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Virulence of *Salmonella enterica* Enteritidis biofilms after exposure to different disinfectants

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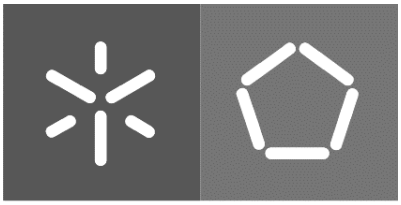

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Virulence of *Salmonella enterica* Enteritidis biofilms after exposure to different disinfectants

Tese de Mestrado
Mestrado em Bioengenharia

Trabalho realizado sob a orientação de
Doutora **Diana Rodrigues** e coorientação de
Professora Doutora **Joana Azeredo**

Outubro 2014

Acknowledgements/

Agradecimentos

Acknowledgements/Agradecimentos

Gostaria de agradecer a todas as pessoas que de alguma forma contribuíram para que a realização desta tese fosse possível.

Em primeiro lugar, gostaria de agradecer às minhas orientadoras, doutora Joana Azeredo e doutora Diana Rodrigues, por me terem aceitado neste projeto, por todas as sugestões dadas, pela partilha de conhecimentos e disponibilidade demonstrada. Gostaria de manifestar especialmente o meu agradecimento à doutora Diana, por toda a atenção e tempo que disponibilizou no decorrer deste trabalho. Por todos os conselhos, incentivo, simpatia e confiança, um muito obrigado!

A todos os colegas do LIBRO, principalmente ao grupo MOP, pelo bom ambiente no trabalho. Especialmente à Sofia Lima, à Ana Silva e à Tânia Rodrigues, por tornarem os longos dias passados no laboratório bem mais leves e divertidos.

Aos meus colegas de curso e amigos, pela partilha de experiências, conselhos e sentimentos. Especialmente à Patrícia, ao Rui e ao Pedro, pela paciência demonstrada principalmente nesta última fase, à Marta pela amizade construída e pelos bons momentos partilhados mesmo durante as longas sessões noturnas, e ao Veloso, pela sua amizade de sempre ao longo destes anos.

Aos meus amigos que me fizeram relembrar como é bom voltar a casa. Especialmente à Márcia, pela força e incentivo mesmo quando distante.

Ao Tiago, por toda a compreensão, carinho, por toda a ajuda demonstrada sempre que necessário, no fundo, por estar sempre presente.

À minha família, principalmente à minha avó, pela preocupação e confiança transmitida, e ao meu avô, que apesar de infelizmente não me ter visto a concluir esta etapa, muito contribuiu para o seu progresso. Ao meu irmão, pelos bons momentos passados na sua companhia e finalmente aos meus pais, por me apoiarem sempre, por acreditarem em mim e por me darem força nos momentos de dificuldade. Obrigada pela nossa união, e por me orientarem o melhor que puderam!

A todos, um muito obrigado!

