkinson et al., 2003, Moon et al., 2006, Ulusoy et al., 2009).

1.4.2.2 Antifungal Activity

The fungicidal and fungitoxic effect of many spice hydrosols against different fungi especially phytopathogenic has also been demonstrated (Boyraz et al., 2003, Boyraz & Özcan, 2006, Boyraz & Özcan, 2005). In all these studies the effects were concentration and time dependent and it was pointed out the utility of specific hydrosols as herbal pesticides to manage fungal diseases of crops. Inouye et al., (2009) reported the inhibitory activity of several hydrosols against filament formation and the growth of *Candida albicans*. In another study, hydrosols of lavender (*Lavandula stoechas*), thyme (*Thymus vulgaris*) showed strong activity, those of chamomile (*Matricaria chamomilla*), eucalyptus leaf (*E. globulus*), laurel leaf (*Laurus nobilis*) and myrtle leaf (*Myrtus communis*) displayed moderate activity and hydrosols of balm leaf (*Melissa officinalis*), heather leaf (*Calluna vulgaris*) exhibited weak activity against wood decay brown rot and white rot fungi when tested for 3 weeks signifying their wood protection potential (Sen & Yalçin, 2010)

1.4.2.3 Antioxidant Activity

Hydrosols of Bosnian *Pelargonium graveolens* leaves and stems along with their essential oils exhibited prominent antioxidant activity (Æavar & Makasimoviæ, 2012).

1.4.2.4 Specification and future research needs

Despite the current popularity enjoyed by herbal and floral hydrosols, no legal definitions of hydrosol and its grades and no specifications or standards for aromatherapy, food, cosmetic and other applications are available. Scientific validation of several claims made by aromatherapy practitioners based on their experience has yet to be carried out. Though it is claimed that hydrosols can safely be stored for 12 months or more, shelf-life and safety studies on long term use of stored hydrosols are lacking. The composition of hydrosols produced from the same species or its plant parts in diverse countries or geographical locations within a country or in varied seasons or at different plant growth stages or under changing management practices is likely to vary, consequently altering their therapeutic efficiencies.

The beneficial effect of hydrosols can be in 2-ways: 1. Suppressing/ inhibiting the growth and activities of harmful/pathogenic bacteria/fungi or 2. Promoting the multiplication and activities of the beneficial bacteria e.g. human probiotic intestinal

bacteria. The second aspect has largely been ignored. Haddadin (2010) demonstrated that water, ethanol, methanol extracts of olive (*Olea europaea*) leaves containing polyphenol compounds increased the cell numbers and the production of short-chain fatty acids by human probiotic intestinal bacteria *Bifidobacterium infantis* and *Lactobacillus acidophilus*. Moreover toxicological studies should definitely be carried out to investigate the side effects on human health and environment equilibria.

1.5 Aim of the study

Foodborne diseases have always been a threat to human health. They are considered an emergent public health concern throughout the world. Many outbreaks have been found to be associated with biofilms (Srey et al., 2013). Thus, biofilms have been of considerable interest in food industry hygiene since they may serve as a reservoir of spoilage and pathogenic bacteria which increases post-processing contamination and risk to public health.Much research has been performed to gain deeper understanding of biofilms and to identify a solution in order to avoid contamination of foodstuffs.

To this direction the first objective of the present study was to monitor biofilm formation of one of the most significant enteric foodborne bacterial pathogens, *Salmonella* Typhimurium, under conditions simulating partially an industrial environment. Specifically a case scenario regarding the behavior of this pathogen throughout the different steps in the production line of a water bottling plant was conducted. In a water bottling plant, the major health risk is the contamination of bottled water with bacteria that are present in the previous production steps, tubes and filters.

According reports describing microbial composition of biofilms formed on surfaces of the processing equipment in various food industries (Shi and Zhu, 2009) it was revealed that biofilms were mainly multi- species, established by various microorganisms. Thus, biofilm forming ability and the arising gene expression was studied in both mono- and multi-species cultures of *Salmonella* Typhimurium with mixed bacterial communities isolated from the bottling plant. Except for simulating the microbial composition by using the indigenous microbiota of water, an abiotic surface (stainless steel) that resembles the specific industrial environment was also chosen for biofilm formation.

Regarding gene expression study, it was aimed to evaluate the expression of genes concerning biofilm formation, cell's resistance and virulence *in situ* in 6 days mature biofilms by usingfluorescence-based bioreporters. Although molecular techniques constitutesone of the most powerful and sensitive gene analysis methods, *in situ* monitoring of gene expression in biofilms is now emerging in an effortto take

into account the differences in gene expression patterns of subpopulations within biofilms (Hermans et al., 2011). Thus, in the present study, bioreporters in combination with fluorescence microscopy are used to study genes expression.

In recent years, much research has been focused on the resistance of biofilms to antimicrobials and other environmental stresses and has led to the search and development of novel antimicrobial strategies capable of displaying both high efficiency and safety (Bridier et al., 2011a). To this direction, the second purpose of this study is to determine the disinfection activity ofhydrosol of the Mediterranean spice *Thymbra capitata*, which is a completely natural antimicrobial agent, against *S.* Typhimurium biofilm cells, in comparison with the commonly used benzalkonium chloride (BC).

More specifically, except for applying the traditional method, which determines log reductions in number of cells after a certain disinfection treatment, the evaluation of bactericidal effect towards biofilms was studied real time and *in situ* by confocal laser scanning microscopy. This direct investigation of biocide reactivity within the native structure of the biofilm aims to provide information on the spatial and temporal dynamics of the biocide action and likely a better understanding of biofilm resistance mechanisms.

2. MATERIALS AND METHODS

2.1 Bacterial strains

In this study, gene expression as well as disinfection was evaluated in biofilms formed by *Salmonella enterica* subsp. enterica serovar Typhimurim strain CDC 6516-60 (ATCC 14028)(FMCC B-415), isolated from animal tissue of 4-week-old chickens.

Gene expression was studied using ten different *S*. Typhimurium fluorescence-based bioreporters, each carrying a plasmid construct with translational fusion of the gene encoding the Enhanced Yellow Fluorescent Protein (EYFP) and a biofilm or virulence relatedgene. Laboratory of General & Agricultural Microbiology (Department of Agricultural Biotechnology, Agricultural University of Athens) engineered GFP (marker-positive control) and EYFP reporters variants and transformed *Salmonella* Typhimurium ATCC 14028. The constructs used for gene expression study are presented in Table 2.1.

The strains and constructs belong to the microorganisms collection of the laboratory (-80° C) and are preserved during the experiments at-20° C in Luria-Bertani (LB) broth containing 20% glycerol and appropriate concentrations of antibiotics if necessary.

Table 2.1:*Salmonella*Typhimurium strains used for the study.

FMCC Code[1]	Description	Genes function	Reference
B-415	*Salmonellaenterica* subsp. *enterica* serovar Typhimurium CDC 6516-60 (ATCC 14028), parent strain		
B-416	*Salmonella enterica* subsp. *enterica* serovar Typhimurium4/74, marker - GFP reporter[2]		
B-417	CDC 6516-60, clpv - EYFP reporter[3]	type VI secretion system (T6SS) encoded in Salmonella pathogenicity island 6 and 19 (SPI-19) , (SPI-6)	Pezoa et all. 2013
B-418	CDC 6516-60, csgB - EYFP reporter[3]	Nucleator in the assembly of curli (coiled surface structures) on the cell surface	Steenackers et al. 2012
B-419	CDC 6516-60, rpoH - EYFP reporter[3]	heat shock regulatory gene	Ban et all. 2015
B-420	CDC 6516-60, osmC - EYFP reporter [3]	defense against oxidative stress	Rosenkrantz et all. 2013
B-421	CDC 6516-60, csrA - EYFP reporter[3]	general regulator: motility, biofilm formation, virulence	Steenackers et al. 2012
B-422	CDC 6516-60, prgH - EYFP reporter[3]	SPI-1 secretion apparatus protein (invasion)	Klein et all. 2000
B-423	CDC 6516-60, sspH2 - EYFP reporter[3]	virulence factor	Bhavsar et all. 2013
B-424	CDC 6516-60, flic - EYFP reporter[3]	motility regulatoror	Bogomolnaya et all. 2014
B-425	CDC 6516-60, ssrA - EYFP reporter[3]	SPI-2 regulator	Tomljenovic-Berube et all. 2010

[1]Laboratory of Food Microbiology and Biotechnology of Foods Culture collection [2]Growth on LB supplemented with 40μg/mL kanamycin [3]Growth on LB supplemented with 100μg/mL ampicillin

2.2 Experimental procedure

2.2.1 Water samples collection and bacterial identification

Samples' collection

Water samples were collected during summer from the following points in the bottling plant(Figure 2.1):

1) Water source (WS)
2) Water distribution network (WD)
3) Final product (FP)

Samples' collection was realized according the protocol below (Figure 2.2 – 2.4):

Figure 2.1: Water bottling plant

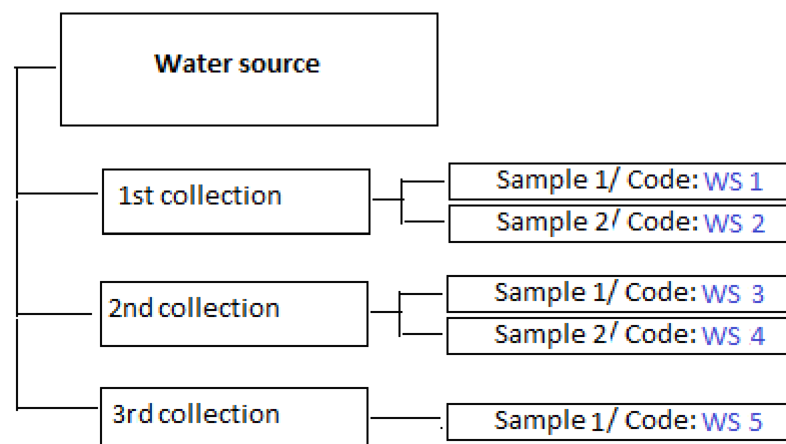

Figure 2.2: Sample collection from the water source

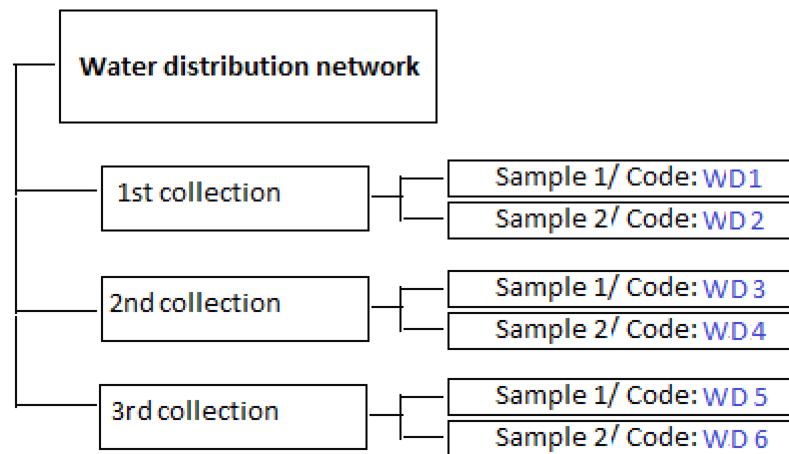

Figure 2.3: Sample collection from the water distribution network

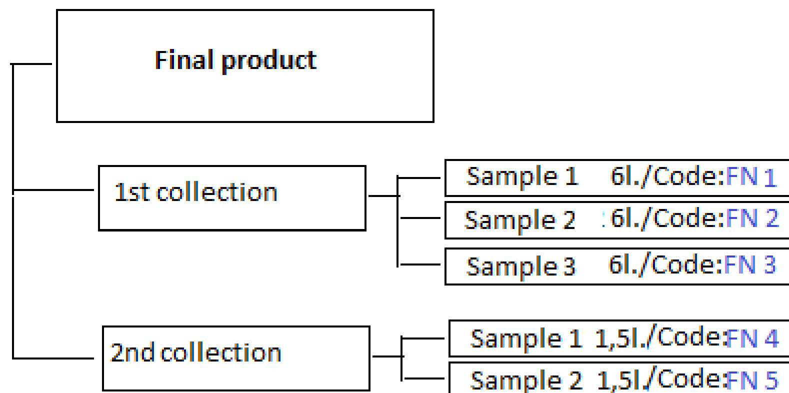

Figure 2.4: Sample collection among final products

Among final products, they were collected samples from the production line of 6 l. bottled water and 1.5 l. bottled water.For the production of 6 L bottled water, a semi-automic with manual bottles feeding system. In contrast, the production of 1.5 L bottled water take place in a fully automatic system.

All samples from water source and distribution network were collected in glass sterilized bottles.

Samples' analysis: Membrane filter method

Water samples were analyzed, without been diluted, with membrane filter method (Figure 2.5).

Briefly, the membrane filter (0.45μm) was removed from the sterile package with a forceps that had been flamed, and was placed into the funnel assembly. The sample was poured into the funnel and the vacuum was turned on to allow the sample to draw completely through the filter. The membrane filter was removed from the funnel and placed into the petri dish.The petri dish was incubated in the appropriate temperature for the appropriate period of time.Colony forming units were counted. In Table 2.2, the nutrient media which used for water analysis are presented.

Figure 2.5: Membrane filter method procedure.

Table 2.2: Nutrient media used for water analysis.

Nutrient media	Selective for:
Pseudomonas selective agar	*Pseudomonas aeruginosa*
m Endo Agar LES	Total coliforms
Slanetz and Bartley medium	Enterococci
Plate count agar	Total viable count

Colonies isolation and storage

The membrane with formed colonies was held vertically with the forceps into a sterilized tube and it was rinsed with 5 ml Ringer. The membrane was removed, 1 ml glycerol was added and tube was stored at -70° C, temporarily. After collection of all samples, tubes were centrifuged at 5000g for 10 min at 4 °C. Supernatant from each tube was removed and pellet was resuspended with 2 ml of LB broth, glycerol was added stored at – 80°C until further use.

Bacterial identification

PCR DGGE analysis was applied for identification of bacteria isolated from water samples. The procedure is schematicallydescribed in Figure 2.6:

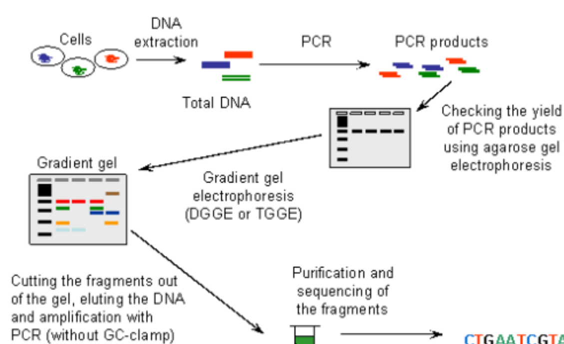

Figure 2.6: Bacterial identification by PCR-DGGE

DNA extraction

The genomic DNA was extracted from each sample according toErcolini et al., (2006)**.**Before DNA extraction 10 µl of each stock sample were added to 10ml LB and incubated at 37 °C for 24 h. Two milliliter of cell overnight culture were centrifuged at 9000 g for 5 min. The resulting pellet was resuspended in 0.5 ml buffer solution consist of 1M sorbitol, 0.1 M EDTA (pH 7,5) containing 25 mg/ml lysozymeand incubated for 120 min at 37°C. After incubation, it was centrifuged at 14000 rpm for 10 min. Pellet was resuspended on 0.5 ml buffer consist of 50mM Tris-HCl, 20 mM EDTA (pH 7.4), mixed with 50 µl 10% SDS and incubated at 65°C for 30 min.After incubation, 0.2 ml potassium acetate 5M were added and left on ice for 30 min. The sample was centrifuged at 14000 rpm for 10 min. The pellet was dried and resuspended on 0.5 ml ethanol 70%. The sample was centrifuged again at 14000 rpm for 10 min. The pellet was dried and resuspended on 50 µl ddH2O. The samples were stored at 20° C until further use.

Polymerase Chain Reaction (PCR)

PCR amplification was performed according to Ercolini et al., (2006). In brief,primers U968 (AACGCGAAGAACCTTAC) and L1401(GCGTGTGTACAAGACCC) were used to amplify the variable V6-V8 region of the16S rRNA gene, giving PCR products of about 450 bp. A GC clamp was added to the forward primer according to a method described previously by Muyzer et al. (1993). PCR amplifications were conducted in a final volume of 25 mL containing 2.5

U of thermostable (Taq) DNA polymerase (New England Biolabs, Ipswich, MA, USA), 2.5 mL Taq buffer, 0.8 mM dNTP's, 0.2 mM of each primer, 1.0 mM $MgCl_2$ and 20 ng of DNA template. PCR reaction realized in the thermal cycler BioRad ProFlex System(Figure 2.7) and consisted of an initial denaturation step at 94 °C for 5 min, followed by 30 cycles (denaturation at 94 °C, 1 min, primer annealing at 56 °C, 45 s, primer extension at 72 °C, 3 min), and a final extension step at 72 °C for 10 min.

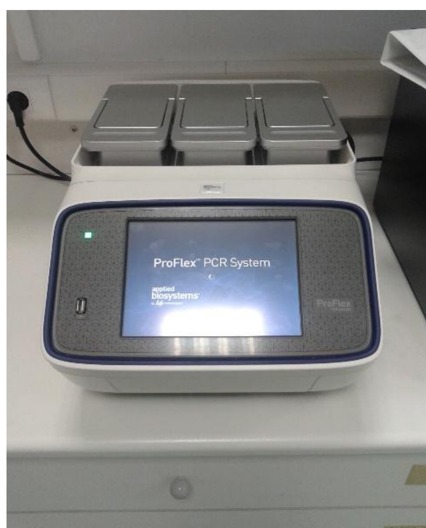

Figure 2.7: Thermal cycler **BioRad** ProFlex PCR System

PCR amplification was checked with simple agarose gel electrophoresis in 2% agarose gel at 80Vfor 1 h. Gel was then stained with ethidium bromide (0.5 mg mL^{-1}) inwater before being photographedusing a GelDoc system (Biorad).

Denaturing Gradient Gel Electrophoresis (DGGE)

PCR products were analyzed by DGGE using a DCode apparatus (Biorad) (Figure 2.8) according to Ercolini et al. (2006). Brieflysamples were applied to 7% (w/v) polyacrylamide gels, containing a 20-50% urea-formamide denaturing gradient (100% corresponded to 7 M urea and 40% (w/v) formamide). Electrophoresis/Temperature Control module was placed, the temperature was set to 60°C and the gels were run for 10 min at 50 V, followed by 4 h at 200 V.

Figure 2.8: DGGE apparatus in action.

The gel was stained with ethidium bromide for 5 to 20 min into a plastic container. Then it was slided onto the UV transilluminator and photographed using a GelDoc system. Differing sequences of DNA (from different bacteria) denatured at different denaturant concentrations resulting in a pattern of bands. Different bands were carefully cut out from the gel and placed in microfuge tube. Sample at that point were stored until sequencing.

Species identification

Bands isolated from DGGE gel were sent to CeMIA, (Department of Immunology & Histocompatibility, Faculty of Medicine, University of Thessaly, Greece) and V6-V8 variable region of the 16S rRNA gene was subjected to sequencing with the primer L1401. Bacterial identification completed using BLAST bioinformatics program for sequence searching and comparing.

2.2.2 Evaluation of biofilm forming ability & Study of gene expression in *Salmonella* Typhimurium monospecies and multispecies biofilms

Abiotic substratum

Stainless steel (SS) coupons (3 x 1 x 0.1 cm, type AISI-304, Halyvourgiki Inc., Athens, Greece) were the abiotic substrates used for biofilm formation, since this material is frequently used for the manufacture of food processing equipment. Prior to use, coupons were cleaned according to the procedure described by Giaouris et al., (2012). Following cleaning, coupons were individually placed in glass test tubes

containing 4 ml Ringer (length 5 cm, diameter 1.5 cm) and autoclaved at 121°C for 15 min.

SS coupons inoculation and biofilms development

During this study gene expression was evaluated in both monospecies and multispecies *Salmonella* 6 daysbiofilms. *S.*Typhimurium biofilms were developed in absence of antibiotics as in trial experiments it was confirmed that it didn't affect the fluorescence ability of reporters. Bacteria isolated from samples collected by the water plant were inoculated in SS coupons and checked for their biofilm forming ability. Subsequently each water sample was inoculated in combination with each *S.* Typhimurium reporter. The procedure followed for biofilm development was according Kostaki et al., (2012). Briefly all strains were resuscitated 2 times (1st 24 h, 2nd 16 h) in LB broth at 37°C or 20 °C before inoculation.2nd subculture was centrifuged at 5000 g for 10 min at 4°C. Supernatant was removed and pellet was resuspended in 10 ml Ringer. Six serial dilutions were prepared in Ringer. 100 μl of the 5th and 6th dilution were spread in TSA and incubated at 20 °C for 24-48 h. CFUs were counted to determine the inoculation level. A volume of 500 μl from the 2nd dilution was added in the tube containing the coupon (Figure 2.9).Adhesion was done by sedimentation for 3 h at 15°C. Subsequently, the coupon was removed from the tube with the planktonic bacterial suspension, and was placed in a tube containing 5 ml LB broth.Coupons were incubated at 20 °C for 6 days without shaking. Two material changes were realized during each incubation period.

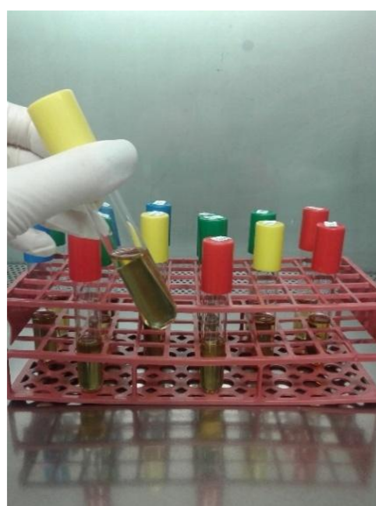

Figure 2.9: Stainless steel coupons in tubes with LB Broth.

Determine cell concentration of 6 d biofilms

Biofilm bacteria on SS coupons were quantified by using the "bead vortexing method" described by Kostaki et al., (2012). Coupon was held vertically with a forceps and each side was rinsed with 5ml Ringer to remove planktonic and loosely attached cells. Coupon was then placed into a falcon containing 6 ml Ringer and 10 glass beads. The falcon was vortexed for 2 min to detach all attached cells (Figure 2.10). Four serial dilutions were prepared in Ringer. 100 µl of the 2nd, 3rd and 4th dilution were spread in TSA and incubated at 20 °C for 24-48 h. CFUs were counted to determine the cell concentration of 6 days biofilm.

Figure 2.10: Cells detachment by vortexing with glass beads.

Study gene expression in 6 d biofilms by fluorescence microscopy

After 6 days biofilm formation, coupon was held vertically with a forceps and each side was rinsed with 5ml Ringer to remove planktonic and loosely attached cells. Coupon was placed on a microscope slide and then it was mounted on the stage of the N-400 FL Epi-fluorescence microscope (Figure 2.11).