Abstract /Resumo

Abstract

Microbial contamination is an ongoing concern in food processing areas, mainly due to its impact in global public health. In recent years, most of the reported foodborne outbreaks have been caused by *Salmonella* Enteritidis. Although there are several disinfectants used in food environments, it has been reported that microbial cells that survive chemical disinfection may express resistance to antibiotics and changes in gene expression. However, there is still a lack of knowledge about these phenomena regarding biofilms cells, which is a matter of concern due to the increased disinfection resistance associated with these microbial communities. Hence, the main goal of this study was to investigate the effect of exposure to chemical disinfectants in the resistance and virulence of *S.* Enteritidis biofilm cells, in order to have some insights about what may occur in case cells released from these biofilms come in contact with a host. To this purpose, in first stage, biofilms susceptibility was evaluated to four disinfectants commonly used in food industry – benzalkonium chloride, triclosan, sodium hypochlorite and peroxide hydrogen –, and then biofilms were periodically exposed to sublethal concentrations of each disinfection agent, in order to get an approach to what may happen in food processing facilities when insufficient cleaning and disinfection take place. After exposure to the disinfectants, biofilm-derived cells were phenotypically characterized in terms of biofilm formation ability and resistance to antibiotics, including four of the most commonly used to treat salmonellosis (ampicillin, ciprofloxacin, cefotaxime and chloramphenicol) and one with a wide range of activity (tetracycline). Moreover, analysis of stress response gene *rpoS* and virulence genes *invA*, *avrA* and *csgD* was also performed through quantitative real-time polymerase chain reaction. The results showed that *S.* Enteritidis biofilm-derived cells were more susceptible to triclosan and less susceptible to sodium hypochlorite and peroxide hydrogen. Regarding antibiotic susceptibility, biofilm-derived cells demonstrated a lower susceptibility than planktonic cells. Moreover, despite exposure to disinfectants led to alterations on antibiotic susceptibility, no resistance was observed. The only exception corresponded to ciprofloxacin, to which both planktonic and biofilm cells, before and after exposure, were considered resistant. Exposure to sodium hypochlorite and peroxide hydrogen enhanced biofilm formation ability and, concerning gene expression, benzalkonium chloride was the disinfectant with the highest influence on the overexpression of *S.* Enteritidis virulence genes. In view of the results obtained in this study, biofilm cells that survived to disinfecting agents may represent an increased public health risk, since they can present decreased susceptibility to antibiotics, enhanced biofilm formation ability, and an overexpression of virulence and stress response genes, which may lead to an increase in *Salmonella* pathogenicity in the case of a possible infection occur by contact of these biofilm-derived cells with a host.

Resumo

A contaminação microbiana constitui um grave problema em áreas de processamento alimentar, principalmente devido ao impacto que apresenta na saúde pública global. Nos últimos anos, a maioria dos surtos alimentares tem vindo a ser causado por *Salmonella* Enteritidis. Embora existam vários desinfetantes disponíveis, tem vindo a ser reportado que células microbianas capazes de sobreviver à desinfeção química podem expressar resistência a antibióticos e mudanças na sua expressão genética. Contudo, há ainda uma falha de conhecimento no que se refere a células de biofilme, os quais representam uma preocupação acrescida no âmbito alimentar dada a sua maior resistência à desinfeção. Assim, o principal objetivo deste estudo foi investigar o efeito da exposição a desinfetantes na virulência de células de biofilmes de *S.* Enteritidis, de modo a obter algumas indicações sobre o que poderá acontecer no caso de células libertadas por estes biofilmes entratarem em contacto com um hospedeiro. Numa primeira etapa, foi avaliada a suscetibilidade dos biofilmes a quatro desinfetantes comummente utilizados na indústria alimentar – cloreto de benzalcónio, triclosan, hipoclorito de sódio e peróxido de hidrogénio -, seguindo-se a exposição periódica dos biofilmes a concentrações subletais de cada um dos desinfetantes, de modo a mimetizar o que acontece em instalações de processamento alimentar quando ocorre limpeza e desinfeção insuficientes. Após exposição aos desinfetantes, as células provenientes de biofilme foram caracterizadas fenotipicamente em termos de capacidade de formação de biofilme e de resistência a quatro dos antibióticos mais comumente usados para tratar salmonelose (ampicilina, ciprofloxacina, cefotaxima e cloranfenicol), assim como a um antibiótico com vasta gama de atividade (tetraciclina). Além disso, também foi realizada a análise de expressão do gene de resposta ao stress *ropS* e dos genes de virulência *invA, avrA, csgD,* através da reação em cadeia da polimerase em tempo real quantitativa. Os resultados obtidos demonstraram que células derivadas de biofilme foram mais suscetíveis ao triclosan e menos suscetíveis ao hipoclorito de sódio e ao peróxido de hidrogénio. Relativamente à suscetibilidade a antibióticos, as células provenientes de biofilme revelaram uma menor suscetibilidade do que as células planctónicas. Apesar de a exposição ter levado a alterações da suscetibilidade a antibióticos, não foi observada a ocorrência de resistência. A única exceção correspondeu à ciprofloxacina, para a qual células planctónicas e de biofilme, antes e após exposição, foram consideradas resistentes. O hipoclorito de sódio e o peróxido de hidrogénio provocaram um aumento da capacidade de formação de biofilme e o cloreto de benzalcónio foi o desinfetante com uma maior influência na sobre-expressão de genes de virulência. Face aos resultados obtidos é possível concluir que células de biofilme sobreviventes a agentes de desinfeção podem representar um risco acrescido para a saúde pública, uma vez que podem apresentar uma diminuição da suscetibilidade a antibióticos, maior capacidade de formação de biofilme e uma sobre-expressão de genes de virulência, que por sua vez podem levar a um aumento da patogenicidade de *Salmonella* no caso de uma possível infeção ocorrer pelo contacto destas células provenientes de biofilme com um hospedeiro.