Figure 2.11 Study genes expression in SS coupons by epifluorescence microscopy.

The 100 W Hg lamp, was used as light source in the fluorescence microscope. The blue excitation filter (excitation wavelength 450-480 nm) was selected, as excitation peaks for GFP and EYFP are close to these wavelengths. The 100X immersion oil objective was used for specimen's observation.Each observation started from the left edge of the coupon, then covered the whole length (30 mm) and end up in the right edge of the coupon (Figure 2.12). This observation method was applied in all coupons to ensure that the sampling area (0,13mm x 30mm = 3,9mm^2) was the same for each case.

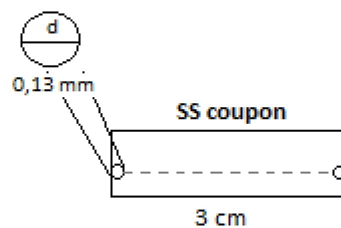

Figure 2.12: Standard observation area on each coupon.

Gene expression was studied qualitative by recording the presence or absence of fluorescent cells. Points into the biofilms where fluorescent cells could be detected were photographed using the software Pinnacle Studio, a video editing program, developed by Pinnacle Systems (http://www.pinnaclesys.com/).

2.2.3 Disinfection of *Salmonella* Typhimurium planktonic cells and biofilms with hydrosol of *Thymbra capitata* and benzalkonium chloride

Two different strategies were followed in the present study to investigate the antimicrobial action of the two biocides. Disinfectant tests using both traditional method and confocal laser scanning microscopy were conducted in AgroParisTech, INRA, Micalis Joint Unit, laboratory Bioadhesion, Biofilm and Hygiene of Materials, Massy.

A) Disinfectant tests on stainless steel coupons and planktonic cells

i. Planktonic cells

24 h culture

S. Typhimurium was resuscitated in 2 successive subcultures (1st 7 h, 2nd 16 h) in TSB at 37°C. 100 µl of the second subculture were added in 10 ml Tryptic Soy Broth (TSB) and incubated at 20°C for 24h.

Disinfectants preparation

Different concentrations of hydrosol (from 25% to 75%) were prepared by mixing appropriate quantities of hydrosol 100% with sterilized deionized water. Likewise different concentrations of benzalkonium chloride (BC) were prepared (from 6 ppm to 200 ppm).

The following conversion was useful during disinfectant preparation 100 ppm (parts per million) = 100 mg/l = 0.01%

Disinfection procedure against planktonic cells

Planktonic cells were challenged with the disinfectants using the EN 1040 standard protocol (Anonymous, 1997). Briefly, 10 µl of the 24h *Salmonella* culture were added in 10 ml 150 mM NaCl (100 fold dilution). 1 ml of this bacterial suspension was added in eppendorf tubes and centrifuged at 5000 g for 10 min. Supernatant was carefully removed. Planktonic cells were exposed to disinfectants for 6 min at 20°C (Giaouris et al., 2013) by adding the disinfection solution directly to the pellet. The action of the antimicrobial was halted by transfer (1:9) to a quenching

solution (3 g/L L-α-phosphatidyl cholin, 30 g/L Tween 80, 5 g/L sodium thiosulfate, 1 g/L L-histidine, 30 g/L saponine) for 10 min. Serial dilutions were prepared and survivors were enumerated using the 6 × 6 drop count method (Chen et al., 2003) on Tryptic Soy Agar (TSA). The control was performed in the same way with sterile deionized water instead of the disinfectant. The logarithm reduction achieved was the difference between the log10 survivors after the test with deionized water and the log10 survivors after the test with the antimicrobial agent.

ii. Biofilms on stainless steel coupons

Abiotic substratum

Stainless steel (SS) coupons of 1 cm^2 (type AISI 316 2R, Goodfellow, Cambridge Science Park, UK) were chosen for biofilm development. Before use, the coupons were placed for 10 min under stirring at 50 °C in 2 % v/v solution of surfactant RBS, after which they were rinsed with sterile deionized water five times at 50 °C and five times at ambient temperature (Neyret et al., 2014). Subsequently, they were stored in sterile deionized water for a maximum of 24 h before use.

SS coupons inoculation and biofilm development

S. Typhimurium was resuscitated in 2 successive subcultures (1st 7 h, 2nd 16 h) in TSB at 37°C. 2nd subculture was centrifuged at 5000 g for 10 min at 4°C. Supernatant was removed and pellet was suspended in 10 ml 150 mM NaCl. Two serial dilutions were prepared in 150 mM Nacl. Coupons were settled in the wells of a polystyrene 24-well microtiter plate (Techno Plastic products, Switzerland) and 1 ml of 2nd serial dilution of prepared suspension was poured into the wells. Adhesion was done by sedimentation for 2 h at 20 °C. Subsequently, the planktonic bacterial suspension was removed and 1 mL of TSB was added. Coupons were incubated at 20 °C for 24 h without shaking.

Preparation of disinfectants

Three different concentrations of hydrosol (100%, 75% and 50%) were prepared by mixing appropriate quantities of hydrosol 100% with sterilized deionized water. Likewise four different concentrations of BC were prepared (0,5%, 0,25%, 0,125% and 0,02%).

Disinfection procedure against biofilms

Following biofilm formation, planktonic suspension was removed from the wells while inclining the plate to 45°. Subsequently, 1 ml Ringer was added to each well and it was again removed inclining the plate to 45° (coupons rinsing). Coupons were immediately challenged with the disinfectant solutions for 6 min at 18°C (Giaouris et al., 2013) by pouring each well with 1 ml of each disinfectant. Control was performed in the same way with sterile deionized water instead of the disinfectant. Coupons were removed from the disinfectant solutions and placed for 10 min at 20°C in the quenching solution (3 g/L L-α-phosphatidyl cholin, 30 g/L Tween 80, 5 g/L sodium thiosulfate, 1 g/L L-histidine, 30 g/L saponine). Coupons were placed in 45-mm Petri dishes with 5 ml of Ringer, and the strongly adherent (biofilm) cells were detached by scratching. Serial dilutions were prepared. The survivors were enumerated and log reductions were determined as previously described (see section for planktonic cells disinfection).

B) Real time visualization of disinfection activity in biofilms

Time lapse Confocal Laser Scanning Microscopy (CSLM) analysis of disinfection action was performed in *S.* Typhimurium biofilms. This technique permitted the direct visualization of cell inactivation patterns in biofilm structures during disinfectant action. The method was based on monitoring of fluorescence loss caused by the leak of an unbound fluorophore outside the cells after disruption of the bacterial membrane by antimicrobial agents.

Inoculation of microtiter plates and biofilm development

Biofilms were grown in a polystyrene 96-well microtiter plates (Greiner Bio-One, France) with a Clear base (polystyrene; 190±5 µm thick), which enabled high-resolution imaging as previously described by Bridier et al., (2010). Briefly, 10 µl of the 2nd subculture were added in 10 ml TSB (100 fold dilution, 10^6 CFU/ml). A volume of 250 µl of this bacterial suspension were added to the wells of the polystyrene microtiter plate. After 2 h of adhesion at 20°C, the planktonic bacterial suspension was removed and 250 µL of TSB was added. The plate was then incubated for 24 h at 20°C to allow for biofilm development

Fluorescent Labeling

According Bridier et al., 2011, the biofilms were stained with Chemchrome V6 (AES Chemunex, Ivry-sur-Seine, France) which is an esterase marker that can penetrate passively into a cell where it is cleaved by cytoplasmic esterases, leading to the intracellular release of fluorescent residues (green fluorescence). The commercial solution of V6 was diluted (1:100) in Chemsol B16 Buffer. Planktonic cells (100 µl) were removed gently from each well and 100 µl of V6/B16 solution were added. Microplate was incubated in the dark for 17 min at 37 °C in order to reach fluorescence equilibrium. The maximum volume of supernatant was removed gently, and biofilms were rinsed once with 100 µl Ringer to eliminate any excess Chemchrome V6. Wells were refilled with 100 µl of Ringer, except for wells were the action of hydrosol 100% would be checked (no dilution).

Time lapse CLSM analysis

Time lapse CLSM analysis of disinfection action was performed in *S.* Typhimurium biofilms as previously described (Bridier et al., 2011). The microtiter plate was mounted on the stage of Leica SP2 AOBS Confocal laser scanning microscope at the MIMA2 microscopy platform (Figure 2.13).

Figure 2.13: Leica SP2 AOBS Confocal laser scanning microscope at the MIMA2 microscopy platform

The following acquisition parameters were adjusted as these had been shown to avoid photo bleaching of the sample during control tests (using deionized water instead of biocide) in previous experiments.

- Objective: 63x oil with 1.4 numerical aperture

- Series of time lapse image scans: 256 x 256 pixels
- Speed of scan: 400 Hz
- Excitation: 488 nm argon laser, 10% of its maximum intensity
- Emission: from 500 nm to 600 nm

The terminals (bottom and top) of the biofilm were settled by defining begin and end. Firstly a xyz stack was done (z-step 1 μm) to quantify the structure parameters of the biofilm which would be disinfected (biovolume, thickness, density etc). Subsequently a xyzt scan was done every 15 s during 25 min in five different sections into the biofilm.

After the launch of the time series images the following quantities of hydrosol 100% and BC 1% were gently added to the well just after completion of the first scan.

- 200 μl hydrosol 100% to wells without NaCl (to achieve final concentration 100%).
- 150 μl hydrosol 100% to wells containing 50 μl NaCl (to achieve final concentration 75%)
- 100 μl hydrosol 100% to wells containing 100 μl NaCl (to achieve final concentration 50%)
- 100 μl BC 1% to wells containing 100 μl NaCl (to achieve final concentration 0,5%)

Image analysis

The intensity of green fluorescence was quantified by using the LCS Lite confocal software (Leica microsystems). Intensity curves showing fluorescence loss were extracted for the five different sections into the biofilm separately as well as for their maximum 2 dimensional projection of the 3 dimensional biofilm structure (5 sections in 1) representative for fluorescence loss 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 biofilms structure were reconstructed using the Easy 3D function of the IMARIS 7.0 software (Bitplane, Switzerland). Quantitative structural parameters of the biofilms, such as 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/). The biovolume represented the overall volume of

cells (μm^3) in the observation field. Thickness (µm) of biofilms was also determined directly from the confocal stack images.

Application of bacterial destruction models to fluorescence intensity curves.

GinaFiT, a freeware add-in for Microsoft Excel developed by Geeraerd et al., (2007) was used to model inactivation kinetics. This tool can test nine different types of microbial survival models, and the choice of the best fit depends on five statistical measures (i.e., the sum of squared errors, the 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, and kmax, the inactivation rate (min^{-1}).

Statistical analysis

All statistical analysis (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.

2.2.4 Chemical analysis of *Thymbra capitata* hydrosol and essential oil

Chemical composition of essential oil and hydrosol was determined by Gas Chromatography (GC) and Gas chromatography- Mass spectrometry (GC-MS) analyses. Hydrosol is an aqueous solution and only its hydrophobic compounds could be analyzed with GC and GC-MS. Hydrophobic compounds were extracted from hydrosol with the following procedure. Ten ml hydrosol were added in the back part of a manual extraction apparatus. Two ml ether were added in the front part of the apparatus which was opened and closed continuously to mix ether with hydrosol. The sample was left until hydrophobic and hydrophilic phases were separated. Hydrophobic phase (upper phase) was carefully removed and a few Na2SO4 was added to bind remaining H2O forming white aggregates. Supernatant was carefully removed and used for GC. The analytical GC and GC-MS was carried out in the chemical laboratory from AgroParisTech INRA GENIAL joint unit, in Massy. . Most

constituents were tentatively identified by GC by comparison of their retention indices with those of authentic standards with retention indices in close agreement with references. Further identification achieved by GC-MS. The mass spectra fragmentation were compared with those stored in the spectrometer database (Willey built in database).

3. RESULTS AND DISCUSSION

3.1 Water samples collection and bacterial identification

A set of 16 samples were collected from water source, water distribution network and final products of a water bottling plant. Results from water samples' analysis by membrane filter method are presented in Table 1, Annex A. Among all samples tested, only in WS3 and WD4 total coliforms were detected (<4 cfu/250 ml and <4 cfu/250ml respectively). None of the other bacterial indicators for contamination was detected, whereas in all samples heterotrophic bacteria were grown in heterotrophic plate count (HPC) media.

Bacteria isolated during HPC tests were identified by PCR DGGE analysis. DGGE patterns obtained from water samples are presented in Figure 3.1Six different DGGE fingerprints (B1, B2, B3, B5, B6, B7) were obtained and subsequently subjected to 16S rRNA gene sequencing. Bacterial identification conducted with BLAST sequence similarity tool.

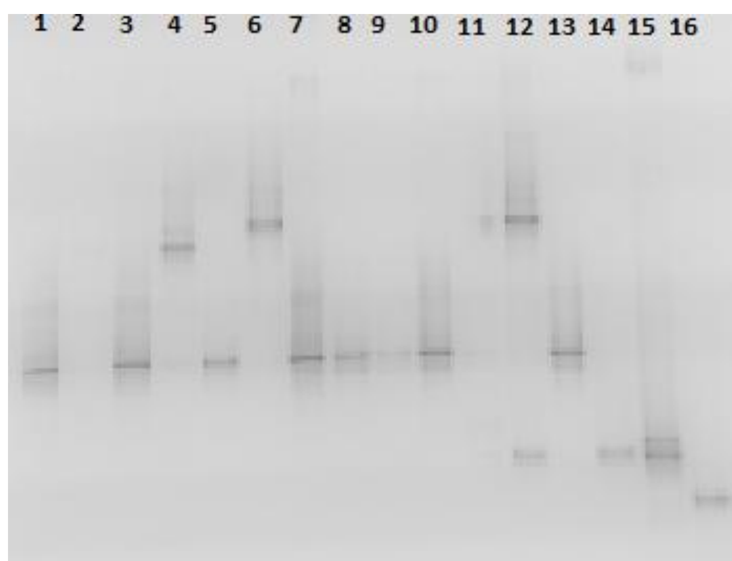

Figure 3.1: DGGE patterns obtained from water samples

The DGGE fingerprint (B1) was assigned to *Citrobacter* spp., (B2) to *Staphylococcus* spp., (B3) to *Staphylococcus* spp., (B5) to *Pseudomonas* spp., (B6) to *Bacillus* spp. and (B7) to *Exiguobacterium* spp. The distribution of these bacteria throughout the three most important stages in the production line into the water bottling plant is presented in Table 3.1

Table 3.1: Distribution of species obtained from HPC medium according to PCR-DGGE profiling throughout production line in water bottling plant.

		Citrobacter **spp**	*Staphylococcu s* **spp**	*Staphylococcu s* **spp**	*Pseudomona s* **spp**	*Bacillu s* **spp**	*Exiguoba cterium* **spp**
Water source	WS1	+					
	WS2	+					
	WS3	+					
	WS4		+	+			
	WS5	+					
Water distribution network	WD1			+			
	WD3	+					
	WD4	+					
	WD6	+					
	WD7	+					
	WD10	+		+			
Final products	FN1			+	+		
	FN2	+					
	FN3					+	
	FN4				+	+	
	FN5						+

The persistence of *Citrobacter* spp. which belongs to *Enterobacteriaceae* family and it is considered as one type of coliform bacteria (Stevens et al., 2003, Fricher et al., 2009), was detected in all three basic stages of the production line (10 of 16 samples). It is noteworthy that during membrane filter method, only in two samples total coliforms were grown. This can be attributed to the fact that drinking water constitutes an oligotrophic environment where bacteria undergo starvation stress and it has been demonstrated that recovery on selective media is restricted (Bjergbaek et Roslev 2005, Maheux et al., 2014).

Coliform bacteria are not a single type of bacteria, but a group of bacteria that includes many genera such as *Escherichia, Klebsiella, Enterobacter, Citrobacter*, *Hafnia* etc (Frichier et al., 2009). The total coliform group remains a water quality indicator in many countries and continues to be used as a regulator parameter despite reservations about its usefulness as indicator of fecal contamination (World Health Organization 2004, Mesquita et al., 2013). Total coliforms and especially genera *Citrobacter, Klebsiella* and *Enterobacter* are present not only in the intestines of mammals and fecal matter, but also in the aquatic environment, soil and vegetation (Cabral et al., 2010, Fielo et al., 2014). They are ubiquitous in nature, and many types are harmless, so considering them as good indicators of fecal contamination may lead

to erroneous conclusions (Pindi et al., 2013). However, members of *Citrobacter* can cause opportunistic infections in human i.e *Citrobacterfreundii* (Badger et al., 1999) so its persistence throughout the bottling plant is alarming. It is necessary to identify the exact species and subsequently conduct attentive sampling, microbiological and virulence tests to confirm the problem occurrence, determine its origin and make appropriate correction actions.

Staphylococcus spp. was the second most common bacteria detected in the bottling plant (4 of 16 samples). Staphylococci in potable water may be regarded as a natural biota or one of the genera that are commonly found in water supplies as HPC bacteria (Allen et al., 2004, Abulreesh et al., 2014). Despite the suggestion that it is not possible to establish health based standards for the presence of such HPC bacteria indrinking water, the exact species should be identified. For example the incidence of high concentration of *S. aureus* in water intended for human consumption may represent potential health hazards, especially if these strains possess determinants of antibiotic resistance and are able to produce enterotoxins (Percival et al., 2004).

Bacillus spp. were detected in two samples which were both final products. Although most *Bacillus* spp. are harmless, a few are pathogenic to human and animals i.e. *Bacillus cereus*. They commonly occur in a wide range of natural environments, such as soil and water and they form part of the HPC bacteria, which are detected in most drinking-water supplies (WHO, 2011). They have the important feature of producing spores that are exceptionally resistant to unfavorable conditions. *Bacillus* spp was the main isolate in final products that have undergone an intense ozone treatment (6 l bottles are loaded in bottling line manually, higher risk) so its prevalence could be linked with its ability to survive in extreme environments. Owing to a lack of evidence that waterborne *Bacillus* spp. are clinically significant, specific management strategies are not required.

Exiguobacterium is a genus of bacilli class, which is widespread in the environment and can be found in a variety of environments including microbialites, ocean, freshwater lakes, Himalayan ice, Himalayan soil, hydrothermal vents, brine shrimp etc. (Raichand et al., 2012). The occurrence of *Exiguobacterium* spp in drinking water has also been reported by Pindi et al., 2013 without any clinical impact in human.

Pseudomonas spp is one of the most diverse and ecologically important group of bacteria on the planet (Spiers et al., 2000, Mena and Gerba, 2009). The prototrophic character and metabolic flexibility, the plasticity of the genome and the ability to survive under different stressful conditions (physical, chemical and antibacterial compounds) are remarkable characteristics of the members of this genus (Palleroni, 2010). Such characteristics are the driving forces for adaptability, justifying the presence of pseudomonads in all the major natural terrestrial and aquatic environments. In particular, drinking water and especially bottled water is considered a relevant habitat of *Pseudomonas* spp. (Vantarakis et al., 2013, Jayasekara et al., 1998, Palleroni 2010). However according supplementary tests that conducted with selective media the occurrence of *Pseudomonas* in the sample FN1 wasn't confirmed.

3.2 Evaluation of biofilm forming ability & Study of gene expression in *Salmonella* Typhimurium monospecies and multispecies biofilms

3.2.1 Biofilm forming ability of *S.* Typhimurium and bacteria isolated from the bottling plant.

The viable biofilm bacteria after 6 days of development on SS coupons were quantified by using the bead vortexing method. Results regarding the populations (log CFU/cm^2) of monospecies biofilm developed in this study are presented in Figure 3.2.

Citrobacter spp. and *Bacilus* spp. displayed the greater biofilm populations (7.3 and 7 logcfu/cm^2 respectively) followed by *Exiguobacterium* spp, S. *Typhimurium* spp. and *Staphylococcus* spp (6, 5.64 and 4.8 respectively). In previous studies the biofilm forming ability of *S. enterica* on stainless steel surfaces (Giaouris et al., 2005 Chia et al., 2009, Kim et al., 2009, Chorianopoulos et al., 2010), *Staphylococcus* spp. (Malheiros et al., 2010, Michu et al., 2011), *Bacillus* spp. (Faille et al., 2014, Nam et al., 2014) and *Citrobacter* spp. (Gunduz et al., 2006) has been reported. To date, there is no reference to our knowledge demonstrating the biofilm forming ability of *Exiguobacterium* on stainless steel and general food processing surfaces.

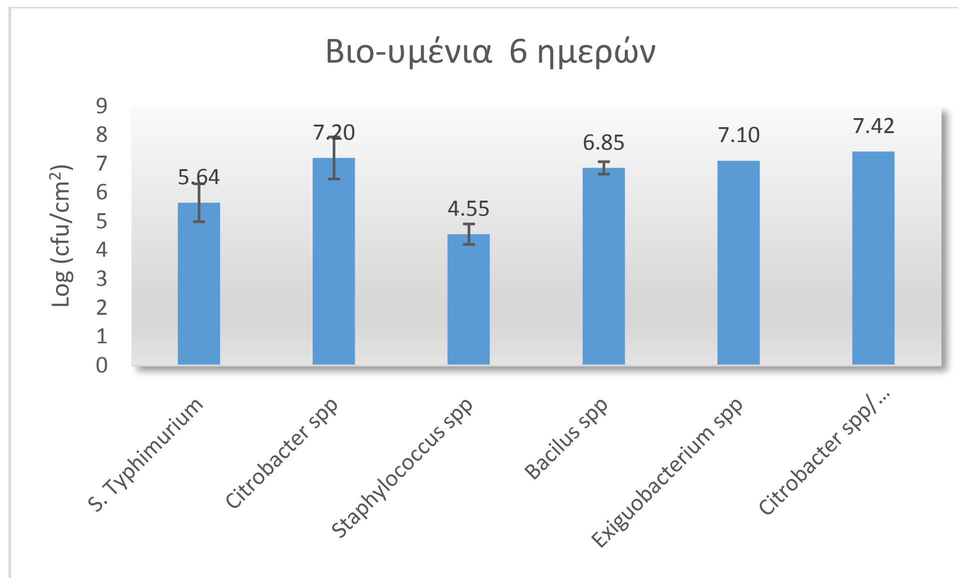

Figure 3.2: Cell density (log cfu/cm^2) in 6 days monospecies biofilms developed by *S.* Tyhimurium and bacteria from the bottling plant.

In recent years, there has gradually been a shift in focus towards examining the complexity and interactions in multispecies biofilms (Zengler et al., 2012). Therefore in this study multispecies bacteria consisting of bacteria isolated from the bottling plant as well as the foodborne pathogen *S.* Typhimurium were developed. Results regarding the populations (log cfu/cm^2) of multispecies biofilms are presented in Figure 3.3-3.5.