Outline of this thesis

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This thesis is organized in eight chapters.

In **chapter 1** a literature review is conducted, providing an overview of aspects related with the central subjects of this thesis. Concern about microbial contamination in food processing areas, disinfection procedures and its possible effects in the survival or acquirement of resistance by foodborne pathogens are addressed. In the last topic of this chapter the scope and aims of this thesis are described.

Chapter 2 concerns to fundamental principles of the techniques used in the present work, as well as the rationale for their employment.

In **chapter 3** the materials and methods used in experimental work are described.

The results obtained during this study are presented in **chapter 4**, and the relation and discussion of these data are addressed in **chapter 5**.

Chapter 6 corresponds to the main conclusions obtained with this study, and **chapter 7** provides an overview of future approaches in this research area.

Finally, **chapter 8** includes a list of the references cited in this thesis.

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Abbreviations

AMP – Ampicillin

BAC - Benzalkonium chloride

CBD - Calgary Biofilm Device

CDC - Centers for Disease Control and Prevention

cDNA - Complementary Deoxyribonucleic Acid

CEF – Cefotaxime

CFU – Colony Forming Units

CIP - Ciprofloxacin

CLO - Chloramphenicol

Cq - Quantification cycle

CV - Crystal Violet

DNA – Deoxyribonucleic Acid

dsDNA - Double-stranded Deoxyribonucleic Acid

ECDC - European Centre for Disease Prevention and Control

EFSA - European Food Safety Authority

EPS - Extracellular Polymeric Substances

EU – European Union

EUCAST - European Committee on Antimicrobial Susceptibility Testing

FDA - Food and Drug Administration

gDNA - Genomic Deoxyribonucleic Acid

HACCP - Hazard Analysis Critical Control Point

HP - Hydrogen peroxide

LB - Luria Bertani Broth Miller

MBEC - Minimum Biofilm Eradication Concentration

MIC - Minimum Inhibitory Concentration

mRNA - Messenger ribonucleic acid

NRT - No Reverse Transcriptase

NTC - No Template Control

OD - Optical density

PAA - Peracetic acid

PCR - Polymerase Chain Reaction

QAC - Quaternary Ammonium Compound

qPCR – Quantitative real-time Polymerase Chain Reaction

RNA - Ribonucleic acid

rRNA - Ribosomal Ribonucleic Acid

SH - Sodium Hypochlorite

SPI - *Salmonella* Pathogenicity Island

Tafi - Thin Aggregative Fimbriae

TC – Triclosan

TET – Tetracycline

TSA - Tryptic Soy Agar

Chapter 1 - Introduction

1.1. Microbial food contamination

Microbial contamination is an ongoing concern in food industries and food processing areas (Evans *et al.*, 2004; Reij and Aantrekker, 2004; Le Gentil *et al.*, 2010; Carlin, 2011; Carrasco *et al.*, 2012). In all stages of food manipulation, from the product harvesting to the consumers, microorganisms may influence both the human health and the quality of food. Of all microorganisms, bacteria are of greater importance for food contamination (CDC/MMWR, 2013), since they have the ability to persist on several environmental conditions. The consumption of food contaminated with particular microorganisms and microbial products may cause serious illness and food poisoning, having a strong impact on global public health. Moreover it also leads to high economic losses, decreasing food quality and shelf life.

To ensure food safety principles, preventive and safety measures have been taken in food industries, as the use of effective programs of quality control, implementation of Hazard Analysis Critical Control Point (HACCP) programs, and the use of increasingly safe methods during processing, transportation, storage and distribution of food. In addition, it is fundamental the training of food handlers and education of consumers in order to avoid foodborne hazards (Todd, 2003; Sofos, 2008; Todd *et al.*, 2009; Havelaar *et al.*, 2010; Ravishankar *et al.*, 2010; Seaman, 2010).

1.2. Foodborne diseases and main pathogens

Foodborne diseases can occur from incorrect storage of foods, consumption of raw or insufficiently cooked foods, contaminated ingredients and through incorrect food handling, such as contact with utensils carrying pathogens or an improper hand hygiene (Bracket, 1999; Harris *et al.*, 2003; Todd *et al.*, 2009; Newell *et al.*, 2010; Ravishankar *et al.*, 2010; Kalyoussef and Feja, 2014). There are several foodborne diseases caused by viruses, bacteria, parasites, toxins, metals and prions, which are responsible for high rates of morbidity and mortality. Table 1.1 shows the major foodborne pathogens and the main characteristics of the diseases they cause.

Table 1.1 Bacterial agents responsible for common foodborne illness. Adapted from FDA, 2014.

Etiologic agent	Disease	Signs and symptoms	Foods sources
Bacillus cereus (diarrheal toxin)	Intoxication	Abdominal cramps, diarrhea, nausea	Meats, stews, gravies, vanilla sauce
Clostridium botulinum	Intoxication	Weakness, dizziness, blurred vision, difficulty speaking, swallowing and breathing, paralysis, diarrhea, possible death.	Inadequately processed, home-canned foods, sausages, seafood products, chopped bottled garlic, honey
Campylobacter jejuni	Infection	Diarrhea (may be bloody), cramps, fever, vomiting	Raw and undercooked poultry, unpasteurized milk, contaminated water
Escherichia coli	Infection	Diarrhea, abdominal cramps, vomiting	Water or food contaminated with human feces, undercooked beef, unpasteurized milk and juice, raw fruits and vegetables
Listeria monocytogenes	Infection	Fever, muscle aches, nausea, diarrhea; pregnant women may have mild flu-like illness and infection can lead to premature delivery; elderly or immunocompromised patients may have meningitis	Fresh cheeses, unpasteurized or inadequately pasteurized milk, ready-to-eat deli meats
Salmonella spp.	Infection	Diarrhea, fever, abdominal cramps, vomiting	Contaminated eggs, poultry, unpasteurized milk or juice, cheese, contaminated raw fruits, vegetables

Tabela 1.1 (Cont.) Bacterial agents responsible for common foodborne illness.

Etiologic agent	Disease	Signs and symptoms	Foods sources
***Shigella* spp.**	Infection	Abdominal cramps, fever, diarrhea (may be bloody)	Food or water contaminated with human fecal material, ready-to-eat foods touched by infected food workers (raw vegetables, salads, sandwiches)
Staphylococcus aureus (preformed enterotoxin)	Intoxication	Sudden onset of severe nausea and vomiting, abdominal cramps, diarrhea, fever	Unrefrigerated or improperly refrigerated meats, potato and egg salads, cream pastries

Just in 2012, 5363 foodborne outbreaks were reported in European Union (EU), causing a high number of human cases and hospitalizations. According to European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), the three main food vehicles in the reported foodborne outbreaks were eggs and egg products (22%), followed by mixed food (15.6%) and finally fish and fish products (9.2%). Campylobacteriosis was, once again, the most frequently reported zoonotic disease in 2012. Nevertheless, *Salmonella* outbreaks were the ones that accounted for the majority of hospitalizations and deaths, being Portugal one of the three countries with the highest hospitalization rates (EFSA and ECDC, 2014a). Between 2008 and 2012, most of the reported outbreaks in EU remain to be caused by *Salmonella*, followed by bacterial toxins, viruses and *Campylobacter* (Figure 1.1). Indeed, in spite of it has been observed a decline in the number of outbreaks caused by *Salmonella* from 2008 to 2011, it has observed a slight increase in 2012.