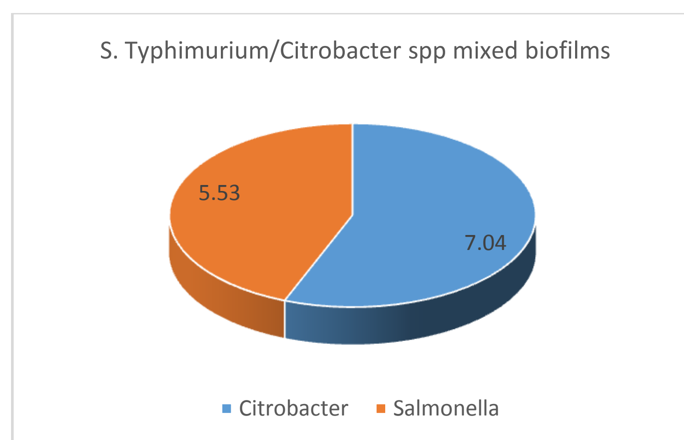

Figure 3.3: Cell densities (log cfu/cm^2) in 6 d *S.* Typhimurium and *Citrobacter* spp. mixed biofilms

In multispecies *S.* Typhimurium biofilms with *Citrobacter* spp, *Citrobacter* spp dominated the bacterial biofilm community. It has previously been reported that *Citrobacter* spp. is a strong competitor in mixed biofilms with *E. coli* and *L. monocytogenes* (Pereira et al., 2010, Weiler et al., 2013). However, cell densities of both bacteria are almost the same as in mono species biofilms (5.53 and 7.03 logcfu/cm^2 respectively) so competitive interactions cannot be easily presumed in this case as both bacteria do not face a reduction in biomass when coexist.

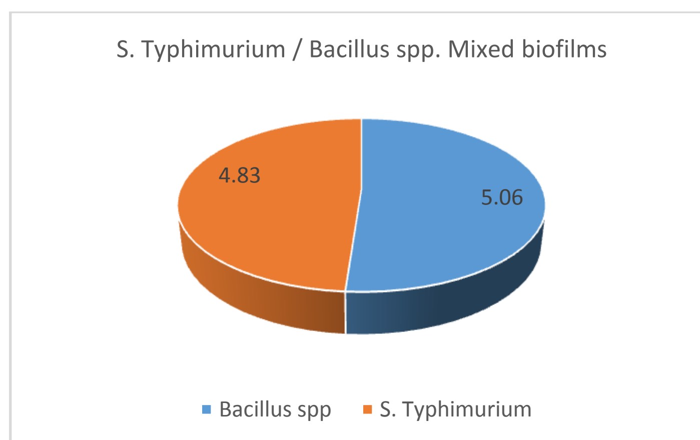

Figure 3.4: Cell densities (log cfu/cm^2) in 6 d *S.* Typhimurium and *Bacillus* spp. mixed biofilms.

In multispecies *S.* Typhimurium biofilms with *Bacillus* spp, cell densities of the two species are almost the same (4.84 and 5.06 logcfu/cm^2 respectively) but lower compared to monospecies biofilms. Especially for *Bacillus* spp. 1 log reduction was observed. Despite the low population of *Bacillus* compared to *Citrobacter* (2 log difference) which would imply an advantage to *S.* Typhimurium growth into this kind of multispecies biofilm, it was observed that *S.* Typhimurium population was limited too. Thus slight competitive interactions may occur in this case.

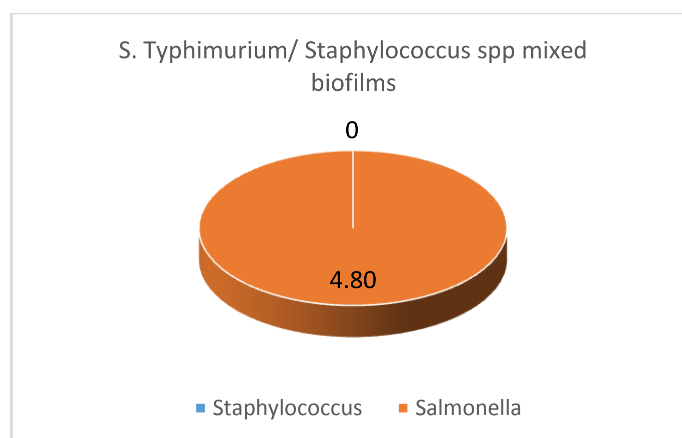

Figure 3.5: Cell densities (log cfu/cm^2) in 6 d *S.* Typhimurium and *Staphylococcus* spp. mixed biofilms

Cell densities of *Staphylococcus* spp. in multispecies *S.* Typhimurium biofilms were below the detection limit of the plate count method (1 logcfu/cm^2). It hasn't previously been reported such an inhibitory impact of *S. enterica* on *Staphylococcus* spp. growth into mixed biofilms. The precise *Staphylococcus* species should be identified and temporal and spatial distributions into the biofilms should be studied by advanced microscopic techniques to figure out this unconventional finding.

Results concerning biomass loss and gains are often not enough to characterize interactions as cooperative or competitive, as many factors should be taken into consideration when exploring complex systems. For example one species may face a reduction in biomass by joining a multispecies community, but it may gain increased protection from various stresses or an expanded niche, resulting in an overall fitness gain (Burmolle et al 2014).

3.2.2 Evaluation of biofilm forming ability as well as plasmid retainment of *S.* Typhimurium strains containing reporters.

The stability of pRH008 plasmid containing EYFP reporter gene into *S.* Typhimurium inoculum and biofilm cells was assayed. The concentration of bacterial suspension used for biofilm inoculation was determined by plate count method in XLD agar and XLD agar with ampicillin. Colonies grown on XLD agar with ampicillin are representative for cells containing the plasmid pRH008 (Figure 3.6).

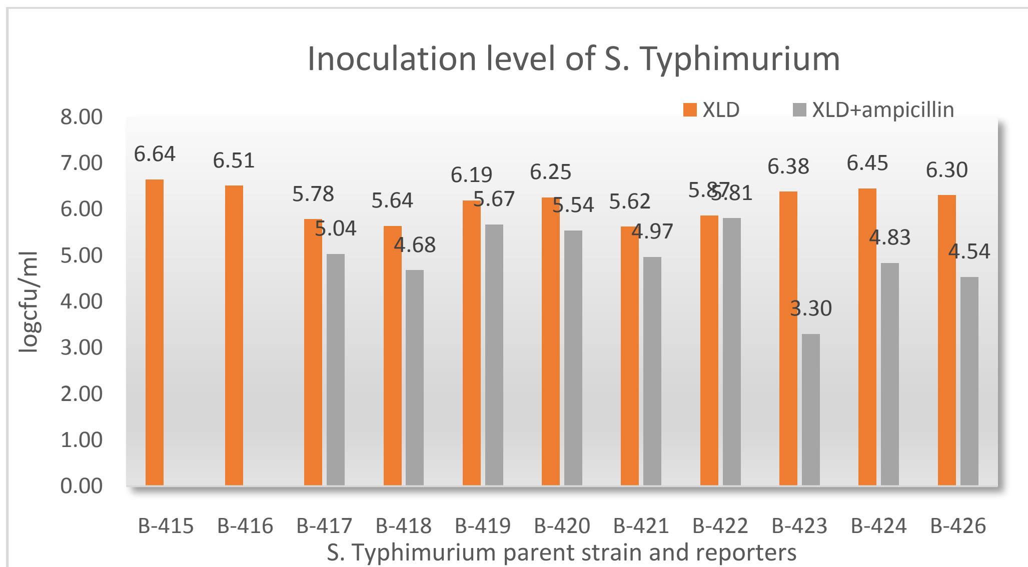

Figure 3.6: Inoculation level (log cfu/cm^2) of *S.* Typhimurium parent strain and reporters.

In order to better understanding the extent of pRH008 attainment by the different *S.* Typhimurium strains, the percentage (%) of cells containing the plasmid in each case was evaluated and is presented in Figure 3.7.

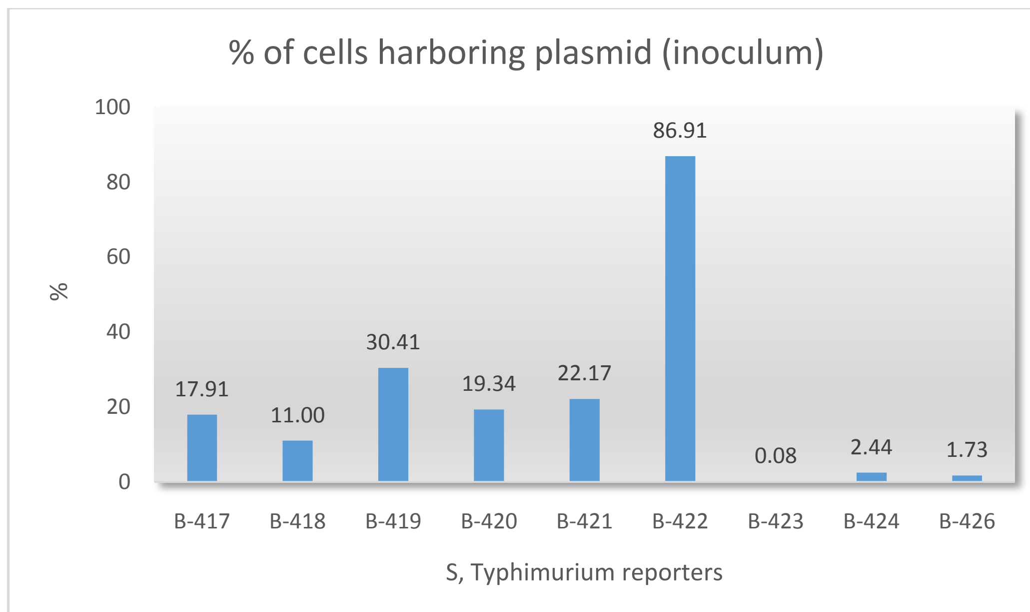

Figure 3.7: Percentage of *S.* Typhimurium cells harboring pRH008 plasmid.

It is noteworthy that plasmid is extremely unstable, and in most cases only a small part of the total population contained the plasmid despite the fact that inoculum had previously resuscitated in 100 μg/ml ampicillin.

Biofilm forming ability of *S.* Typhimurium reporters as well as plasmid stability was evaluated into 3 days and 6 days biofilms. Results regarding cell densities of *S.* Typhimurium biofilms enumerated in XLD and XLD with ampicillin as well as percentage of cells containing pRH008 are presented in Figures 3.2.8-3.2.11.

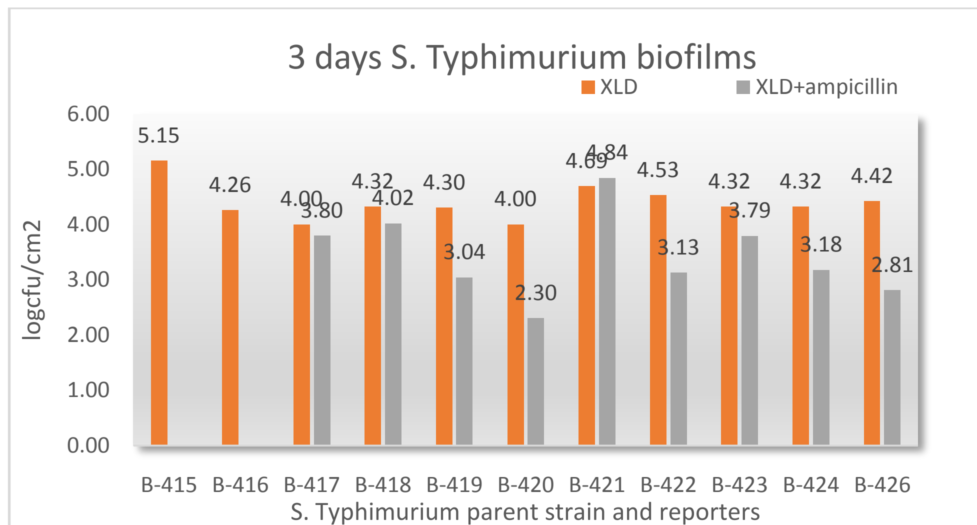

Figure 3.8: Cell density (log cfu/cm^2) in *S.* Typhimurium monospecies biofilms after 3 days of development.

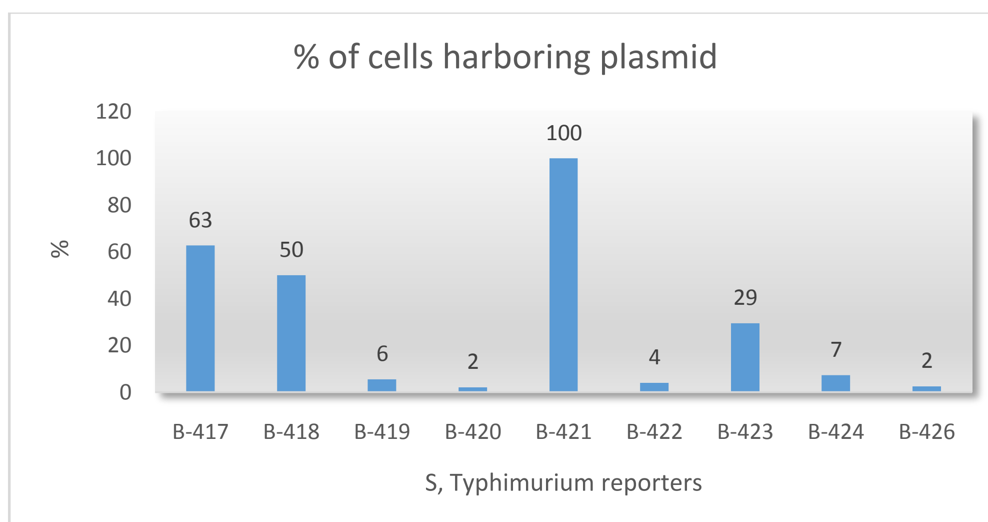

Figure 3.9.: Percentage of *S.* Typhimurium cells harboring pRH008 plasmid into 3 days biofilm

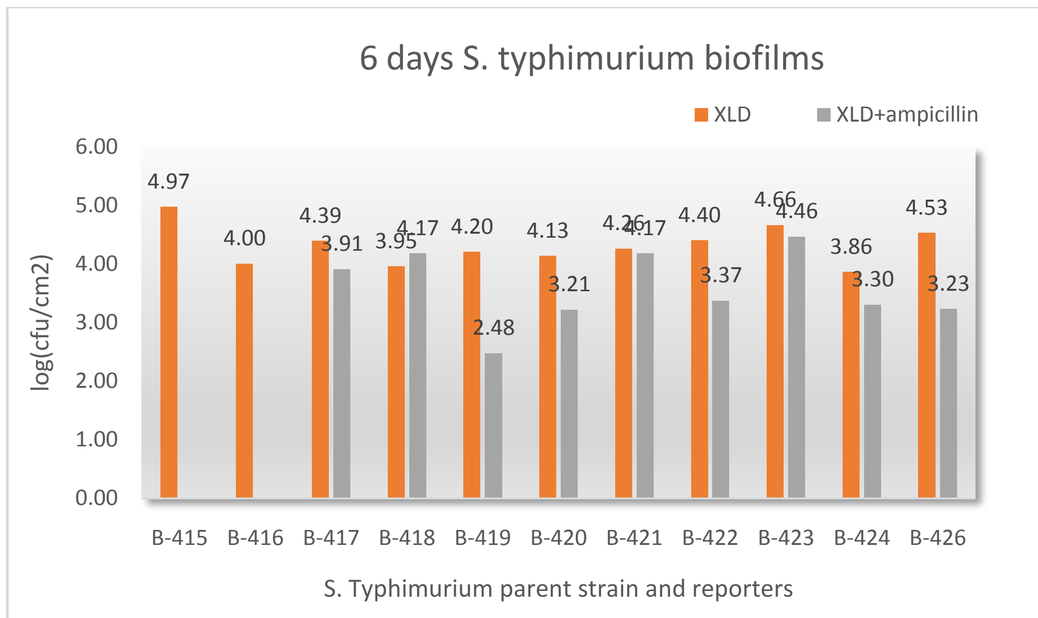

Figure 3.10: Cell density (log cfu/cm^2) in I. Typhimurium monospecies biofilms after 6 days of development.

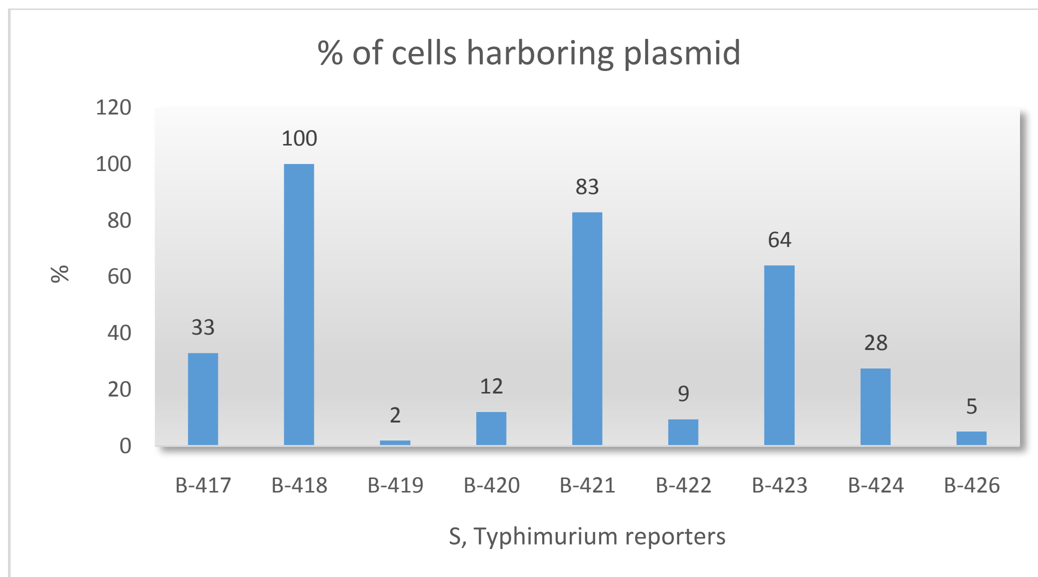

Figure 3.11: Percentage of S. Typhimurium cells harboring pRH008 plasmid into 6 days biofilm.

A reduced biofilm forming (1.2 log difference) ability of *S.* Typhimurium transformed strains compared to parent strain was observed. *S.* Typhimurium parent strain had an average population 5.64 ± 0.64 in 6 days biofilms whereas its reporters 4.26 ± 0.26. There is no reference to our knowledge demonstrating an impact of

transformation with plasmid containing reporter gene to the biofilm forming ability of the cell.

As gene expression was studied in both monospecies and multispecies biofilms it wasn't possible to develop biofilms in culture media containing ampicillin. Fortunately, this didn't have an extra negative impact on plasmid stability. Comparing percentages of cells attained plasmid pRH008 in the beginning (inoculation), middle and end of biofilm development (Figure 3.12) revealed that plasmid loss was not a result of consecutive cell divisions in medium without ampicillin but it was rather a random event. Certain strains i.e. B-419, B-420, B-422 and B-426 displayed extremely low percentages of plasmid attainment (2 to 12%) in 6 days biofilms. Other strains displayed more satisfactory percentages in 6 d biofilms i.e. B-418, B-421, B-423.

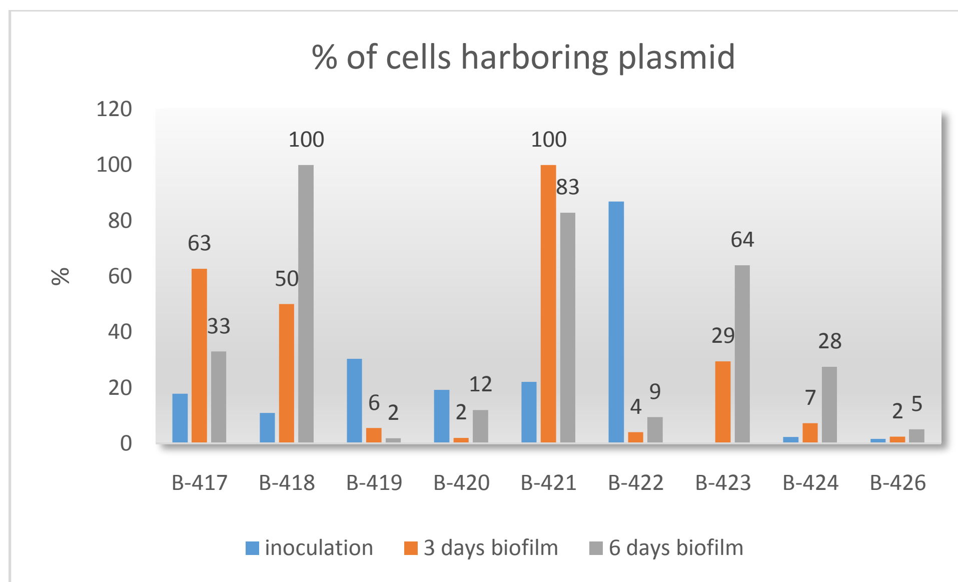

Figure 3.12: Percentage (%) of cells harboring plasmid at the initial inoculum, in 3 days and 6 days biofilm.

The average population of *S*. Typhimurium cells with reporter gene in 6 days biofilms was 3.59 ± 0.63 log cfu/cm^2. This is a relatively low cell density indicating that only few small aggregates of transformed cells will be developed on stainless steel coupons.

3.2.3 Study gene expression in 6 days *S*. Typhimurium monospecies and multispecies biofilms by fluorescence microscopy.

Study of gene expression was conducted by fluorescence microscopy. Gene expression was evaluated by recording florescence signal from individual cells where EYFP reporter gene was inducible expressed. Six days biofilms developed on stainless steel coupons by *S*. Typhimurium parent strain B-415 were stained with acridine orange fluorescent dye and biofilm structure was observed with N-400 FL Epi-fluorescence microscope (Figure 3.13).

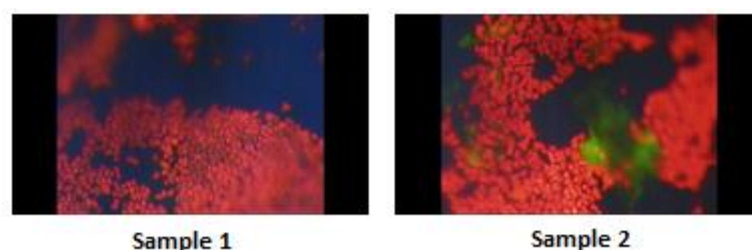

Figure 3.13: *S*. Typhimurium B-415 monospecies biofilms stained with acridine orange fluorescent dye.

Strain B-416 was transformed with GFP reporter plasmid that enable constitutive fluorescent labeling of *S*. Typhimurium cells. For this reason B-416 was considered as fluorescence marker ensuring that procedure followed each time was not inhibitory to fluorescent signaling of reporters. Illustrative photos showing expression of GFP reporter in monospecies and multispecies *S*. Typhimurium 6 days biofilms are presented in Figure 3.14.