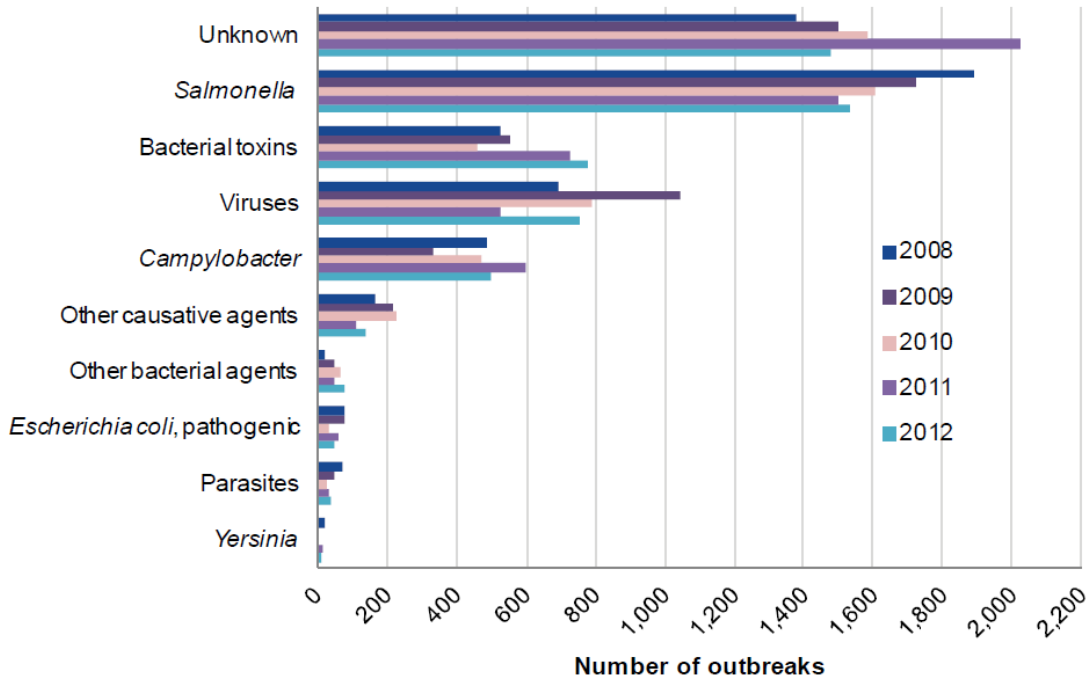

Figure 1.1 Total number of foodborne outbreaks in Europe Union, 2008 - 2012. Adapted from EFSA and ECDC, 2014a.

1.2.1. *Salmonella enterica* – characteristics and pathogenicity

Salmonella spp. corresponds to members of the family *Enterobacteriaceae* and the genus is divided into two species, *Salmonella enterica* and *Salmonella bongori*. The specie *Salmonella enterica* is subdivided into six subspecies: *enterica, salamae, arizonae, diarizonae, hutnae* and *indica*, based on biochemical differences. The species *Salmonella enterica* subspecies *enterica* has a large variety of serotypes such as Enteritidis, Typhimurium, Heidelberg, Typhi, Agona, Infantis, Virginia and others. Differences in lipopolysaccharide and flagellar structures generate the antigenic variation that is reflected in the more than 2500 serotypes, considered as potential pathogens in both animals and humans (Norhana *et al.*, 2010). In order to avoid confusion between serotypes and species, and to shorten reports, the serotype name starts with a capital letter, is not italicized, and the nomenclature may be written with the genus followed directly by the serotype name. For example, *Salmonella enterica* subspecie *enterica* serotype Enteritidis may be written as *Salmonella* Enteritidis or *S.* Enteritidis (Brenner *et al.*, 2000).