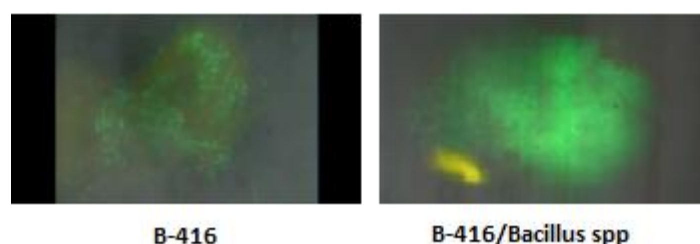

Figure 3.14: Expression of fluorescent GFP reporter in *S*. Typhimurium monospecies and multispecies biofilms

Illustrative photos showing the inducible expression of EYFP reporters in *S*. Typhimurium monospecies and multispecies 6 days biofilms are presented in Figure

3.15. B-423 harbors plasmid with sspH2 promotor, B-418 with csgB promotor and B-424 with flic promotor. In Figure 3.16 photos showing the same biofilms stained with acridine orange fluorescent dye are presented.

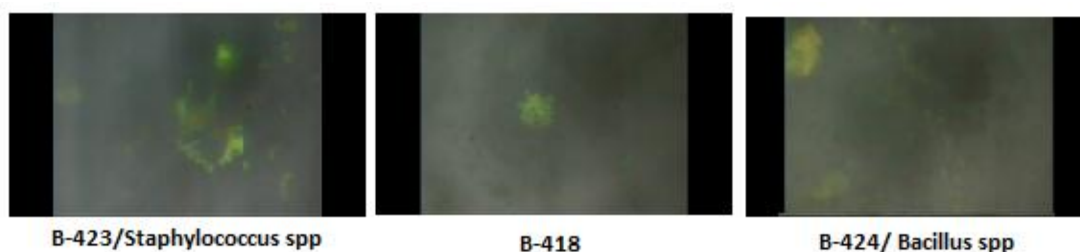

Figure 3.15: Expression of fluorescent EYFP reporters in *S*. Typhimurium monospecies and multispecies biofilms

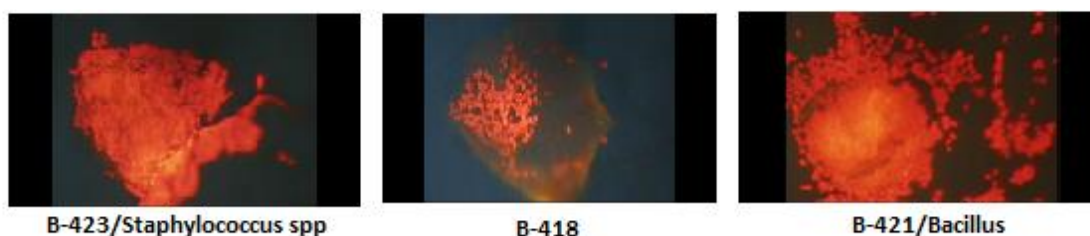

Figure 3.16: *S*. Typhimurium reporters' monospecies and multispecies biofilms stained with acridine orange fluorescent dye.

Illustrative photos cited above were some of the most discrete photos acquired during this study in regions with most fluorescent cells observed.

Three main problems were experiencing during this study. First, in some biofilms only few fluorescent cells could be detected whereas in most biofilms no fluorescent cells were observed. Second, perceived brightness of EYFP was very weak and sometimes hardly distinguished even by human eye. Third, there was a deficient imprinting of the microscope image into the computer screen leading to very bad quality photos and consequently no substantial results.

Gene expression is temporal and spatial differentiated which means that one gene can be expressed only in different stages during biofilm formation and only by a certain subpopulation of the whole biofilm. To ensure that eliminated expression of EYFP did not occur only in 6 days biofilms, 2 d., 3 d. and 8d. biofilms were also

studied. Similar almost zero EYFP expression patterns were observed. Another compilation during this study was the absence of repeatability (except for B-421) in results obtained from the same biofilms.

Although EYFP reporters were used for gene expression study, constitutive fluorescent marker was GFP. It is known (Malone et al., 2009) that EYFP is detectable with common GFP filters although they have different excitation peaks. However it is critical to choose specific, appropriate optical properties of the imaging setup in order to achieve greater brightness and photostability for a certain fluorescent protein. Unfortunately this wasn't possible due to lack of an EYFP marker and limitations of the epifluorescence microscope used. In most reported studies using fluorescent reporters in biofilms, confocal laser scanning microscopy was conducted (Yarwood et al., 2004, Malone et al., 2009). GFP has previously been reported as an effective marker in *Salmonela* studies (Corcoran et al., 2012, Laughlin et al., 2014, Hermans et al., 2011). However no reference to our knowledge have reported the efficiency of EYFP in *Salmonela* cells. Moreover transformed strain used in this study weren't verified for their efficacy with molecular techniques (pcr, restriction analysis, sequencing etc) as commonly required (Hermans et al., 2011). All statements cited above point out weaknesses of the experimental procedure however the lack of fluorescent cells should rather been attributed to the low densities of cells harboring plasmid into the biofilm.

An exception to what mentioned above was the B-421 strain. Interestingly enough, it was observed that there were many fluorescent cells in multispecies 6d biofilms with *Citrobacter* spp. (6 replications). But there were no fluorescent cells in monospecies biofilms or multispecies biofilms with another microorganism. Illustrative photos showing expression of EYFP reporter 6d multispecies biofilms are presented in Figure 3.17.

Strain B-421 possessed two very important advantages compared to other strains. First, it was demonstrated a very good plasmid stability in this strain (Figure 3.12) and second, fluorescence signal was localized in a small region into the cell results in more intense fluorescence compared to other EYFP reporters.

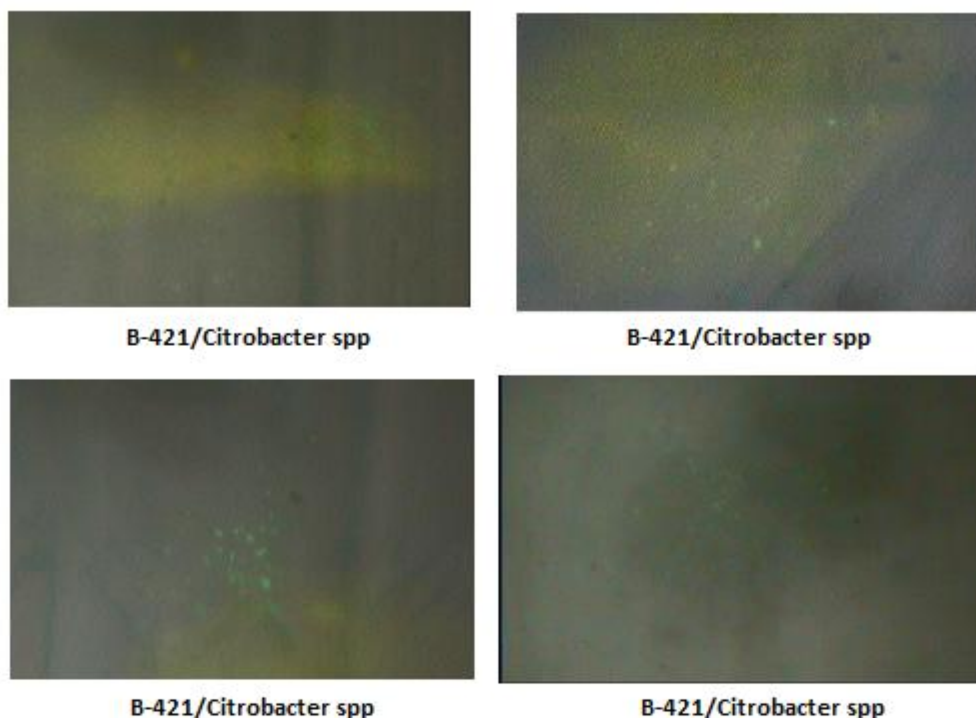

Figure 3.17: Expression of csrA EYFP reporter in *S*. Typhimurium multispecies biofilms with *Citrobacter* spp.

B-421 harbors plasmid with csrA gene promoter regulating EYFP expression. CsrA (RsmA) proteins are a family of RNA binding proteins that are widely distributed central components of the global carbon storage regulatory system (Csr) involved in the control of many cellular functions and virulence traits, like motility, quorum sensing, carbon metabolism, interaction with hosts and biofilm production (Lenz et al., 2005, Heroven et al., 2008, Brencic and Lory, 2009). Concerning biofilm formation it has been shown to repress biofilm formation post-transcriptionally by direct or indirect effects in EPS production, indirect effects by inducing motility, direct effect on levels of C-di-GMP: a central molecule switch planktonic to sessile mode and activation of biofilm dispersal (Martinez et Vadyvalo 2012). It is noteworthy that expression of this gene seems to be induced in multispecies biofilms with *Citrobacter* spp. CsrA tightly control the switch between sessility and motility in *Salmonella* cells at multiple levels (Jonas et al., 2010). The induction of CsrA expression causes release of viable planktonic cells from the biofilm, reflecting a role for CsrA in activation of biofilm dispersal. It could be presumed that biofilm

dispersion is activated early in multispecies biofilms with *Citrobacter* spp probably because of its high cell densities which cause unfavorable conditions such as starvation. CsrA also plays an important role in the regulation of *Salmonella* virulence genes so it would be of great interest if it was demonstrated that there is a *Citrobacte*r – dependent induction of Salmonella virulence factors indicating that this foodborne pathogen could be more dangerous in mixed biofilms with a common bacteria isolated by food industry. Findings concerning upregulation of csrA in mixed biofilms with *Citrobacter* spp derived from fluorescence microscopy study should be verified and further with molecular techniques such as real time PCR.

3.3 Disinfection of *Salmonella* Typhimurium planktonic cells and biofilms with hydrosol of *Thymbra Capitata* and benzalkonium chloride

3.3.1 Chemical Analysis of Essential oil and Hydrosol

Both essential oil (EO) and hydrosol of *Thymbra capitata* were analyzed chemically by gas chromatography (GC) and gas chromatography- mass spectrometry (GC-MS) to determine their qualitative composition.

The constituents were identified according their retention indices during GC and GC-MS and are presented in Table 3.2.The main constituents of *Thymbra capitata* EO according Bakhy et al., 2013, Hortigon et al., 2014, Blanco et al., 2010 were oxygenated phenolic monoterpene carvacrol, monocyclic monoterpene γ-terpinene and the alkylbenzene p-cymene. These components were also detected in the present study. In addition, other important components (Salgueiro et al., 2005., Blanco et al., 2010, Galego et al., 2008) 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 found in this analysis.

Table 3.2: Main chemical components of *Thymbra capitata* essential oil and hydrosol. The common ones are pointed in red.

Essential oil (EO) *Thymbra capitata*	**Hydrosol *Thymbra capitata***
a-pinene	ethanol
a-thujene	toluene
b-myrcene	hexanol
a-terpinene	1,4-cineol
limonene	methyl butanol
sabinene	**limonene**
(Z)-beta-ocimene	cineol1-8
g-terpinene	1-hexanol
p-cymene	3-hexen-1-ol
d 3-carene	3-octanol
3 ocbanol	nonanal
1-octen-3ol	alpha_thujonc
linalool	**1-octen-3ol**
E-caryophylene	linalool oxyde
cis dihydr carvone	**linalool**
borneol	terpin-4-ol
b-bisabolene	4-methylbenzaldehyde
carvone	beta-fenchol
a-bisabolene	**borneol**
isothymol	**carvone**
caryophylenne oxide	C18
thymol	p-cymen-8-ol
carvacrol	ethyl_hexyloctanoate
	BHT
	2 phenyl-ethanol
	phenylmethanol
	thymol
	carvacrol

The chemical composition of hydrosol of aromatic herbs which is produced in the same extraction process as essential oils, is not commonly studied. Briefly, are their chemical analysis were the subject of a limited number of publications (Paolini et al., 2008, Inouye et al., 2008, Garneau et al., 2012, Gaoming et al., 2014, Garneau et al., 2014). However, in our knowledge the chemical composition of *T. capitata* hydrosol hadn't been reported until now.

The chemical analysis of the present study reveals that *T. capitata* hydrosol is a complex mixture containing part of the essential oil components. Among the three major constituents of *T. capita* EO, only carvacrol becomes dissolved in the hydrosol during distillation. The other two major components γ-terpinene and p-cymene as well as α-terpinene, α-thujene and myrcene are absent in hydrosol and this is in accordance with Garneau et al., 2014, who reported that none of these hydrocarbon compounds of EO were observed in the hydrosol of *Melissa officinalis* and *Asarum canadense*. Most of these compounds are completely insoluble in water (US National Library of Medicine, 2014)while some others disappear quickly from the acidic hydrosol with diverse degradation mechanisms (Gaoming et al., 2014).

Except from carvacrol, the other constituents of hydrosol that are in common with EO are carvone, borneol, linalool, 1 octen-3-ol, limonene and thymol. It is noteworthy the fact that hydrosol of *T. capitata* contains carvacrol and thymol which are compounds of essential oils that have been shown to possess (either alone or especially in combination) multiple biological properties such as wide spectrum antimicrobial activity as well as antifungal, anti-inflammatory, antioxidant, hepatoprotective and anti-tumoral activities (Nostro et al., 2004, Nostro et al., 2007, Patel., 2015). Carvacrol and thymol are phenols that are slightly soluble in water and chemically unstable (Coimbra et al., 2015). Nevertheless hydrosol of *T. capitata* is a stable natural water solution of these two significant active components. Except from carvacrol and thymol, the constituents limonene, carvone, borneol and linalool also have biological properties and uses i.e. insecticides (Sun., 2007, Enviromental Protection Agency., 2009). Thus its possible applications in research, pharmaceutical and industrial fields are rising.

Several new oxygenated compounds as well as several unidentified compounds were observed in hydrosol, comparing to EO. Similar findings were

reported by other researchers concerning hydrosols of different plants (Paolini et al., 2008,Garnaeu et al., 2012 and Gaoming et al., 2014). Specifically 1-hexanol, 3-hexen-1-ol, linalool oxide, terpin-4-ol, and p-cymen-8-ol are compounds that are also found in the hydrosols of *Melissa officinalis* and*Asarum canadense* (Garneau et al., 2014).Many ofthese components are volatile, water soluble, second metabolites derived from chemical reactions occurring during hydro distillation or extraction procedure before GC. For example, during the hop steam distillation linalool is transformed into isomeric furan and pyran linalool oxides (Garneau et al., 2014).

It has been reported that, it is of great importance to carry out analysis by GC equipped with flame ionization detector (GC-FID) following by GC-MS in order to determine the exact % composition of both essential oil and hydrosol taking into consideration that composition of hydrosol may be altered during storage (Garneau et al., 2014). As the significant bactericidal effect of hydrosol will be demonstrated subsequently in this study, it is necessary to consider the concentration of key compounds like carvacrol or thymol to compare them with concentrations of respective EO.

3.3.2 Disinfectant tests on planktonic cells and stainless steel coupons

Disinfectant efficacy of BC and *T. capitata* hydrosol was tested against 24 h *S.* Typhimurium planktonic cells and mono species biofilms on stainless steel coupons, by determining the logarithm reduction in the number of viable cells achieved after a 6 min treatment. In brief, the logarithm reduction was calculated by the difference between the log_{10} survivors counted after treatment with deionized water (control) and log_{10} survivors after treatment with the antimicrobial agent.

Regarding the obtained results, a density of 7.47 ± 0.46 log (cfu/cm$^{2)}$) was attained in *S.*Typhimurium ATCC 14028 biofilms after 24 h of development. The cell suspension density was adjusted to the same population level 7.96 ± 0.23 log (cfu/ml) in order to determine planktonic cell resistance so that the eradication concentrations could be compared in both states (biofilm and planktonic).Log_{10} survivors of planktonic and biofilm cells after treatment with BC and *T. capitata* hydrosol counted by 6X6 drop method are presented in Table 2,3,4,5 Annex A.

Log reductions (log cfu/ml) of planktonic cells achieved after 6 min exposure to different concentrations of BC and *T. capitata* hydrosol are presented in the following Figure 3.18. The bars represent the mean values ± standard errors (n=6, three independent experiments, each performed two times).

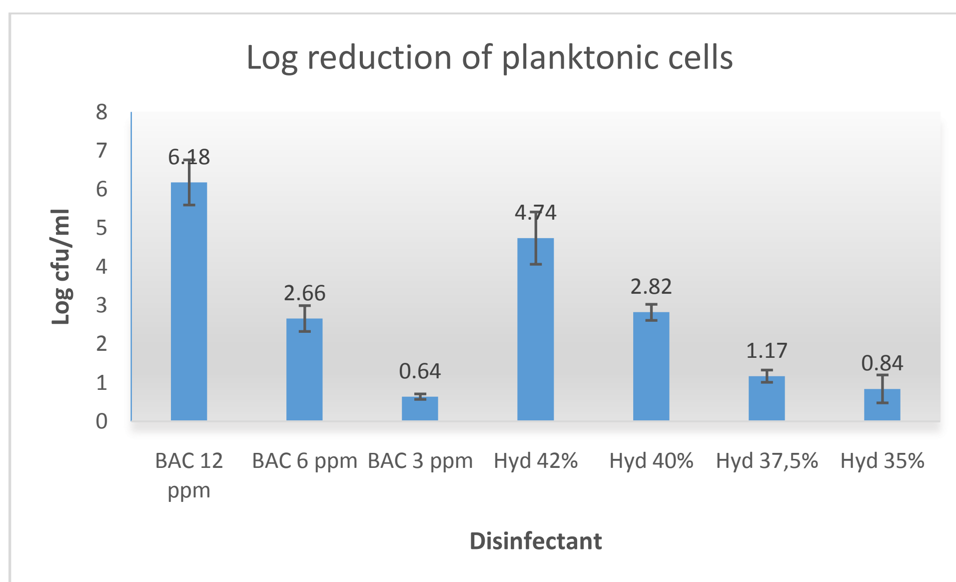

Figure 3.18: Log reductions (log cfu/ml) of planktonic cells after 6 min treatment with BAC and *T. capitata* hydrosol

BC 22 ppm as well as hydrosol 45 % were also used in disinfectant tests but resulted in log-reductions at levels below the detection limit of the plate counting method (1 log cfu/ml). With these concentrations complete eradication (>7log cfu/ml) of planktonic cells was achieved.

Despite numerous studies have been associated with the bactericidal activity of biocides, it is often difficult to compare results because of differences in test methods and strains. In the present study, approximate 6, 2.5 and 0.5 log reductions were achieved with the application of 12 ppm, 6 ppm and 3 ppm BC respectively. In a previous study, it had been reported that *Salmonella* planktonic cells failed to grow when BC was applied at recommended user (0.07%) following incubation for 5 min (Wong et al., 2010). In the present study, it was demonstrated that complete eradication of *S.* Typhimurium planktonic cells can also be achieved after 6 min exposure with a lower concentration of BC (22 ppm = 0.0022%). According to a

previous study (Bridier et al., 2011b) 5 min treatment with 42, 22 and 13 ppm resulted in 5, 3 and 2 log reductions of *Salmonella enterica* ser S24 (St. Paul),respectively. The strain used in the present study, seems to be more susceptible to BC than *Salmonella enterica* ser S24, although this can't be safely presumed as slightly different methods were followed (i.e. overnight culture instead of 24 h culture). Likewise, Mangalappalli-Illathu et al., 2008 showed that 5 log reduction of *Salmonella enterica* Serovar Enteritidis planktonic cells could be achieved with a relatively high concentration of BC (30 ppm)but after 10 min treatment.

In the case of *T. capita* hydrosol, approximate 5, 3 and 1 log reductions were achieved with 42 %, 40 % and 37,5 % (v/v), respectively. It has already been reported the antimicrobial activity of *T. capita* essential oil against *Listeria monocytogenes* (MIC[*1]= 0.05-0.2 μl/ml) , multidrug resistant *Enterococcus faecalis* (MIC=0.063% v/v), *Candida* spp preformed biofilms (MIC = 0.32 μl/ml) and *Yiardia* spp. (IC_{50} [*2]=71 μg/ml) (Faleiro et al., 2005, Benbelaïd et al., 2014, Palmeira-de-Oliveira et al., 2012, Machado et al., 2010). MIC is the minimum inhibitory concentration whereas IC_{50} is the Concentration that inhibit growth at 50%.

As previously mentioned in chapter 1.4.2.1 of this study, information on the evaluation of different hydrosols as disinfectants are available (Chorianopoulos et al., 2011,Saodic 2003, Saodic et Ozcan 2003, Verma et al., 2011 and Tornuk et al., 2011). However in our knowledge, the antimicrobial action of *T. capita* hydrosol fraction has not been recorded yet. In the present study, it was demonstrated that *T. capita* hydrosol was bactericidal against *S.* Typhimurium planktonic cells even at low concentrations (42% (v/v) resulted in 5 log reduction).These antibacterial properties can be attributed either to a presumably high content of carvacrol or to the synergistic action of several active compounds (carvacrol, thymol, linalool etc) (Bakali et al., 2008). However, the quantification of its chemical composition seems to be necessary for a more sufficient conclusion.

Except from planktonic cells, disinfectant efficacy was also tested against *S.* Typhimurium biofilms on stainless steel coupons. Log reductions (log cfu/cm^2) of 24 h biofilm cells achieved after 6 min exposure to different concentrations of BC and *T. capitata* hydrosol are presented in Figure 3.19. The bars represent the mean values ± standard errors (n=6, three independent experiments, each performed two times).

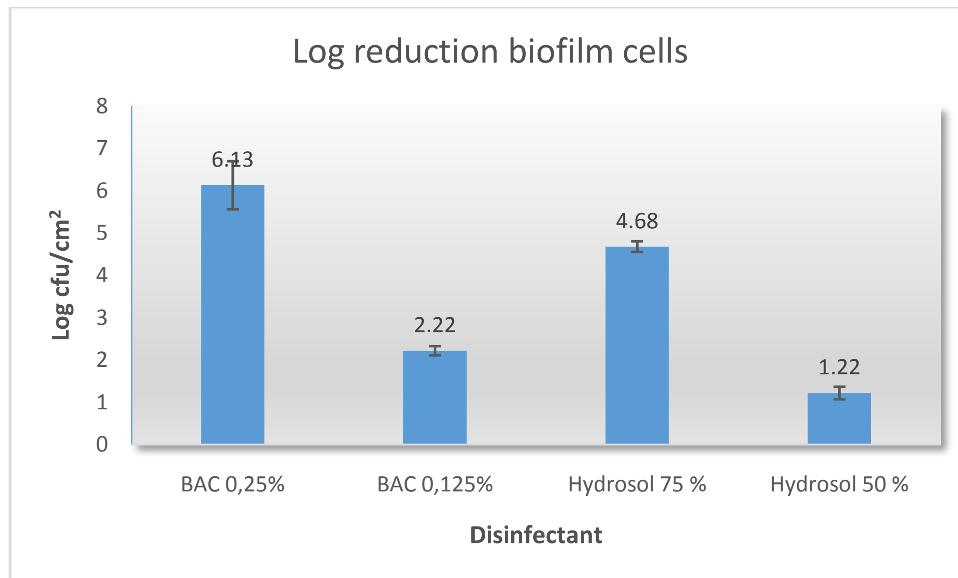

Figure 3.19: Log reductions (log cfu/cm^2) of biofilm cells after treatment with BAC and *T. capitata* hydrosol during 6 min

BC 0.5 % as well as hydrosol 100 % were also used in disinfectant tests but resulted in log-reductions at levels below the detection limit of the plate counting method (1 log cfu/ml).. The application of these concentrations resulted in complete eradication of biofilm cells (> 7log(cfu/ml)).

BC recommended user concentration is 0.07% (v/v) (Wong et al., 2010) whereas according Gaulin et al., 2011 typical sanitizing concentration of Quaternary ammonium compounds ranges from 0,02% to 0,1%. (v/v) (generally used at 0.02% (v/v). In the present study, it was demonstrated that BC 0.125% has an inadequate bactericidal effect on *S.* Typhimurium biofilm cells because it results only in a 2 log reduction whereas a sufficient 6 log reduction was achieved with BC 0.25%. Similarly, according to Corcoran et al., 2013, 0.02% BC failed to eradicate 48 h *S.* Typhimurium biofilm on concrete (only 0.22 log reduction). Wong et al., reported that 3 days *S.* Typhimurium biofilms were eradicated with BC 0.75 %. These findings along with results from the present study point out that BC which is a very common industrial disinfectant is not effective against *S.* Typhimurium biofilms at recommended or generally used concentrations. This constitutes a common phenomenon for many industrial sanitizers as efficiency testing of disinfectants such as european EN 1040 method, Koch's method, Rideal–Walker method and Kelsey–Sykes, all utilize planktonic bacterial cultures which do not reflect the efficacy against

bacteria in the biofilm state. However use of BC at high concentrations required for biofilm disinfection cannot be easily applied in food industry. BC maximum residue level (MRL) for food and feed laid down in Regulation (EC) No 396/2005 was enhanced from 0.1 mg/kg to 0.5 mg/kg (June 2014) because of unexpected exceeding levels of BC residues. Moreover 0.1% is the maximum concentration of BAC that does not produce primary irritation on intact skin or act as a sensitizer (Seymour et al., 2001)

T. capitata hydrosol has a significant antibiofilm action (complete eradication with 100% (v/v), approximate 5 log reduction with 75% (v/v). Chorianopoulos et al., (2011) have shown antibacterial action of *Satureja thymbra* hydrosol against monospecies and multispecies *Pseudomonas putida*, *Salmonella enterica* and *Listeria monocytogenes* 5 days biofilms. In our knowledge, this is the only reference reporting antibiofilm action of hydrosol. *T. capitata* and *S. thymbra* are similar species which belong to the same family *Lamiaceae* (also called Labiatae). In the present study, not only antibiofilm action of a different hydrosol was proven but also important further information was demonstrated. Although, it is difficult and often misleading to compare different studies, according this study lower exposure time is actually required for the complete eradication of biofilm compared to Chorianopoulos et al., 2011 (6 min instead of 60 min) and a lower dose (75% (v/v) instead of 100% (v/v)) was also sufficient.

Comparing disinfectant tests against planktonic cells and biofilms, the resistance coefficient (Rc) was determined for BC and *T. capitata* hydrosol. 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. Rc for the two disinfectants used in this study are presented in the Table 3.3.

Table 3.3: Resistance coefficient (Rc) for BC and *T. capitata* hydrosol

Resistance coefficient					
	Log reduction (approximate values)	$C_{biofilm}$	$C_{planktonic}$	Rc	AVG Rc
BC	6	0,25%	12 ppm	208,33	208,33
	2,5	0,125%	6 ppm	208,33	
T. capitata hydrosol	5	75%	42%	1,79	1,56
	1	50%	37,5%	1,33	

Hydrosol Rc is much lower than BC Rc, showing that hydrosol is almost equally active against planktonic and biofilm cells whereas BC need to be 200 times more concentrated to have the same effect on biofilm cells as planktonic. According Bridier at al., 2011a depending on the species and the biocide considered, Rc values could range from 1 to 1000. Especially for BC, Rc values ranges from 10 to 1000 but in most cases it was >50. More recently Bridier et al., 2011c demonstrated that Rc for BC 0.5% against *Pseudomonas aeruginosa* biofilms was 100. Among all biocides reported by Bridier et al., 2011a only hydrogen peroxide and phenol exhibited the minimum Rc=1. Biocides with the second lowest Rc(=4) were of plant origin (oregano, carvacrol, thymol and eucalyptus oil). It is noteworthy that hydrosol had a lower Rc than its main components carvacrol or thymol alone (however testing in different species biofilms) revealing a very promising antibiofilm agent.

3.3.3 Real time visualization of disinfectant activity in 24 h *S.* Typhimurium biofilms by confocal laser scanning microscopy.

Confocal laser scanning microscopy (CSLM) constitutes an innovative optical microscopy technique which in combination with improvements in fluorescent labeling, has provided the opportunity for the direct investigation of biocide reactivity within thenative structure of biofilms (Bridier et al. 2011c). *S.* Typhimurium biofilms were stained with an esterase marker that can penetrate passively into the cell where it

is cleaved by cytoplasmic esterases, leading to the intracellular release of fluorescent residues (fluorescein, having a green fluorescence when excited at 488 nm). As soon as cell's membrane permeabilization occurs fluorescence is lost, representing cell's death. This staining proved to be stable for several hours with *Salmonella enterica* and other Gram negative species (Joux et al 2000). The three- dimensional structure (5 sections) of the biofilm was scanned by CLSM at 15 sec intervals during exposure to the biocides.

During control experiments (treatment with distilled water), we observed a loss of fluorescence of less than 10% ± 3% of initial fluorescence, after 25 min of treatment. Illustrative experiments showing the spatial and temporal patterns of fluorescence loss in 24 h *S.* Typhimurium biofilms treated with BC 0.5% and three different concentrations of *T. capitata* hydrosol are presented below.

Using GinaFIT add-in, the "shoulder log-linear tail", "log-linear tail", or "log-linear" inactivation models were fitted to the fluorescence intensity curves obtained from the CLSM during biocide treatment. Two inactivation kinetic parameters were determined: Sl, the shoulder length (min) that corresponded to the length of the lag phase, and kmax, the inactivation rate (min-1).

Kinetics of biofilm eradication by T. capitata hydrosol 100% (v/v)

Projection image as well as structure parameters of the biofilm part where the bactericidal activity was studied during the disinfectant challenge is presented in Figure 3.20 and Table 3.4.

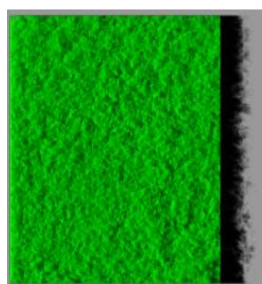

Figure 3.20: Two dimensional projection of the three dimensional S. Typhimurium biofilm structure derived from the initial xyz stack (z-step 1 µm)

Table 3.4: Quantification of biofilm main structure parameters (ICY community platform)**.**

Biovolume	Vol/Surf	Thickness Max	Thickness Avg
(μm^3)	(μm)	(μm)	(μm)
853172.2	15.04996	45	25.68379

Spatial and temporal pattern of fluorescence loss in cell clusters treated with hydrosol 100% (v/v) is presented in Figure 3.21. Each image corresponds to the 2 dimensional (D) projection of the 3D biofilm structure (5 sections) after 0, 1.75, 3.5, 5.25, 7, 8.75, 10.5 and 12.25 min of biocide addition.

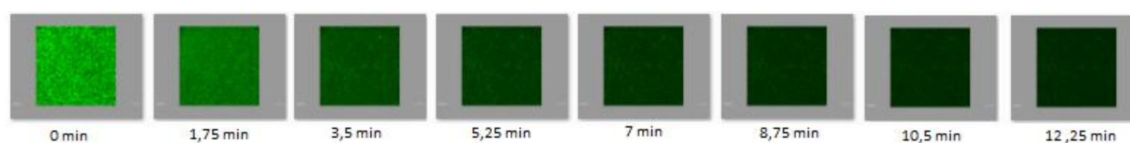

Figure 3.21: Visualization of Chemchrome V6 fluorescence loss (cell membrane permeabilization) in *S.* Typhimurium biofilms during treatment with *T. capitata* hydrosol 100% (v/v).

In Figure 3.22the fluorescence intensity of the 2D max projection of the 3 dimensional biofilm structure is presented. The fluorescence intensity curves in Figure 3.6 correspond to the intensity recorded at four different sections into the biofilm measured from the surface during treatment. The "log-linear tail" GInaFIT inactivation model (solid line) was applied to the experimental data (distinct points).

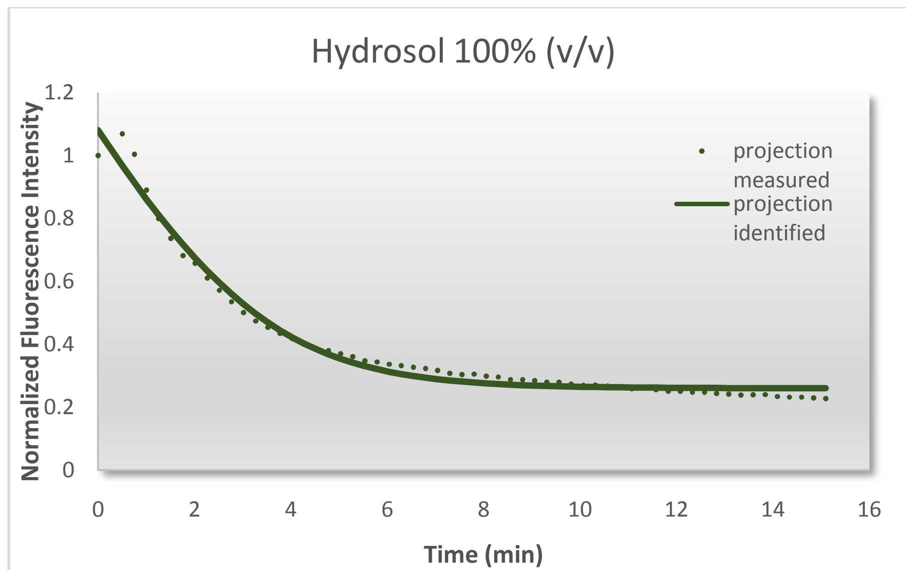

Figure 3.22: Quantification of fluorescence intensity during *T. capitata* hydrosol 100% (v/v) treatment. The values shown represent the loss of fluorescence at the max projection of the 5 biofilm sections. The inactivation parameter kmax (inactivation rate) was obtained after fitting GInaFIT inactivation model (kmax = 0.63 $R^2 = 0.98$)

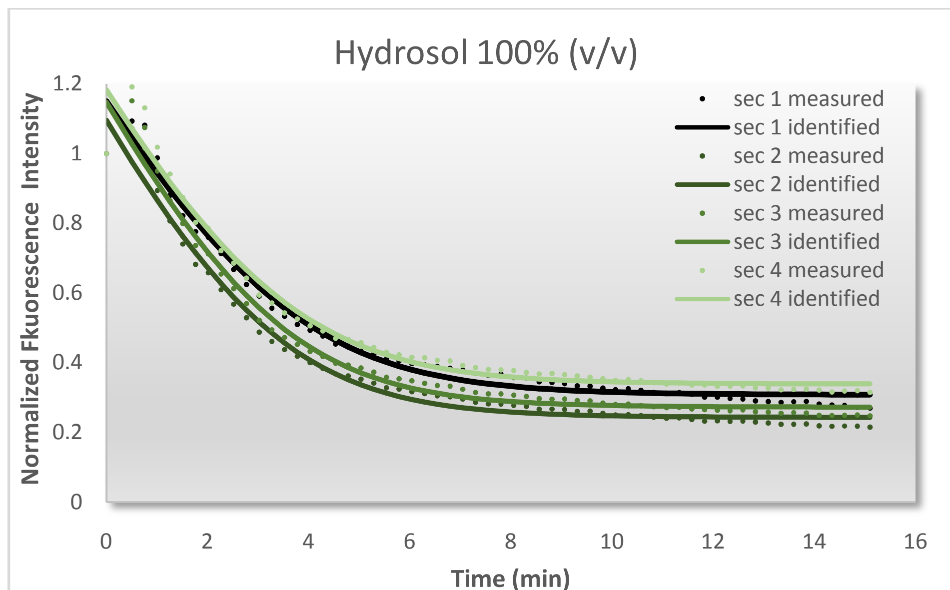

Figure 3.23: Quantification of fluorescence intensity during *T. capitata* hydrosol 100% (v/v) treatment. The values represent the loss of fluorescence at four sections: 1 (0 µm, bottom), 2 (11.25 µm), 3 (22.5 µm) and 4 (33.75 µm) into the biofilm. Inactivation rate kmax, was obtained after GInaFIT modeling (**sec 1**: kmax = 0.58, $R^2 = 0.98$, **sec 2**: kmax = 0.64, $R^2 = 0.98$, **sec 3**: kmax = 0.64, $R^2 = 0.98$, **sec 4**: kmax = 0.60, $R^2 = 0.97$)

T. capitata hydrosol 75% (v/v)

Respective results obtained from treatment with hydrosol 75 % are presented in Figure 3.24-3.27.

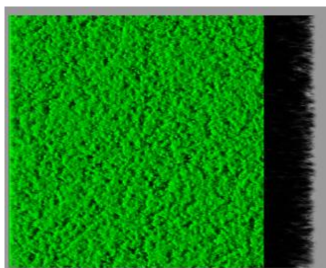

Figure 3.24: Two dimensional projection of the three dimensional S. Typhimurium biofilm structure derived from the initial xyz stack (z-step 1 μm).

Table 3.5: Quantification of biofilm main structure parameters (ICY community platform)**.**

Biovolume	Vol/Surf	Thickness Max	Thickness Avg
($μm^3$)	(μm)	(μm)	(μm)
873367.6	15.4062	56	30.82846

Figure 3.25: Visualization of Chemchrome V6 fluorescence loss (cell membrane permeabilization) in *S.* Typhimurium biofilms during treatment with *T. capitata* hydrosol 75% (v/v).

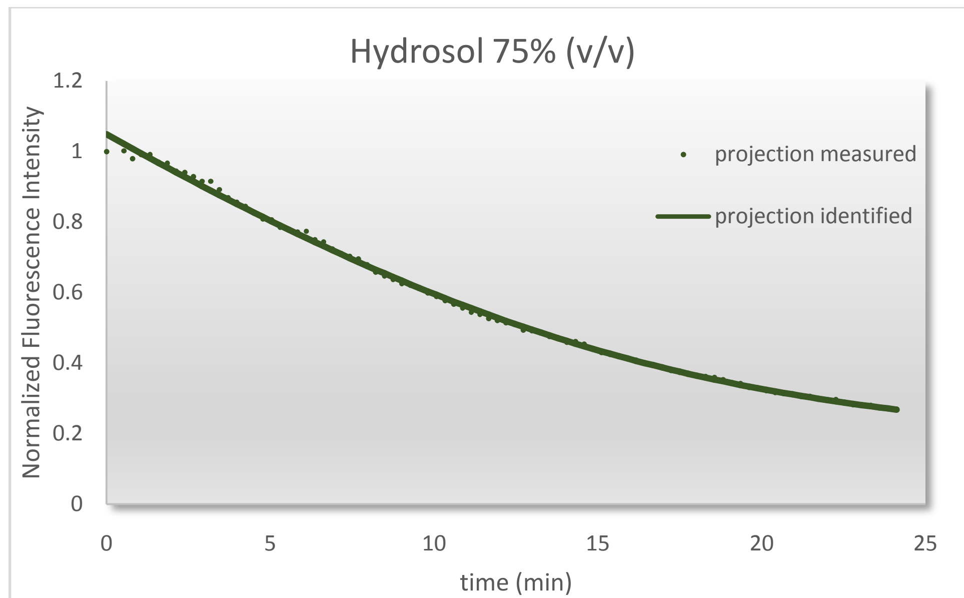

Figure 3.26: Quantification of fluorescence intensity during *T. capitata* hydrosol 75% (v/v) treatment. The values shown represent the loss of fluorescence at the max projection of the 5 biofilm sections. Inactivation rate kmax was obtained after GInaFIT modeling (kmax = 0.14 R^2 = 0.99, SI=0 R^2 = 0.99)

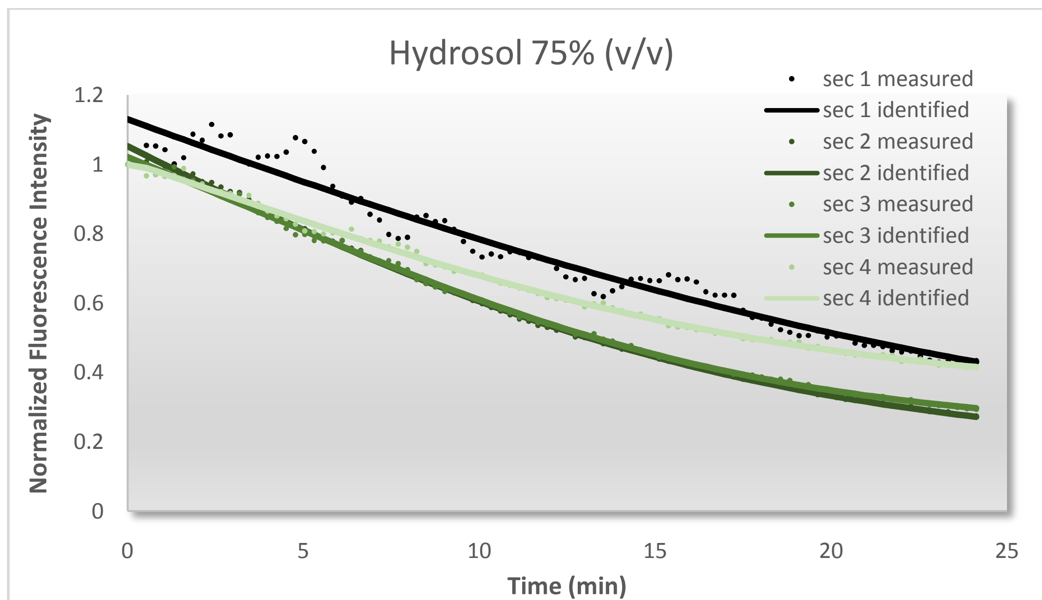

Figure 3.27:. Quantification of fluorescence intensity during *T. capitata* hydrosol 75% (v/v) treatment. The values represent the loss of fluorescence at four sections: 1 (0 µm, bottom), 2 (14 µm), 3 (28 µm) and 4 (42 µm) into the biofilm. Inactivation rate kmax, was obtained after GInaFIT modeling (**sec 1**: kmax = 0.10, R^2 = 0.96, **sec 2**: kmax = 0.14, R^2 = 0.99, **sec 3**: kmax = 0.13, R^2 = 0.99, **sec 4**: kmax = 0.11, R^2 = 0.99)

T. capitata hydrosol 50% (v/v)

Respective results obtained from treatment with hydrosol 50% are presented Figure 3.28-3.31.

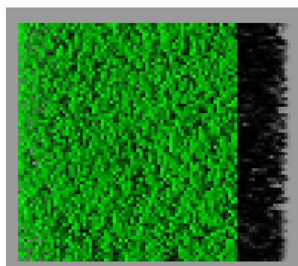

Figure 3.28: Two dimensional projection of the three dimensional S. Typhimurium biofilm structure derived from the initial xyz stack (z-step 1 μm).

Table 3.6: Quantification of biofilm main structure parameters (ICY community platform)**.**

Biovolume	Vol/Surf	Thickness Max	Thickness Moy
(μm^3)	(μm)	(μm)	(μm)
613423.2	10.82079	64	30.2802

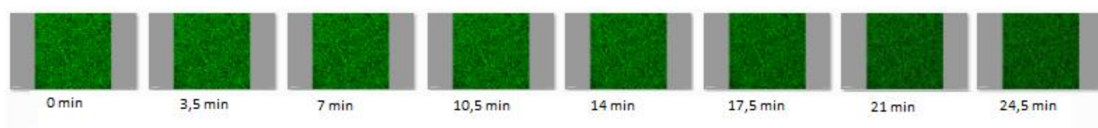

Figure 3.29: Visualization of Chemchrome V6 fluorescence loss (cell membrane permeabilization) in *S.* Typhimurium biofilms during treatment with *T. capitata* hydrosol 50% (v/v).

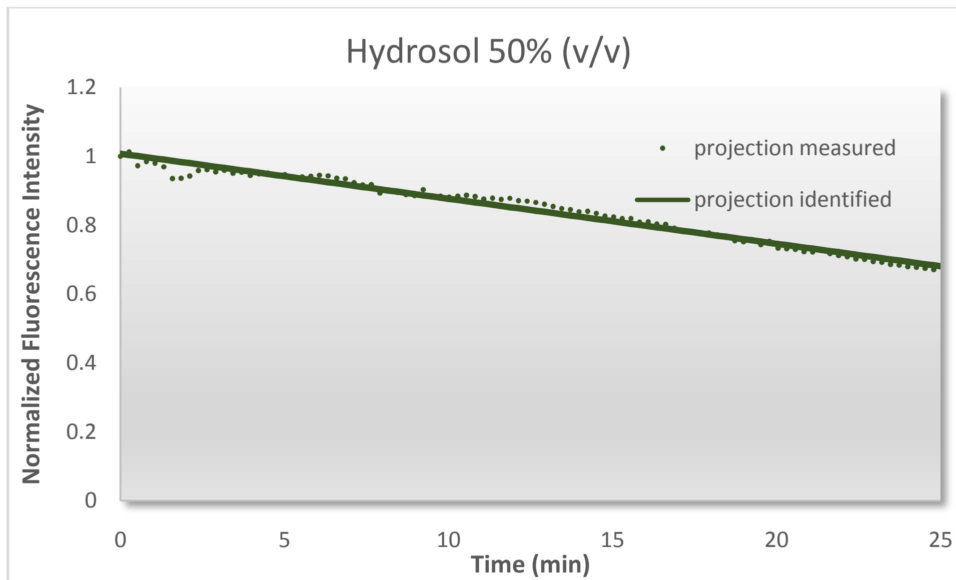

Figure 3.30: Quantification of fluorescence intensity during *T. capitata* hydrosol 50% (v/v) treatment. The values shown represent the loss of fluorescence at the max projection of the 5 biofilm sections. Inactivation rate kmax was obtained after GInaFIT modeling (kmax = 0.03 R^2 = 0.98)

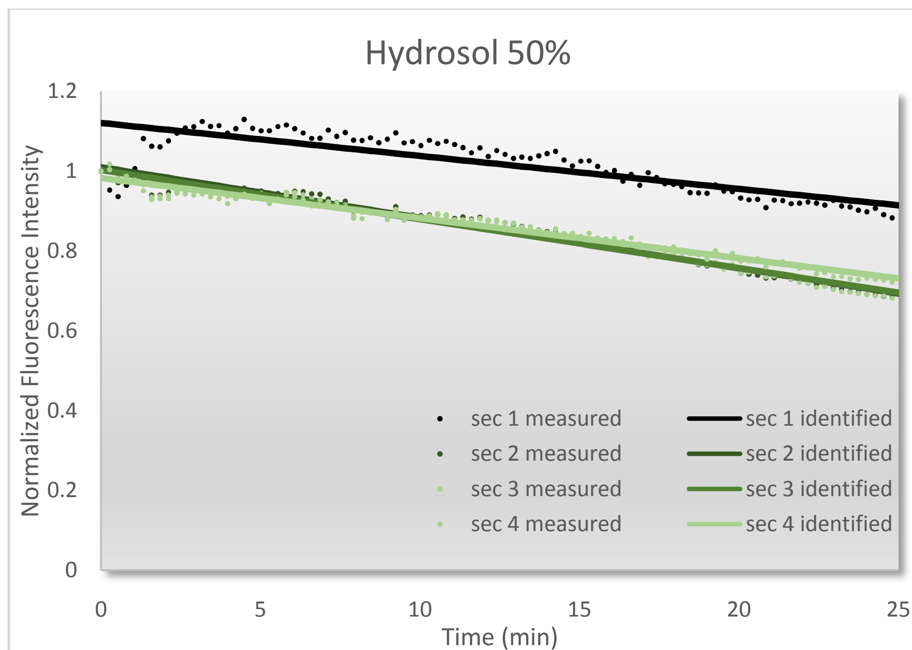

Figure 3.31: Quantification of fluorescence intensity during *T. capitata* hydrosol 50% (v/v) treatment. The values represent the loss of fluorescence at four sections: 1 (0 µm, bottom), 2 (16 µm), 3 (32 µm) and 4 (48 µm) into the biofilm. Inactivation rate kmax, was obtained after GInaFIT modeling (**sec 1**: kmax = 0.02, R^2 = 0.98, **sec 2**: kmax = 0.03, R^2 = 0.98, **sec 3**: kmax = 0.03, R^2 = 0.97, **sec 4**: kmax = 0.02, R^2 = 0.97)

BC 0,5% (v/v)

Respective results obtained from treatment with BAC 0.5 % are presented in Figures 3.32-3.35.