Salmonellae are gram negative, facultative anaerobes and mesophilic, with optimum growth temperature between 35 and 37 °C and optimum pH around 7. These bacteria are oxidase negative,

catalase positive and chemoorganotrophic, with ability to metabolize nutrients by the respiratory and fermentative pathways. The genus comprises bacilli from 0.5 to 3 micrometres, nonsporulating and most strains are motile by flagella. They are unable to grow under dehydration conditions and are killed by pasteurization (Norhana *et al.*, 2010). Widely distributed in nature, and with humans and animals as their primary reservoirs, their usual habitat is the intestine of both cold and warm blooded animals. Although unable to multiply outside of the host digestive tract, these bacteria have the ability to live in water and in soil when conditions are favorable, being a versatile harmful pathogen in food processing areas.

Several studies have evaluated bacterial contamination by *Salmonella* in different food sources and in different countries (Arguello *et al.*, 2012; Bouzidi *et al.*, 2012; Finstad *et al.*, 2012; Kuijpers and Mooijman, 2012; Lambertini *et al.*, 2012; Paulsen *et al.*, 2012; Zweifel and Stephan, 2012; Kotzekidou, 2013). Centers for disease control and prevention (CDC) report that *Salmonella* is the most important foodborne bacterium causing disease in humans in industrialized countries (CDC, 2011). In 2012, among the confirmed *Salmonella* outbreaks with a serotype reported, Enteritidis was the most common serotype (CDC/MMWR, 2013; EFSA and ECDC, 2014a) associated to human confirmed cases, followed by *S.* Typhimurium, as in previous years (Figure 1.2). *S.* Enteritidis was a rare serotype until the mid-late 1980s when it emerged as a frequent cause of salmonellosis in European countries and across the world (Cogan and Humphrey, 2003).

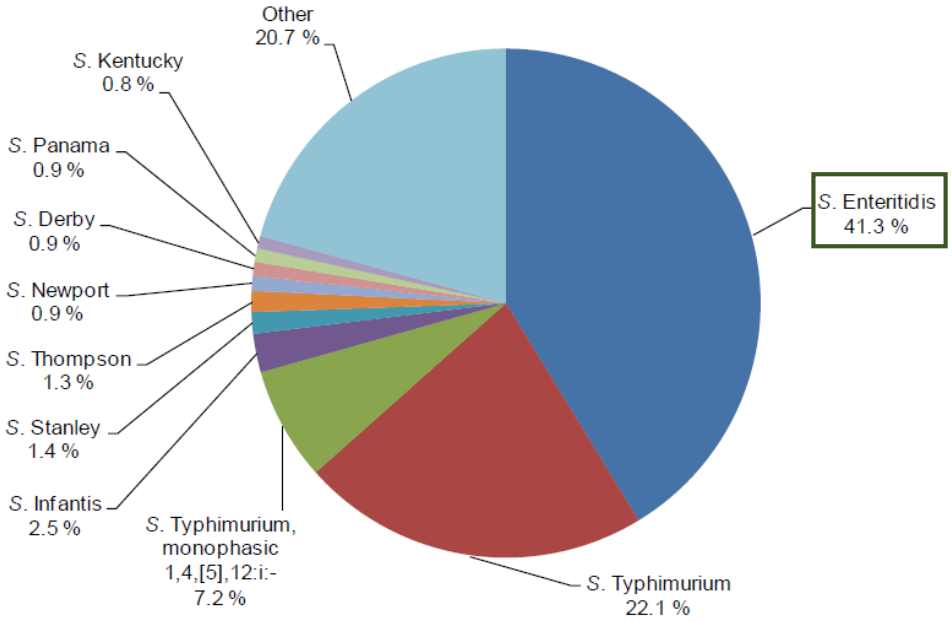

Figure 1.2 Distribution of the 10 most common *Salmonella* serotypes in humans, in EU, 2012. Adapted from EFSA and ECDC, 2014a.