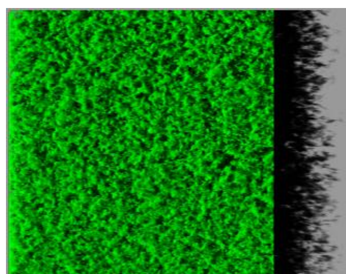

Figure 3.32: Two dimensional projection of the three dimensional *S.* Typhimurium biofilm structure derived from the initial xyz stack (z-step 1 μm)

Table 3.7: Quantification of biofilm main structure parameters (ICY community platform)

Biovolume	Vol/Surf	Height Avg	Height Max
(μm^3)	(μm)	(μm)	(μm)
752843.9	13.28017	35.92827	68

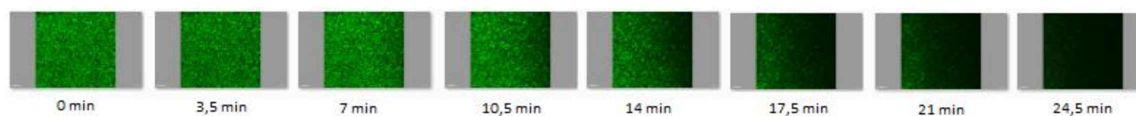

Figure 3.33: Visualization of Chemchrome V6 fluorescence loss (cell membrane permeabilization) in *S.* Typhimurium biofilms during treatment with BC0.5% (v/v).

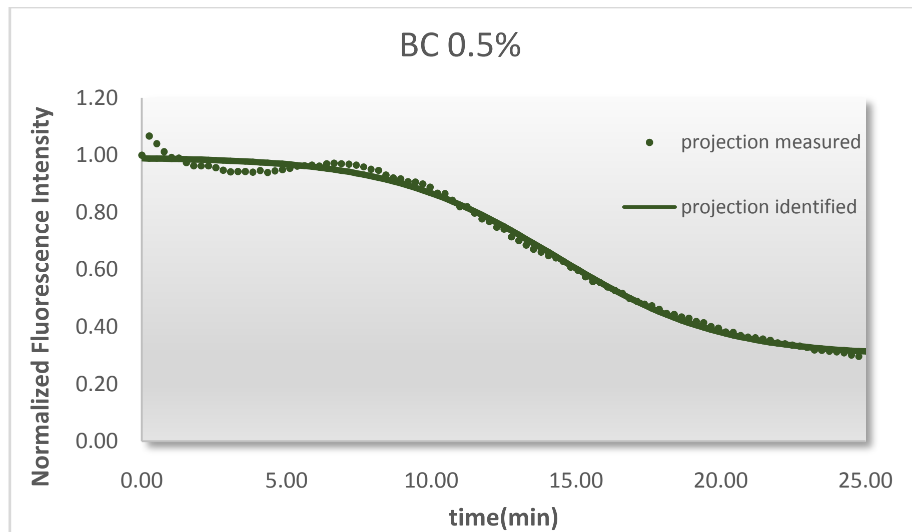

Figure 3.34: Quantification of fluorescence intensity duringBAC 0.5% (v/v) treatment. The values shown represent the loss of fluorescence at the max projection of the 5 biofilm sections. "Shoulder log linear tail" GInaFIT inactivation model was applied in this case. Two inactivation parameters, Sl (shoulder length) and kmax (inactivation rate), were obtained after GInaFIT modeling (SI=12.15 kmax = 0.37 R^2 = 0.99)

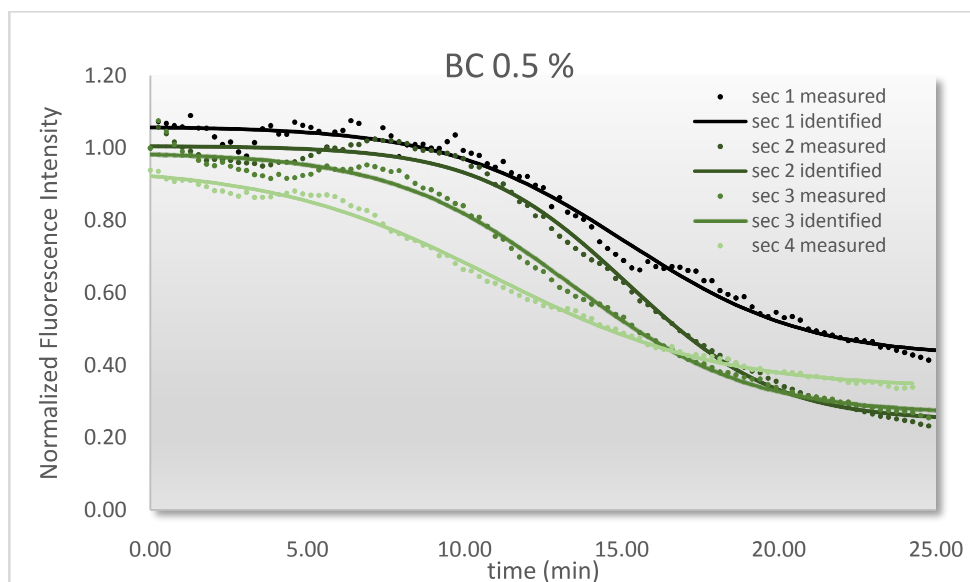

Figure 3.35: Quantification of fluorescence intensity during BAC 0.5% (v/v) treatment. The values represent the loss of fluorescence at four sections: 1 (0 µm,

bottom), 2 (17 μm), 3 (34 μm) and 4 (51 μm) into the biofilm. Shoulder length SI and inactivation rate kmax, were obtained after GInaFIT modeling (**sec 1**: SI=13.14, kmax = 0.36, R^2 = 0.99, **sec 2**: SI=13.23 kmax = 0.45, R^2 = 0.99, **sec 3**: SI=11.14, kmax = 0.37, R^2 = 0.99, **sec 4**: SI=9.79, kmax = 0.02, R^2 = 0.97)

Comparative study of the illustrative experiments presented above as well as results from the statistical analysis of the inactivation parameters regarding all repetitions for each case, demonstrated in Table 3.8, for the two biocides revealed some very interesting findings.

Table 3.8: Inactivation parameters determined for each disinfectant and its respective concentrations after statistical analysis (JMP v8.0 software) of results by all repetitions.Mean values are presented ± standard errors.

Disinfectant		kmax	SI
Hyd 100%	Max projection	0.82±0.09	0.2±0.13
	Sec 1	0.82±0.09	0.56±0.13
	Sec 2	0.85±0.09	0.28±0.13
	Sec 3	0.84±0.09	0.26±0.13
	Sec 4	0.84±0.09	0.27±0.13

Hyd 75%	Max projection	0.24± 0.06	3.44±2,79
	Sec 1	0,25± 0.06	7.04±2,79
	Sec 2	0.25± 0.06	3.69±2,79
	Sec 3	0.24± 0.06	3.59±2,79
	Sec 4	0.21± 0.06	5.05±2,79
Hyd 50%	Max projection	0.03±0.006	
	Sec 1	0,02±0.006	
	Sec 2	0.03±0.006	
	Sec 3	0,03±0.006	
	Sec 4	0,02±0.006	
BC 0.5%	Max projection	0.51±0.09	10,7±1.15
	Sec 1	0.64±0.09	11.34± 1.67
	Sec 2	0.57±0.09	10.93±1.67
	Sec 3	0.48±0.09	10.5±1.71
	Sec 4	0.37±0.09	11.53±1.89

SI for BC (10.7 ± 7, R^2=0.98 ±0.08) was significantly different and markedly higher than the almost 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 (no significant difference between experiments and replications). 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). As far as inactivation rate, k_{max} for BC (0.51 ± 0.40, R^2=0.98 ±0.08) was statistical significant lower than k_{max} for hydrosol (0.82 ± 0.36, R^2=0.99 ±0.04).

In the present study, no significant difference was recorded in SI at the different sections inside the biofilm. Nevertheless, in the 1st section, mean SI (11.34 ± 1.7) was a little higher that 2nd (10.93 ± 1.7) which was a little higher than 3rd (10.58 ±

1.7) implying a slightly more effective action in the periphery than in the bottom of the biofilm. No significant difference was observed in k_{max} values at different biofilm sections.

Comparing the three different concentrations of hydrosol, there was significant difference in both k_{max} and SI values. Hydrosol 50% didn't exhibit sufficient bactericidal activity as it had an almost zero inactivation rate (0.03 ± 0.02). Hydrosol 75% had a low k_{max} (0.24 ± 0.17) but also a relatively low SI (3.44 ± 1.9) compared to 10.7 of BC 0.5%. No significant difference was observed in k_{max} and SI at different biofilm sections in all cases.

Spatial information obtained from CSLM analysis is of great importaance because biofilms are known for their structural and physiological heterogeneity (Stewart et al., 2003, Rani et al., 2007, Joux et al., 2000). Different studies have reported differential biocide action in distinct areas from the periphery to the center in *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* biofilms (Haagensen et al. 2007, Kaneko et al 2007, Davison et al 2010). Especially for BC 0.5%, it has previously been reported (Bridier et al. 2011c) a non-uniform activity pattern in the structure of *P. aeruginosa* ATCC 15442 biofilm. To date, there is no report to our knowledge about the spatial biocide action in *S. enterica* biofilms. In the present study, it was demonstrated that neither for BC nor for hydrosol there was significant difference in the inactivation kinetics at different depths into the biofilm. However there is approximately 40 sec more delay in the biocide action of BC 0.5% at each section which is deeper into the biofilm. The fact that biocides appear to act on the upstream and downstream of the cell cluster in a similar way supports the contention that inside cell clusters solute transport occurs exclusively by diffusion (Rani et al., 2005,Stewart et al., 2003). If convective transport was significant, enhanced action of the antimicrobial agent at the upstream edge of the cell cluster would be expected in comparison to the downstream edge.

Temporal information is important because it 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 will 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 will be concave down

(Dodds et al., 2000, Cogan et al., 2006). BC 0.5% exhibited high SI values which means that there is an important initial period of time 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 (Bridier et al., 2011c). Hydrosol 100% caused a uniform and direct linear loss of fluorescence in cell clusters of S. Typhimurium, suggesting that the slightly greater resistance of the biofilm compared to planktonic cells observed in this study could not be due to limitations affecting penetration of the biocide 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.

Comparing results from the two methods applied (disinfection and counting on coupons versus CLSM kinetics), some differences are found. BC 0.5 % resulted in total eradication of biofilms on SS coupons after 6 min whereas it was partially effective against biofilms on microplates. Likewise hydrosol 75% resulted in 5 log reduction in SS coupons biofilms within 6 min whereas in microplates its bactericidal activity the first 6 minutes was limited. These alterations can be attributed to the different surface used for biofilm formation which could lead to different biofilm structures and biovolumes. Disinfection kinetics can’t be followed on SS coupons because CLSM observations on this surface can only be done with an inverted microscope. Moreover, it is difficult (although technically feasible) to make the disinfection and counting from biofilms grown on microplates. Unfortunately to date, there is no universal test method to evaluate antimicrobial susceptibilities of bacterial biofilms. Consequently developing a standard method of antimicrobial testing for biofilms is essential, as it could allow valid comparisons of susceptibility data to be made.

Inactivation kinetics obtained from CSLM study as well as “Resistance coefficient” derived from planktonic and coupons disinfection suggests that *T. capita* hydrosol action is limited only by the intrinsic kinetics of inactivation and that biofilm and planktonic cells have almost similar susceptibilities. These findings are of great importance revealing an innovative, completely natural and markedly effective agent against *S.* Typhimurium biofilms. However, more safety studies based on toxicology

data should be carried out before hydrosol is widely used as biofilm disinfectants in food industries as also recommended by (Chorianopoulos et al., 2008)

4. CONCLUSIONS

Biofilm formation is an important issue in food industries constituting an important factor of cross contamination. In the present study, it was monitored the biofilm formation of *Salmonella* Typhimurium in mono- and multi-species cultures with bacteria from a bottling water plant.Among bacteria detected throughout the production line of the water bottling plant, *Citrobacter* spp. was the only one that poses concerns about microbiological quality of the water since it belongs to total coliforms group of bacteria and it was found in all production stages from the water source to the final product. The exact *Citrobacter* species should be identified and further investigated i.e. epidemiological study and pathogenicity tests. Results from the present study rather reveal the inefficiency of total coliforms as good indicators of water contamination. Although a member of total coliforms was detected it was of limited sanitary significance and it doesn't seem representative of fecal contamination as the other species identified were few and all members of the natural water microbiota.

All bacteria detected in the bottling plant demonstrated significant ability to form biofilms on stainless steel surfaces, indicating the risk of biofilm development in tanks and tubes of the water distribution network as well as presumptive food processing surfaces rinsed with this water. Regarding the behavior of the enteric foodborne pathogen *Salmonella* Typhimurium in the specific production line it was demonstrated that its biofilm forming ability was neither enhanced nor inhibited under multi-species culture conditions with indigenous bacteria. In contrast, biofilm forming ability of some indigenous bacteria (*Staphylococcus*, *Bacillus*) was suppressed when co-culture with the pathogen.

Although biofilm forming ability of the pathogen wasn't alteredsignificantly, the observation with fluorescence based bioreporters revealed that gene expression seemed to be affected in multispecies biofilms. Specifically it was observed that under certain multispecies conditions (co-culture with *Citrobacter* spp), expression of csrA gene, which is a key global regulator of a variety of processes in bacteria, was induced in *S*. Typhimurium biofilm cells. This finding is of great interest in order to find out if the induction of this gene, which is linked to biofilm dispersal, quorum sensing and virulence, is species-specific or depends on conditions prevailing in this

multispecies biofilm (i.e. high bacterial density). Howerever, further studies with the application of molecular methods are needed to confirm and further investigate this observation..

Regarding biofilm control strategies, in the present study it was demonstrated the high efficiency of *T. capita* hydrosol as an antibiofilm agent. In contrast BC, which is a common industrial disinfectant proved to have insufficient antibiofilm activity in user's recommended concentration. Both methods followed to determine bactericidal effect on biofilms led to the same conclusion. Although, antibiofilm action of hydrosol has previously been reported in the present study interesting supplementary information are given. Initially,the resistance coefficient of *T. capitata*hydrosol was determined and it was found notable low (Rc=1.56), showing that hydrosol was almost equally active against both planktonic and biofilm cells. According CSLM study of antibiofilm action it was shown that hydrosol has the ability to quickly and uniformly penetrate biofilm structure despite complex EPS matrix. Thus from the present study an innovative, completely natural and environmental safe as well as markedly effective agent against *S.* Typhimurium biofilms is presented.

It has to be noted that the precise chemical composition of hydrosol should be determined to find out if its antibacterial properties should be attributed to a presumably high content of carvacrol or to the synergistic action of several active compounds (carvacrol, thymol, linalool etc). In any case before extensive use as disinfectant in food industry, safety studies about toxicity should be carried out.

In conclusion, it is of great importance to take into consideration that bacteria survive in food processing environments mainly into biofilms which are much more resistant against environmental stresses and antimicrobial agents. Although biofilm study has come into sharp focus in recent years, more research is required to further understand the intricate mechanisms behind biofilm formation and eradication, and advanced microscopy techniques are emerging in studying these highly heterogenic and dynamic bacterial communities. A universal test method to evaluate antimicrobial susceptibilities of bacterial biofilms should be applied as it is essential for developing efficient control strategies.

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International education is our best opportunity
to turn ideologies into human aspirations,
William Fulbright (1905-1995)

5. BIBLIOGRAPHY

1. Abdallah M, Benoliel C, Drider D, Dhulster P, Chihib NE. (2014) Biofilm formation and persistence on abiotic surfaces in the context of food and medical environments. Arch Microbiol. 196(7):453-72.
2. Abulreesh, H. H. (2014). Absence of vancomycin-resistance genes in Staphylococcus aureus isolated from potable water. Journal of Public Health and Epidemiology, 6(2), 70-75.
3. Adams R. Martin, Moss O. Maurice, (2008) Food Microbiology, 3rd Edition, University of Surrey, RSC publishing, UK
4. Allen MJ, Edberg SC, Reasonar DJ (2004). Heterotrophic plate count bacteria – what is their significance in drinking water. Int. J. Food Microbiol. 92:265-274.
5. Annous, B.A.; Fratamico, P.M & Smith, J.L. (2009). Quorum sensing in biofilms: why bacteria behave the way they do. Journal of Food Science, Vol.74, No.1., pp. R24-R37
6. Anonymous, 1997. Chemical disinfectants and antiseptics—basic bactericidal activity: test method and requirements (phase 1). Ed AFNOR: NF EN 1040
7. BadgerJ. L., M. F. Stins, and K. S. Kim, (1999) Citrobacter freundii invades and replicates in human brain microvascular endothelial cells. Infection and Immunity, vol. 67, no. 8, pp. 4208–4215, 1999.
8. Bakhy K1, Benlhabib O, Al Faiz C, Bighelli A, Casanova J, Tomi F. (2013). Wild Thymbra capitata from Western Rif (Morocco): essential oil composition, chemical homogeneity and yield variability.Nat Prod Commun. 8(8):1155-8.
9. Bakkali F, Averbeck S., Averbeck D., Idaomar M. (2008) Biological effects of essential oils—a review. Food and Chemical Toxicology, 46 pp. 446–475
10. Belessi Ce, Gounadaki AS, Psomas An, Skandamis Pn (2011) efficiency of different sanitation methods on Listeria monocytogenes biofilms formed under various environmental conditions. Int J Food Microbiol 1:25.cro.2010.10.020
11. Benamara H, Rihouey C, Jouenne T, Alexandre S (2011) Impact of the biofim mode of growth on the inner membrane phospholipid composition and lipid domains in Pseudomonas aeruginosa. Biochim Biophys Acta 1:98–105.

12. Benbelaïd F, Khadir A, Abdoune MA, Bendahou M, Muselli A, Costa J., (2014) Antimicrobial activity of some essential oils against oral multidrug-resistant Enterococcus faecalis in both planktonic and biofilm state. Asian Pac J Trop Biomed.4(6):463-72
13. Bhavsar AP, Brown NF, Stoepel J, Wiermer M, Martin DDO, et al. (2013) The Salmonella Type III Effector SspH2 Specifically Exploits the NLR Co-chaperone Activity of SGT1 to Subvert Immunity. PLoS Pathog 9(7): e1003518. doi:10.1371/journal.ppat.1003518
14. Bjergbaek LA, Roslev P., (2005) Formation of nonculturable Escherichia coli in drinking water. J Appl Microbiol.99(5):1090-8.
15. Blanco J, Ruiz T, Pérez-Alonso MJ, Vázquez FM, Cases MA, Gervasini C. (2010) Chemical composition and antioxidant activity of the essential oil of Thymbra capitata (L.) Cav. in Spain. Act Bot Gall 157: 55–63
16. Bogomolnaya LM, Aldrich L, Ragoza Y, Talamantes M, Andrews KD, et al. (2014) Identification of Novel Factors Involved in Modulating Motility of Salmonella enterica Serotype Typhimurium. PLoS ONE 9(11): e111513. doi:10.1371/journal.pone.0111513
17. Bos R, van der Mei HC, Busscher HJ (1999) Physico-chemistry of initial microbial adhesive interactions—its mechanisms and methods for study. FeMS Microbiol Rev 23:179–230.
18. Boyen, F.; Eeckhaut, V.; Van Immerseel, F.; Pasmans, F.; Ducatelle, R. & Haesebrouck, F. (2009). Quorum sensing in veterinary pathogens: mechanisms, clinical importance and future perspectives. Veterinary Microbiology, Vol.135, No.3-4, (March 2009), pp.187-195
19. Brencic, A., and Lory, S. (2009). Determination of the regulon and identification of novel mRNA targets of Pseudomonas aeruginosa RsmA. Mol. Microbiol. 72, 612–632.
20. Bridier A, Dubois-Brissonnet F, Greub G, Thomas V, Briandet R. (2011c). Dynamics of biocide action in Pseudomonas aeruginosa biofilms. Antimicrob Agents Chemother 55:2648–2654.
21. Bridier A., Briandet R., Thomas V., Dubois-Brissonnet F. (2011a) Resistance of bacterial biofilms to disinfectants: a review, Biofouling: The Journal of Bioadhesion and Biofilm Research, 27:9, 1017-1032,

22. Bridier A., Briandet R, Thomas V, Dubois-Brissonnet F. (2011b) Comparative biocidal activity of peracetic acid, benzalkonium chloride and ortho phthalaldehyde on 77 bacterial strains. J Hosp Infect. 78(3):208-13
23. Bridier, A., F. Dubois-Brissonnet, A. Boubetra, V. Thomas, and R. Briandet. (2010). The biofilm architecture of sixty opportunistic pathogens deciphered using a high throughput CLSM method. J. Microbiol. Methods 82:64–70.
24. Brooks, J.D. & Flint, S.H. (2008). Biofilms in the food industry: problems and potential solutions. International Journal of Food Science and Technology, Vol.43, (August 2008), pp. 2163-2176
25. Buckingham-Meyer K, Goeres DM, Hamilton MA (2007) Comparative evaluation of biofim disinfectant effiacy tests. J Microbiol Methods 70:236–244.
26. Burmølle M, Ren D, Bjarnsholt T, Sørensen SJ. (2014) Interactions in multispecies biofilms: do they actually matter? Trends Microbiol. 22(2):84-91
27. Cabral JP. 2010. Water microbiology. Bacterial pathogens and water. Int. J. Environ. Res. Public Health 7:3657–3703.
28. Carneiro VA, dos Santos HS, Arruda FVS, Bandeira PN, Albuquerque M, Pereira MO, Henriques M, Cavada BS, Teixeira EH. (2011). Casbane diterpene as a promising natural antimicrobial agent against biofilm-associated infections. Molecules 16:190–201.
29. Cerf O, Carpentier B, Sanders P (2010) Tests for determining in-use concentrations of antibiotics and disinfectants are based on entirely different concepts: “resistance” has different meanings. Int J Food Microbiol 136:247–254.
30. Chaieb K, Kouidhi B, Jrah H, Mahdouani K, Bakhrouf A. (2011). Antibacterial activity of thymoquinone, an active principle of Nigella sativa and its potency to prevent bacterial biofilm formation. BMC Complement Altern Med 11:6.
31. Chen CY, Nace GW, Irwin PL, (2003). A 6 × 6 drop plate method for simultaneous colony counting and MPN enumeration of Campylobacter jejuni, Listeria monocytogenes, and Escherichia coli. J Microbiol Methods 55, 475–479

32. Cherchi C, Gu AZ (2011) Effect of bacterial growth stage on resistance to chlorine disinfection. water Sci Technol 64:7–13. doi:10.2175/193864710798193608
33. Chia, T.W.R.; Goulter, R.M.; McMeekin, T.; Dykes, G.A. & Fegan, N. (2009). Attachment of different Salmonella serovars to materials commonly used in a poultry processing plant. Food Microbiology, Vol.26, (May 2009), pp. 853-859
34. Chorianopoulos NG, Giaouris ED, Skandamis PN, Haroutounian SA, Nychas GJ. (2008) 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. J Appl Microbiol. 104(6):1586-96
35. Chorianopoulos, N.G.; Giaouris, E.D.; Kourkoutas, Y. & Nychas, G.-J. (2010). Inhibition of the early stage of Salmonella enterica serovar Enteritidis biofilm development on stainless steel by cell-free supernatant of a Hafnia alvei culture. Applied and Environmental Microbiology, Vol.76, No.6, (January 2010), pp. 2018-2022
36. Cloete, T. E., & Jacobs, L. (2001). Surfactants and the attachment of Pseudomonas aeruginosa to 3CR12 stainless steel and glass. Water SA, 27, 21–26.
37. Cogan, N. G. (2006). Effects of persister formation on bacterial response to dosing. J. Theor. Biol. 238:694–703.
38. Coimbra M1, Isacchi B, van Bloois L, Torano JS, Ket A, Wu X, Broere F, Metselaar JM, Rijcken CJ, Storm G, Bilia R, Schiffelers RM. (2011) Improving solubility and chemical stability of natural compounds for medicinal use by incorporation into liposomes Int J Pharm. 20;416(2):433-42
39. Corcoran CP, Podkaminski D, Papenfort K, Urban JH, Hinton JC, Vogel J. (2012) Superfolder GFP reporters validate diverse new mRNA targets of the classic porin regulator, MicF RNA. Mol Microbiol. 84(3):428-45. doi: 10.1111/j.1365-2958.2012.08031.x. Epub 2012 Mar 28.
40. Corcoran M., Morris D., De Lappe N., O'Connor J., Lalor P., Dockery P., Cormican M., (2014) Commonly Used Disinfectants Fail To Eradicate

Salmonella enterica Biofilms from Food Contact Surface Materials. Appl Environ Microbiol. 80(4):1507-14

41. Crump, JA; Mintz, ED (2010). "Global trends in typhoid and paratyphoid Fever.". Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 50 (2): 241–6.
42. D'Aoust, J-Y. and Maurer, J. (2007) *Salmonella* Species. In Food Microbiology ed. Doyle, M.P. and Beuchat, L.R. pp. 187–236. Washington DC: ASM Press.
43. Davison, W. M., B. Pitts, and P. S. Stewart. 2010. Spatial and temporal patterns of biocide action against Staphylococcus epidermidis biofilms. Antimicrob. Agents Chemother. 54:2920–2927.
44. Davison, W. M., B. Pitts, and P. S. Stewart. 2010. Spatial and temporal patterns of biocide action against Staphylococcus epidermidis biofilms. Antimicrob. Agents Chemother. 54:2920–2927.
45. de Rezende, C. E., Anriany, Y., Carr, L. E., Joseph, S. W., & Weiner, R. M. (2005). Capsular polysaccharide surrounds smooth and rugose types of Salmonella enterica serovar Typhimurium DT104. Applied and Environmental Microbiology, 71 (11), 7345 −7351.
46. Dodds, M. G., K. J. Grobe, and P. S. Stewart. 2000. Modeling biofilm antimicrobial resistance. Biotechnol. Bioeng. 68:456–465.
47. Donlan RM (2009) Preventing biofims of clinically relevant organisms using bacteriophage. Trends Microbiol 17:66–72. doi:10.1016/j.tim.2008.11.002
48. Donlan RM, Costerton Jw (2002) Biofims: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 15:167–193. doi:10.1128/CMR.15.2.167-193.2002
49. EFSA, (2015) Trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013, EFSA Journal 13(1):3991
50. ENVIRONMENTAL PROTECTION AGENCY (March 4, 2009). "Pesticide Products; Registration Application". Federal Register 74 (41): 9396–9397
51. Ercolini, D., Russo, F., Torrieri, E., Masi, P., Villani, F., 2006. Changes in the spoilage related microbiota of beef during refrigerated storage under different packaging conditions. Applied and Environmental Microbiology 72, 4663e4671.

52. Faille C1, Bénézech T2, Midelet-Bourdin G3, Lequette Y2, Clarisse M2, Ronse G2, Ronse A2, Slomianny C4. (2014) Sporulation of Bacillus spp. within biofilms: a potential source of contamination in food processing environments. Food Microbiol. 40:64-74.
53. Faleiro L, Miguel G, Gomes S, Costa L, Venancio F, Teixeira A, FigueiredoAC, Barroso JG, Pedro LG. (2005) Antibacterial and antioxidant activities of essential oils isolated from Thymbra capitata (L.) Cav. and Origanum vulgare L. J. Agric Food Chem 53: 8162–8168
54. Fiello M., Mikell Jr., Moore MT., Cooper CM., (2014) Variability in the Characterization of Total Coliforms, Fecal Coliforms and Escherichia coli in Recreational Water Supplies of North Mississippi, USA M. Bull Environ Contam Toxicol 93:133–137
55. Flemming HC, Wingender J (2010) The biofim matrix. nat Rev Microbiol 8:623–633. doi:10.1038/nrmicro2415
56. Fricker CR, Eldred BJ. 2009. Identification of coliform genera recovered from water using different technologies. Lett. Appl. Microbiol. 49:685–688.
57. Ga-Hee Ban, Dong-Hyun Kang, Hyunjin Yoon, (2015) Transcriptional response of selected genes of Salmonella enterica serovar Typhimurium biofilm cells during inactivation by superheated steam. International Journal of Food Microbiology. Vol. 192: 117-123
58. Galego L, Almeida V., Goncalves V., Costa M., Monteiro I., Matos F., Miguel G., (2008) Antioxidant activity of the essential oils of Thymbra capitata, Origanun vulgare Thymus mastichina and Calamintha Baetica. Acta Hort. (ISHS) 765:325-334.
59. Gaoming Lei, Longhu Wang, Xuesong Liu & Anyun Zhang (2014) Chemical composition of essential oils and hydrosols from fresh flowers of Cerasus subhirtella and Cerasus serrulata from East China, Natural Product Research: Formerly Natural Product Letters, 28:21, 1923-1925
60. Garneau FX, Collin G, Gagnon H, Pichette A. (2012) Chemical composition of the hydrosol and the essential oil of three different species of the Pinaceae family: Picea glauca (Moench) Voss., Picea mariana (Mill.) B.S.P. and Abies balsamea (L.) Mill. J Essent Oil Bearing Plant 15(2):227-236.
61. Garneau FX, Collin G, Gagnon H. (2014) Chemical composition and stability of the hydrosols obtained during essential oil production. I. The case of

Melissa officinalis L. and Asarum canadense L.American Journal of Essential Oils and Natural Products 2 (1): 54-62

62. Gaulin C., Lê M.L., Shum M., Fong D. (2011) Disinfectants and sanitizers for use on food contact surfaces. National Collaborating Centre for Environmental Health at the British Columbia Centre for Disease Control in August 2010; revised August 2011.
63. Geeraerd, A. H., V. P. Valdramidis, and J. F. Van Impe. (2005). GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves. Int. J. Food Microbiol. 102:95–105.
64. Gerstel, U., & Römling, U. (2003). The csgD promoter, a control unit for biofilm formation in Salmonella Typhimurium. Research in Microbiology, 154(10), 659 −667.
65. Gianotti A, Serrazanetti D, Sado Kamdem S, Guerzoni Me (2008) Involvement of cell fatty acid composition and lipid metabolism in adhesion mechanism of Listeria monocytogenes. Int J Food Microbiol 123:9–17.
66. Giaouris E, Chorianopoulos N, Doulgeraki A, Nychas G-J (2013) Co-Culture with Listeria monocytogenes within a Dual-Species Biofilm Community Strongly Increases Resistance of Pseudomonas putida to Benzalkonium Chloride . PLoS ONE 8(10): e77276. doi:10.1371/journal.pone. 0077276
67. Giaouris E, Samoilis G, Chorianopoulos n, ercolini D, nychas GJ (2013) Differential protein expression patterns between planktonic and biofim cells of Salmonella enterica serovar enteritidis PT4 on stainless steel surface. Int J Food Microbiol 162:105–113.
68. Giaouris E., Chorianopoulos N., Skandamis P. and Nychas G.J (2012). Attachment and Biofilm Formation by Salmonella in Food Processing Environments, Salmonella - A Dangerous Foodborne Pathogen, Dr. Dr. Barakat S M Mahmoud (Ed.), ISBN: 978-953-307-782-6
69. Giaouris Efstathios, Giaouris Georgios, Chorianopoulos Nikos, Ercolini Danilo, Nychas George-John, 2012. Differential protein expression patterns between planktonic and biofilm cells of Salmonella enterica serovar Enteritidis PT4 on stainless steel surface. Int. J. of Food Microbiology 162 (2013) 105–113
70. Giaouris, E.; Chorianopoulos, N. & Nychas, G.-J.E. (2005). Effect of temperature, pH, and water activity on biofilm formation by Salmonella

enterica Enteritidis PT4 on stainless steel surfaces as indicated by the bead vortexing method and conductance measurements. Journal of Food Protection, Vol.68, No.10, (May 2005), pp. 2149-2154

71. Giaouris, E.D. & Nychas, G.-J.E. (2006). The adherence of Salmonella Enteritidis PT4 to stainless steel: the importance of the air-liquid interface and nutrient availability. Food Microbiology, Vol.23, pp. 747-752
72. Grimont, P., and Weill, F., (2007) Antigenic Formulae of the Salmonella Serovars. WHO Collaborating Centre for Reference and Research on Salmonella.
73. Gunduz GT1, Tuncel G., (2006) Biofilm formation in an ice cream plant. Antonie Van Leeuwenhoek. 89(3-4):329-36. Epub 2006 Apr 25
74. Haagensen, J. A. J., M. Klausen, R. K. Ernst, S. I. Miller, A. Folkesson, T. Tolker-Nielsen, and S. Molin. 2007. Differentiation and distribution of colistinand sodium dodecyl sulfate-tolerant cells in Pseudomonas aeruginosa biofilms. J. Bacteriol. 189:28–37.
75. Haddadin, M.S.Y. (2010). Effect of olive leaf extracts on the growth and metabolism of two probiotic bacteria of intestinal origin. Pakistan J. Nutr., 9: 787–793.
76. Hall-Stoodley, L., & Stoodley, P. (2009). Evolving concepts in biofilm infections. Cellular. Microbiology, 11 (7), 1034 −1043.
77. Hamilton, S., Bongaerts, R. J., Mulholland, F., Cochrane, B., Porter, J., Lucchini, S., et al. (2009).The transcriptional programme of Salmonella enterica serovar Typhimurium reveals a key role for tryptophan metabolism in biofilms. BMC Genomics, 10,599
78. Hassett DJ et al (1999) Quorum sensing in Pseudomonas aeruginosa controls expression of catalase and superoxide dismutase genes and mediates biofim susceptibility to hydrogen peroxide. Mol Microbiol 34:1082–1093.
79. Hengge, R. (2009). Principles of c-di-GMP signalling in bacteria. Nature Reviews. Microbiology, 7(4), 263 −273.
80. Hermans K, Nguyen TL, Roberfroid S, Schoofs G, Verhoeven T, De Coster D, Vanderleyden J, De Keersmaecker SC. (2011) Gene expression analysis of monospecies Salmonella typhimurium biofilms using differential fluorescence induction.J Microbiol Methods.;84(3):467-78.

81. Heroven, A. K., Sest, M., Pisano, F., Scheb-Wetzel, M., Steinmann, R., Bohme, K., et al. (2012). Crp induces switching of the CsrB and CsrC RNAs in Yersinia pseudotuberculosis and links nutritional status to virulence. Front. Cell. Infect. Microbiol. 2:158. doi: 10.3389/fcimb.2012.00158
82. Hortigón-Vinagre María P., Blanco José, Ruiz Trinidad, Henao Fernando. (2014). Thymbra capitata Essential Oil Prevents Cell Death Induced by 4-Hydroxy-2-Nonenal in Neonatal Rat Cardiac Myocytes. Planta Med. 80(15):1984-90.
83. Inouye S, Takahashi M, Abe S. (2008) A comparative study on the composition of forty four hydrosols and their essential oils. Intern J Essent Oil Therap 2(3):89- 104.
84. Inouye, S., Takahashi, M. and Abe, S. (2009). Inhibitory activity of hydrosols, herbal teas and related essential oils against filament formation and the growth of Candida albicans. Jpn. J. Med. Mycol., 50: 243–251.
85. Irie, Y. & Parsek, M.R. (2008). Quorum sensing and microbial biofilms. Current Topics in Microbiology and Immunology, Vol.322, pp. 67-84
86. Jay M. James., (2000) Modern Food Microbiology, 6th Edition, Aspen Publishers, Inc. Gaithersburg, Maryland
87. Jayasekara NY, Heard GM, Cox JM, Fleet GH. (1998) Populations of pseudomonads and related bacteria associated with bottled non-carbonated mineral water. Food Microbiol 15:167–76.
88. Jonas K1, Edwards AN, Ahmad I, Romeo T, Römling U, Melefors O (2010) Complex regulatory network encompassing the Csr, c-di-GMP and motility systems of Salmonella Typhimurium.Environ Microbiol. 12(2):524-40.
89. Jonas, K., Edwards, A. N., Ahmad, I., Romeo, T., Römling, U., & Melefors, O. (2010). Complex regulatory network encompassing the Csr, c-di-GMP and motility systems of Salmonella Typhimurium. Environmental Microbiology, 12(2), 524 −540.
90. Joux, F., and P. Lebaron. 2000. Use of fluorescent probes to assess physiological functions of bacteria at single cells level. Microbes Infect. 2:1523–1535.
91. Kaneko, Y., M. Thoendel, O. Olakanmi, B. E. Britigan, and P. K. Singh. 2007. The transition metal gallium disrupts Pseudomonas aeruginosa iron

metabolism and has antimicrobial and antibiofilm activity. J. Clin. Investig. 117:877–888.

92. Karatan e, watnick P (2009) Signals, regulatory networks, and materials that build and break bacterial biofims. Microbiol Mol Biol Rev 73:310–347. doi:10.1128/MMBR.00041-08
93. Kelly D, McAuliffe O, Ross RP, Coffey A (2012) Prevention of Staphylococcus aureus biofim formation and reduction in established biofim density using a combination of phage K and modified derivatives. Lett Appl Microbiol 54:286–291. doi:10.1111/j.1472-765X.2012.03205.x
94. Khan R, Zakir M, Khanam Z, Shakil S, Khan AU. 2010. Novel compound from Trachyspermum ammi (Ajowan caraway) seeds with antibiofilm and antiadherence activities against Streptococcus mutans: a potential chemotherapeutic agent against dental caries. J App Microbiol 109:2151–2159.
95. Kim, S.-H. & Wei, C.-I. (2009). Molecular characterization of biofilm formation and attachment of Salmonella enterica serovar Typhimurium DT104 on food contact surfaces. Journal of Food Protection, Vol.72, No.9, (September 2009), pp. 1841-1847
96. Klein R. Joanna, Fahlen F. Thomas, Jones D. Brandley (2000) Transcriptional Organization and Function of Invasion Genes within Salmonella enterica Serovar Typhimurium Pathogenicity Island 1, Including the prgH, prgI, prgJ, prgK, orgA, orgB, and orgC Genes. Infect Immun. 2000 Jun;68(6):3368-76.
97. Knowles JR, Roller S, Murray DB, Naidu AS. 2005. Antimicrobial action of carvacrol at different stages of dual-species biofilm development by Staphylococcus aureus and Salmonella enterica serovar Typhimurium. Appl Environ Microbiol 71:797–803.
98. Kostaki M, Chorianopoulos N, Braxou E, Nychas G-J, Giaouris E (2012) Differential biofilm formation and chemical disinfection resistance of sessile cells of Listeria monocytogenes strains under monospecies and dual-species (with Salmonella enterica) conditions. Appl Environ Microbiol 78: 2586-2595. doi:10.1128/AEM.07099-11. PubMed: 22307304.
99. Kulasakara, H., Lee, V., Brencic, A., Liberati, N., Urbach, J., Miyata, S., et al. (2006). Analysis ofPseudomonas aeruginosa diguanylate cyclases and

phosphodiesterases reveals a role for bis-(3’-5’)-cyclic-GMP in virulence. Proc. Natl. Acad. Sci. U.S.A. 103, 2839–2844. doi: 10.1073/pnas.0511090103

100. Lamprokostopoulou, A., Monteiro, C., Rhen, M., & Römling, U. (2010). Cyclic di-GMP signalling controls virulence properties of Salmonella enterica serovar Typhimurium at the mucosal lining. Environmental Microbiology, 12(1), 40 −53.

101. Latasa, C., Roux, A., Toledo-Arana, A., Ghigo, J. M., Gamazo, C., Penades, J. R., et al. (2005). BapA, a large secreted protein required for biofilm formation and host colonization of Salmonella enterica serovar Enteritidis. Molecular Microbiology, 58(5), 1322 −1339

102. Laughlin RC, Knodler LA, Barhoumi R, et al. (2014) Spatial Segregation of Virulence Gene Expression during Acute Enteric Infection with Salmonella enterica serovar Typhimurium. mBio.5(1):e00946-13. doi:10.1128/mBio.00946-13.

103. Lazar, V. (2011). Quorum sensing in biofilms - How to destroy the bacterial citadels or their cohesion/power? Anaerobe, in press

104. Lenz, D. H., Miller, M. B., Zhu, J., Kulkarni, R. V., and Bassler, B. L. (2005). CsrA and three redundant small RNAs regulate quorum sensing in Vibrio cholerae. Mol. Microbiol. 58, 1186–1202.

105. Lewis K, Ausubel FM. 2006. Prospects for plant-derived antibacterials. Nature Biotechnol 24:1504–1507.

106. Lewis R J Sr (2004). Sax's Dangerous Properties of Industrial Materials (11 ed.). Wiley-Interscience, Wiley & Sons, Inc. Hoboken, NJ. p. 104. doi:10.1002/0471701343

107. Lin C et al. (2011) effect of superhydrophobic surface of titanium on Staphylococcus aureus adhesion. J nanomater. doi: 10.1155/2011/178921

108. Lindsay, D. & von Holy, A. (2006). What food safety professionals should know about bacterial biofilms. British Food Journal, Vol.108, No.1, pp.27-37

109. Litrup, E.; Torpdahl, M.; Malorny, B.; Huehn, S.; Christensen, H. & Nielsen, E.M. (2010).Association between phylogeny, virulence potential and serovars of Salmonella enterica. Infection, Genetics and Evolution, Vol.10, No. 7, (July 2010), pp. 1132-1139

110. Loo CY, Young PM, Lee wH, Cavaliere R, whitchurch CB, Rohanizadeh R (2012) Superhydrophobic, nanotextured polyvinyl chloride fims for delaying Pseudomonas aeruginosa attachment to intubation tubes and medical plastics. Acta Biomater 8:1881–1890.

111. Luppens SBI, Kara D, Bandounas L, Jonker MJ, Wittin FRA, Bruning O, Breit TM, ten Cate JM, Crielaard W. 2008. Effect of Veillonella parvula on the antimicrobial resistance and gene expression of Streptococcus mutans grown in a dual-species biofilm. Oral Microbiol Immunol 23:183–189.

112. Machado M, Dinis AM, Salgueiro L, Cavaleiro C, Custódio JB, Sousa Mdo C(2010) Anti-Giardia activity of phenolic-rich essential oils: effects of Thymbra capitata, Origanum virens, Thymus zygis subsp. sylvestris, and Lippia graveolens on trophozoites growth, viability, adherence, and ultrastructure. Parasitol Res. 106(5):1205-15.

113. Maheux AF1, Boudreau DK2, Bisson MA3, Dion-Dupont V3, Bouchard S3, Nkuranga M3, Bergeron MG4, Rodriguez MJ5. (2014) Molecular method for detection of total coliforms in drinking water samples.Appl Environ Microbiol. 80(14):4074-84. doi: 10.1128/AEM.00546-14. Epub 2014 Apr 25.

114. Maillard JY (2005) Antimicrobial biocides in the healthcare environment: effiacy, usage, policies, and perceived problems. Ther Clin Risk Manag 1:307–320

115. Malone CL, Boles BR, Lauderdale KJ, Thoendel M, Kavanaugh JS, Horswill AR. (2009) Fluorescent reporters for Staphylococcus aureus. J Microbiol Methods. 77(3):251-60. J

116. Mangalappalli-Illathu AK, Vidović S, Korber DR., (2008) Differential adaptive response and survival of Salmonella enterica serovar enteritidis planktonic and biofilm cells exposed to benzalkonium chloride. Antimicrob Agents Chemother.52(10):3669-80.

117. Marcus SL, Brumell JH, Pfeifer CG, Finlay BB, (2000) Salmonella pathogenicity islands: big virulence in small packages. Microbes Infect. Feb;2(2):145-56. Review

118. Marin, C.; Hernandiz, A. & Lainez, M. (2009). Biofilm development capacity of Salmonella strains isolated in poultry risk factors and their

resistance against disinfectants.Poultry Science, Vol.88, (October 2008), pp. 424-431

119. Martínez C. L. and Vadyvaloo V., (2014) Mechanisms of post-transcriptional gene regulation in bacterial biofilms. Front Cell Infect Microbiol. 2014; 4: 38.

120. Mc Cay PH, Ocampo-Sosa AA, Fleming GT (2010) effect of subinhibitory concentrations of benzalkonium chloride on the competitiveness of Pseudomonas aeruginosa grown in continuous culture. Microbiology 156:30–38.

121. McDonnell G, Russell AD (1999) Antiseptics and disinfectants: activity, action, and resistance. Clin Microbiol Rev 12:147–179

122. McDougald D, Rice SA, Barraud n, Steinberg PD, Kjelleberg S. (2011) Should we stay or should we go: mechanisms and ecological consequences for biofim dispersal. nat Rev Microbiol 10:39–50. doi:10.1038/nrmicro2695

123. Mena KD, Gerba CP. (2009) Risk assessment of Pseudomonas aeruginosa in water. Rev Environ. Contam Toxicol 201:71-115.

124. Mesquita S, Noble RT. 2013. Recent developments in monitoring of microbiological indicators of water quality across a range of water types, water resources planning, development and management.

125. Molbak, K., Olsen, J., Wegener, H. 2006. Salmone*l*la Infections, p. 55-115. In H. Reimann, D. Cliver (eds.), Foodborne Infections and Intoxications. Academic Press.

126. Moon, T., Cavanagh, H.M.A. and Wilkinson, J.M. (2007). Antifungal activity of Australian grown Lavandula spp. essential oils against Aspergillus nidulans, Trichophyton mentagrophytes, Leptosphaeria maculans and Sclerotinia Sclerotium. J. Essent. Oil Res., 19: 171–175.

127. Nam H, Seo HS, Bang J, Kim H, Beuchat LR, Ryu JH., (2014) Efficacy of gaseous chlorine dioxide in inactivating Bacillus cereus spores attached to and in a biofilm on stainless steel. Int J Food Microbiol. 188:122-7.

128. Nostro A1, Sudano Roccaro A, Bisignano G, Marino A, Cannatelli MA, Pizzimenti FC, Cioni PL, Procopio F, Blanco AR. (2007) Effects of oregano, carvacrol and thymol on Staphylococcus aureus and Staphylococcus epidermidis biofilms. J Med Microbiol. 56(Pt 4):519-23.

129. Nostro, A., Blanco, A. R., Cannatelli, M. A., Enea, V., Flamini, G., Morelli, I., Sudano Roccaro, A. & Alonzo, V. (2004). Susceptibility of methicillin-resistant staphylococci to oregano essential oil, carvacrol and thymol. FEMS Microbiol Lett 230, 191–195.

130. Oliveira, K.; Oliveira, T.; Teixeira, P.; Azeredo, J.; Henriques, M. & Oliveira, R. (2006). Comparison of the adhesion ability of different Salmonella Enteritidis serotypes to materials used in kitchens. Journal of Food Protection, Vol.69, No.10, (May 2006), pp.2352-2356

131. Palermo eF, Lee DK, Ramamoorthy A, Kuroda K (2011) Role of cationic group structure in membrane binding and disruption by amphiphilic copolymers. J Phys Chem B 115:366–375.

132. Palleroni NJ. (2010) Pseudomonas. Topley & Wilson's microbiology and microbial infections. John Wiley & Sons, Ltd.

133. Palmeira-de-Oliveira A1, Gaspar C, Palmeira-de-Oliveira R, Silva-Dias A, Salgueiro L, Cavaleiro C, Pina-Vaz C, Martinez-de-Oliveira J, Queiroz JA, Rodrigues AG. (2012) The anti-Candida activity of Thymbra capitata essential oil: effect upon pre-formed biofilm. J Ethnopharmacol.27;140(2):379-83.

134. Paolini J, Leandri C, Desjobert JM, Barboni T, Costa J. (2008) Comparison of liquid-liquid extraction with headspace methods for the characterization of volatile fractions of commercial hydrolats from typically Mediterranean species. J Chromatogr A 1193(1-2):37-49.

135. Patel S., (2015)Plant essential oils and allied volatile fractions as multifunctional additives in meat and fish-based food products: a review.Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 8:1-16.

136. Percival SL, Chalmers RM, Embrey M, Hunter PR, Sellwood J, WynJones P (2004). Microbiology of Waterborne Diseases. Elsevier Academic Press, San Diego, USA.

137. Pereira, A.L., Silva, T.N., Gomes, A.C.M.M., Araújo, A.C.G., Giugliano, L.G., 2010. Diarrheaassociated biofilm formed by enteroaggregative Escherichia coli and aggregative Citrobacter freundii: a consortium mediated by putative F pili. BMC Microbiology 10, 1471 –2180.

138. Pezoa D, Yang H-J, Blondel CJ, Santiviago CA, Andrews-Polymenis HL, et al. (2013) The Type VI Secretion System Encoded in SPI-6 Plays a Role in Gastrointestinal Colonization and Systemic Spread of Salmonella enterica serovar Typhimurium in the Chicken. PLoS ONE 8(5): e63917. doi:10.1371/ journal.pone.0063917

139. Pindi Pavan Kumar, P. Raghuveer Yadav , and A. Shiva Shanker (2013) Identification of Opportunistic Pathogenic Bacteria in Drinking Water Samples of Different Rural Health Centers and Their Clinical Impacts on Humans. Biomed Res Int. 348250.

140. Raichand R, Pareek S, Singh NK, Mayilraj S. (2012) Exiguobacterium aquaticum sp. nov., a member of the genus Exiguobacterium.Int J Syst Evol Microbiol. 62(Pt 9):2150-5.

141. Rani, S. A., B. Pitts, and P. S. Stewart. 2005. Rapid diffusion of fluorescent tracers into Staphylococcus epidermidis biofilms visualized by time lapse microscopy. Antimicrob. Agents Chemother. 49:728–732.

142. Rani, S. A., B. Pitts, H. Beyenal, R. A. Veluchamy, Z. Lewandowski, K. Buckingham-Meyer, and P. S. Stewart. 2007. Spatial patterns of DNA replication, protein synthesis and oxygen concentration within bacterial biofilms reveal diverse physiological states. J. Bacteriol. 189:4223–4233.

143. Rasschaert, G., Houf, K., De Zutter, L., 2007. Impact of the slaughter line contamination on the presence of Salmonella on broiler carcasses. Journal of Applied Microbiology 103, 333–341.

144. Renner LD, weibel DB (2011) Physicochemical regulation of biofim formation. MRS Bull 36:347–355

145. Robbe-Saule, V., Jaumouille, V., Prevost, M. C., Guadagnini, S., Talhouarne, C., Mathout, H., et al. (2006). Crl activates transcription initiation of RpoS-regulated genes involved in the multicellular behavior of Salmonella enterica serovar Typhimurium. Journal of Bacteriology, 188(11), 3983 −3994.

146. Rodrigues, D.; Teixeira, P.; Oliveira, R. & Azeredo, J. (2011). Salmonella enterica Enteritidis biofilm formation and viability on regular and triclosan-impregnated bench cover materials. Journal of Food Protection, Vol.74, No.1, (July 2010), pp. 32-37

147. Römling, U., Bian, Z., Hammar, M., Sierralta, W. D., & Normark, S. (1998). Curli fibers are highly conserved between Salmonella Typhimurium

and Escherichia coli with respect to operon structure and regulation. Journal of Bacteriology, 180(3), 722 −731.

148. Rosenkrantz, J. T., Aarts, H., Abee, T., Rolfe, M. D., Knudsen, G. M., Nielsen, M.- B., et al. (2013). Non-essential genes form the hubs of genome scale protein function and environmental gene expression networks in Salmonella enterica serovar Typhimurium. BMC Microbiol. 13:294. doi: 10.1186/1471-2180-13-294

149. Saðdiç, Ö. (2003). Sensitivity of four pathogenic bacteria to Turkish thyme and oregano hydrosols. LWT Food Sci. mTechnol., 36: 467–473.

150. Saðdiç, Ö. and Özcan, M. (2003). Antibacterial activity of Turkish spice hydrosols. Food Control, 14: 141–143.

151. Salgueiro LR, Pinto E, Gonçalves MJ, Pina-Vaz C, Cavaleiro C, Rodrigues AG, Palmeira A, Tavares C, Costa-de-Oliveira S, Martinez-de-Oliveira J. (2004) Chemical composition and antifungal activity of the essential oil of Thymbra capitata. Planta Med. 70(6):572-5.

152. Schulte S, wingender J, Flemming H-C (2005) effiacy of biocides against biofims. In: Paulus w (ed) Directory of microbicides for the protection of materials. Springer, Berlin, pp 93–120

153. Sen, S. and Yalçin, M. (2010). Activity of commercial still waters from volatile oils production against wood decay fungi. Maderas Cienc. Tecnol., 12: 127–133.

154. Seymour Stanton Block (2001). Disinfection, sterilization, and preservation (5, illustrated ed.). Lippincott Williams & Wilkins. p. 311. ISBN 0-683-30740-1.

155. Shaner NC, Steinbach PA, Tsien RY., (2005) A guide to choosing fluorescent proteins Nat Methods. Dec;2(12):905-9..

156. Shen Y, Stojicic S, Qian w, Olsen I, Haapasalo M (2010) The synergistic antimicrobial effect by mechanical agitation and two chlorhexidine preparations on biofim bacteria. J endod 36:100–104.

157. Shi, Xianming, and Xinna Zhu. (2009) Biofilm formation and food safety in food industries." Trends in Food Science & Technology: 407-413.

158. Simm, R., Remminghorst, U., Ahmad, I., Zakikhany, K., & Römling, U. (2009). A role for the EAL-like protein STM1344 in regulation of CsgD

expression and motility in Salmonella enterica serovar Typhimurium. Journal of Bacteriology, 191 (12), 3928 −3937.

159. Simo˜es, M., Simo˜es, L. C., Machado, I., Pereira, M. O., & Vieira, M. J. (2006). Control of flow-generated biofilms using surfactants – evidence of resistance and recovery. Food and Bioproducts Processing, 84, 338–345.

160. Simões M, Simões L C, Vieira M J (2010) A review of current and emergent biofilm control strategies. LWT-Food Science and Technology 43, 573-583.

161. Solano, C., Garcia, B., Valle, J., Berasain, C., Ghigo, J. M., Gamazo, C., et al. (2002). Genetic analysis of Salmonella enteritidis biofilm formation: Critical role of cellulose. Molecular Microbiology, 43(3), 793 −808.

162. Spiers AJ, Buckling A, Rainey PB. (2000) The causes of Pseudomonas diversity. Microbiology146(Pt 10):2345–50

163. Splendiani, A., Livingston, A. G., & Nicolella, C. (2006). Control membrane-attached biofilms using surfactants. Biotechnology and Bioengineering, 94, 15–23.

164. Srey S., Jahid I. K., Ha S. D., 2013 Biofilm formation in food industries: A food safety concern. Food Control 31:572 :585

165. Steenackers H, Hermans K, Vanderleyden J, De Keersmaecker SCJ. 2012. Salmonella biofilms: an overview on occurrence, structure, regulation and eradication. Food Res Int. 45:502–531.

166. Stevens M, Ashbolt N, Cunliffe D. 2003. Recommendations to change the use of coliforms as microbial indicators of drinking water quality. National Health and Medical Research Council, Canberra, Australia. Capital Territory, Australia.

167. Stewart, P. S. 2003. Diffusion in biofilms. J. Bacteriol. 185:1485–1491

168. Sun, J (2007). "d-Limonene: safety and clinical applications". Alternative medicine review: a journal of clinical therapeutic 12 (3): 259–64

169. Suzuki, K., Babitzke, P., Kushner, S. R., & Romeo, T. (2006). Identification of a novel regulatory protein (CsrD) that targets the global regulatory RNAs CsrB and CsrC for degradation by RNase E. Genes & Development, 20(18), 2605 −2617.

170. Teplitski, M., Al-Agely, A., & Ahmer, B. M. (2006). Contribution of the SirA regulon to biofilm formation in Salmonella enterica serovar Typhimurium. Microbiology, 152(Pt 11), 3411 −3424

171. Teunis PF, Kasuga F, Fazil A, Ogden ID, Rotariu O, Strachan NJ., (2010) Dose-response modeling of Salmonella using outbreak data. Int J Food Microbiol. Dec 15;144(2):243-9.

172. Tomljenovic-Berube, A. M., Mulder, D. T., Whiteside, M. D., Brinkman, F. S. L., & Coombes, B. K. (2010). Identification of the Regulatory Logic Controlling Salmonella Pathoadaptation by the SsrA-SsrB Two-Component System. PLoS Genetics, 6(3), e1000875. doi:10.1371/journal.pgen.1000875

173. Tornuk. F., Cankurt, H., Ozturk, I., Saðdiç, Ö., Bayram, O. and Yetim, H. (2011). Efficacy of various plant hydrosols as natural food sanitizers in reducing Escherichia coli O157:H7 and Salmonella typhimurium on fresh cut carrots and apples. Int. J. Food Microbiol., 148: 30–35.

174. Tsien Y. Roger, (1998) The green fluorescent protein. Annu. Rev. Biochem. 67:509-544

175. Ulusoy, S., Tinaz, G.B. and Canbay, H.S. (2009). Tocopherol, carotene, phenolic contents and antibacterial properties of rose essential oil, hydrosol and absolute. Curr. Microbiol., 59: 554–558.

176. Van der Veen S, Abee T. 2010. Mixed species biofilm's of Listeria monocytogenes and *Lactobacillus plantarum* show enhanced resistance to benzalkonium chloride and peracetic acid. Int J Food Microbiol 144:421–431.

177. Vantarakis A., M. Smaili, I. Detorakis, G. Vantarakis, M. Papapetropoulou Diachronic long-term surveillance of bacteriological quality of bottled water in Greece (1995–2010) Original Research Article Food Control, Volume 33, Issue 1, September 2013, Pages 63-67

178. Verma, R.S., Pandey, V., Padalia, R.C., Saikia, D. and Krishna, B. (2011). Chemical composition and antimicrobial potential of aqueous distillate volatiles of Indian peppermint (Mentha piperita) and spearmint (Mentha spicata). J. Herbs, Spices Med. Pl., 17: 258–267.

179. Verran J, whitehead K (2005) Factors affecting microbial adhesion to stainless steel and other materials used in medical devices. Int J Artif Organs 28:1138–1145

180. Wain, J; Hendriksen, RS; Mikoleit, ML; Keddy, KH; Ochiai, RL (2015). "Typhoid fever.". Lancet 385 (9973): 1136–45.
181. Walters, M. & Sperandio, V. (2006). Quorum sensing in Escherichia coli and Salmonella. International Journal of Medical Microbiology, Vol.296, pp. 125-131
182. Wang, Q., Zhao, Y., McClelland, M., & Harshey, R. M. (2007). The RcsCDB signaling system and swarming motility in Salmonella enterica serovar Typhimurium: dual regulation of flagellar and SPI-2 virulence genes. Journal of Bacteriology, 189(23), 8447 − 8457.
183. Weiler C1, Ifland A, Naumann A, Kleta S, Noll M. Incorporation of Listeria monocytogenes strains in raw milk biofilms. Int J Food Microbiol. 2013 Feb 1;161(2):61-8.
184. White, A. P., & Surette, M. G. (2006). Comparative genetics of the rdar morphotype in Salmonella. Journal of Bacteriology, 188(24), 8395 −8406.
185. White, A. P., Gibson, D. L., Collinson, S. K., Banser, P. A., & Kay, W. W. (2003). Extracellular polysaccharides associated with thin aggregative fimbriae of Salmonella enterica serovar enteritidis. Journal of Bacteriology, 185(18), 5398 −5407.
186. White, A. P., Weljie, A. M., Apel, D., Zhang, P., Shaykhutdinov, R., Vogel, H. J., et al. (2010). A global metabolic shift is linked to Salmonella multicellular development. PLoS ONE, 5(7), e11814
187. White, A.P.; Gibson, D.L.; Kim, W.; Kay, W.W. & Surette, M.G. (2006). Thin aggregative fimbriae and cellulose enhance long-term survival and persistence of Salmonella. Journal of Bacteriology, Vol.188, No.9, (May 2006), pp. 3219-3227
188. Wilkinson, J.M., Hipwell, M., Ryan, T. and Cavangh, H.M.A. (2003). Bioactivity of Backhousia citriodora: Antibacterial and antifungal activity. J. Agric. Food Chem., 51: 76–87.
189. Wong HS, Townsend KM, Fenwick SG, Trengove RD, O'Handley RM., Comparative susceptibility of planktonic and 3-day old Salmonella Typhimurium biofilms to disinfectants. J Appl Microbiol. 2010 Jun;108(6):2222-8.

190. World Health Organization, 2011, Guidelines for drinking-water quality, fourth edition, ISBN: 978 92 4 154815 1

191. World Health Organization. 2004. Guidelines for drinking-water quality,3rd ed, vol 1. Recommendations. World Health Organization, Geneva, Switzerland.

192. Yarwood JM, Douglas J. Bartels2, Esther M. Volper1, and E. Peter Greenberg1,Quorum Sensing in Staphylococcus aureus Biofilms, J. Bacteriol. March 2004 vol. 186 no. 6 1838-1850

193. Zakikhany, K., Harrington, C. R., Nimtz, M., Hinton, J. C., & Römling, U. (2010). Unphosphorylated CsgD controls biofilm formation in Salmonella enterica serovar Typhimurium. Molecular Microbiology, 77(3), 771 −786.

194. Zengler, K. and Palsson, B.O. (2012) A road map for the development of community systems (CoSy) biology. Nat. Rev. Microbiol. 10, 366–372

195. Zhang Y, Hu Z (2013) Combined treatment of Pseudomonas aeruginosa biofims with bacteriophages and chlorine. Biotechnol Bioeng 110:286–295. doi:10.1002/bit.24630

196. Zijnge V, van Leeuwen MBM, Degener JE, Abbas F, Thurnheer T, Gmur R, Harmsen HJM. 2010. Oral biofilm architecture on natural teeth. PLoS One 5(2):e9321.

ANNEX A

Table 1: Bacterial concentrations (cfu/ml) in water samples collected from bottling plant.

A/A	Kind of samples	ΚΩΔ.	ΗΜ/ΝΙΑ ΔΕΙΓΜΑΤΟΛΗΨΙΑΣ	Ps. Aeruginosa (cfu/ml)	Total coliforms (cfu/ml)	Faecal enterococci (cfu/ml)	E. coli (cfu/ml)	Total count (cfu/ml)
1	Water Source	WS1	05/08/14	-	<1	<1	<1	4
2	<<	WS2	05/08/14	-	<1	<1	<1	5
3	<<	WS3	11/08/14	-	1	<1	<1	3
4	<<	WS4	18/08/14	-	<1	<1	<1	29
5	<<	WS5	25/08/14	-	<1	<1	<1	5
6	Water distribution network	WD1	05/08/14	<1	<1	<1	<1	12
7	<<	WD2	11/08/14	<1	<1	<1	<1	7
8	<<	WD3	11/08/14	-	-	-	-	9
9	<<	WD4	11/08/14	<1	2	9*	<1	23
10	<<	WD5	11/08/14	-	-	-	-	26
11	<<	WD6	25/08/14	<1	<1	<1	<1	4
12	Final product	6l FN5	08/08/14	<1	<1	<1	<1	16
13	<<	6L FN4	08/08/14	<1	<1	<1	<1	15
14	<<	6l FN3	08/08/14	<1	<1	<1	<1	30
15	<<	1,5l FN2	18/08/14	<1	<1	<1	<1	1
16	<<	1,5l FN1	18/08/14	<1	<1	<1	<1	3

Table 2: Log_{10} survivors of 24 h *S.* Typhimurium planktonic cells after 6 min disinfection with different concentrations of BAC

Survival of *S.* Typhimurium planktonic cells (logcfu/ml)						
		Control	**BAC 22 ppm**	**BAC 12 ppm**	**BAC 6 ppm**	**BAC 3 ppm**
Experiment 1	Rep. 1	7,91	<DL*	<DL	3,92	7,26
	Rep. 2	7,90	<DL	3,40	6,06	7,26
Experiment 2	Rep. 1	7,97	<DL	2,22	3,54	7,30
	Rep. 2	8,01	<DL	3,07	6,14	6,98
Experiment 3	Rep. 1	7,90	<DL	<DL	5,5	7,30
	Rep. 2	7,91	<DL	1,52	4,45	7,36
	Rep. 3	7,96			5,845	
Average		7,96	<DL	1,70	5,06	7,24
St deviation		0,23	<DL	1,47	1,07	0,13

<DL*: Below detection limit (1 logcfu/ml)

Table 3: Log_{10} survivors of 24 h *S.* Typhimurium planktonic cells after 6 min disinfection with different concentrations of *T. capitata* hydrosol.

Survival of *S.* Typhimurium planktonic cells (logcfu/ml)							
		Control	**Hyd 45%**	**Hyd 42%**	**Hyd 40%**	**Hyd 37,5%**	**Hyd 35%**
Experiment 1	Rep. 1	7,91	<DL*	3,18	5,34	6,22	7,68
	Rep. 2	7,90	<DL	<DL	4,20	6,45	7,63
Experiment 2	Rep. 1	7,97	<DL	3,30	4,81	6,88	7,72
	Rep. 2	8,01	<DL	3,71	5,62	7,06	8,12
Experiment 3	Rep. 1	7,91	<DL	4,30	5,48	6,92	7,76
	Rep. 2	7,90	<DL	4,65	5,17	7,05	6,50
Average		7,96	<DL	3,19	5,10	6,76	7,56
St deviation		0,23	<DL	1,66	0,52	0,34	0,551667

Table 4: Log_{10} survivors of 24 h *S.* Typhimurium biofilm cells after 6 min disinfection with different concentrations of BAC.

Survival of *S.* Typhimurium biofilm cells (logcfu/cm^2)					
		Control	**BAC 0,5%**	**BAC 0,25%**	**BAC 0,125%**
Experiment 1	Rep. 1	8,12	<DL*	<DL	4,74
	Rep. 2		<DL	3,39	4,26
Experiment 2	Rep. 1	7,70	<DL	3,39	4,65
	Rep. 2		<DL	3,90	4,58
Experiment 3	Rep. 1	7,24	<DL	<DL	4,68
	Rep. 2		<DL	<DL	
Average		7,68	<DL	3,19 or 1,78	4,58
St deviation		0,44	<DL	0,29 or 1,95	0,19

Table 5: Log_{10} survivors of 24 h *S.* Typhimurium biofilm cells after 6 min disinfection with different concentrations of BAC.

Survival of *S.* Typhimurium biofilm cells (logcfu/cm^2)					
		Control	**Hyd 100 %**	**Hyd 75%**	**Hyd 50%**
Experiment 1	Rep. 1	8,12	<DL*	4,57	7,58
	Rep. 2		<DL	4,63	7,64
Experiment 2	Rep. 1	7,70	<DL	4,26	6,94
	Rep. 2		<DL	4,52	7,02
Experiment 3	Rep. 1	7,24	<DL	4,97	6,52
	Rep. 2		<DL	5,10	6,52
Average		7,68	<DL	4,67	7,04
St deviation		0,44	<DL	0,30	0,49