The infectious dose of *Salmonella* necessary to induce infection can be influenced by several factors, according to host susceptibility, bacterial strain, growth condition, and virulence of the serotype (Fluit, 2005). Some reports point out that 1 to 10 cells may be sufficient to cause salmonellosis on persons more vulnerable to infections (Yousef and Carlstrom, 2003; Bhunia, 2008). Children and elderly individuals are at a greater predisposition for disease and should be treated presumptively (Gill and Hamer, 2001). In fact, salmonellosis results from a true foodborne infection because the bacteria multiply and invade the intestinal mucosa where they produce an enterotoxin and cytotoxin that destroy the epithelial cells (Alouf and Freer, 1999). The symptoms usually appear 12 to 36 hours after ingestion of contaminated food and the most common symptoms are characterized by the appearance of diarrhea, nausea, abdominal pain, fever and vomiting, which usually persist for 2 to 5 days but can last for several weeks (Kit *et al.*, 2011). However, in some patients the infection may be more serious, developing bacteremia, and also the associated dehydration can be life threatening but mortality is usually low (Hohmann, 2001).

The initial source of *Salmonella* is the intestinal tract of a wide range of domestic and wild animals which may result in a variety of foodstuffs of both animal and plant origin becoming contaminated with faecal organisms (Prescott *et al.*, 2002). Transmission often occurs when organisms are introduced in food preparation areas, due to inadequate storage temperatures, inadequate cooking or cross contamination of ready-to-eat food. In addition, the microorganisms can also be transmitted by direct contact with either humans, animals or faecally contaminated environments. Infected food handlers may also act as a source of contamination for foodstuffs. In relation to salmonellosis treatment, although usually therapy through antibiotics not be required, fluoroquinolones, third generation cephalosporins and ampicillin are the main choices for antibiotic therapy (Gill and Hamer, 2001; Kit *et al.*, 2011).

1.3. Adhesion and biofilm formation by *Salmonella enterica* on food processing surfaces

For *Salmonella* to persist in a food producing facility it is important that adhesion occurs and, consequently, biofilm production. Bacterial adhesion is a complex process caused by balance between different interactions, which depends of the surrounding medium, the attachment surface and the bacterial cells (Abdallah *et al.*, 2014). Biofilms are complex microenvironments formed by populations developed from a single or multiple species (Davies, 2003), and which can be found in

a variety of biotic or abiotic surfaces, usually immersed in a liquid medium. They are the most common form of organization of microorganisms, mainly due to the set of advantages that this lifestyle features compared to the planktonic lifestyle (Mah and O'Toole, 2001; Hall-Stoodley *et al.,* 2004; Fux *et al.*, 2005; Scher *et al.,* 2005; Marin *et al.,* 2009; Monds and O'Toole, 2009; Van Houdt and Michiels, 2010; Giaouris *et al.*, 2012; Abdallah *et al.*, 2014).

The development of biofilms involves cell–surface and cell–cell interactions which determine structure, function and composition of biofilm (Bridier *et al.*, 2011; Wong and O'Toole, 2011). In biofilms, microorganisms are involved in a gelatinous polymeric matrix consisting mainly by water and their excretion products (extracellular polymeric substances) (Sutherland, 2001; Donlan and Costerton, 2002; Yang *et al.*, 2012). Moreover, the spatial localization of cells within biofilm matrix may be responsible to different behaviours and expression patterns (Bridier *et al.*, 2011), and even a development of a dormant state (Monds and O'Toole, 2009). According to Srey *et al.* (2013), biofilm formation is a complex and dynamical process consisting of five phases: initial attachment, irreversible attachment, development of biofilm architecture, maturation and dispersion (Figure 1.3). These formation stages are common to many different microorganisms that have biofilm formation capability.

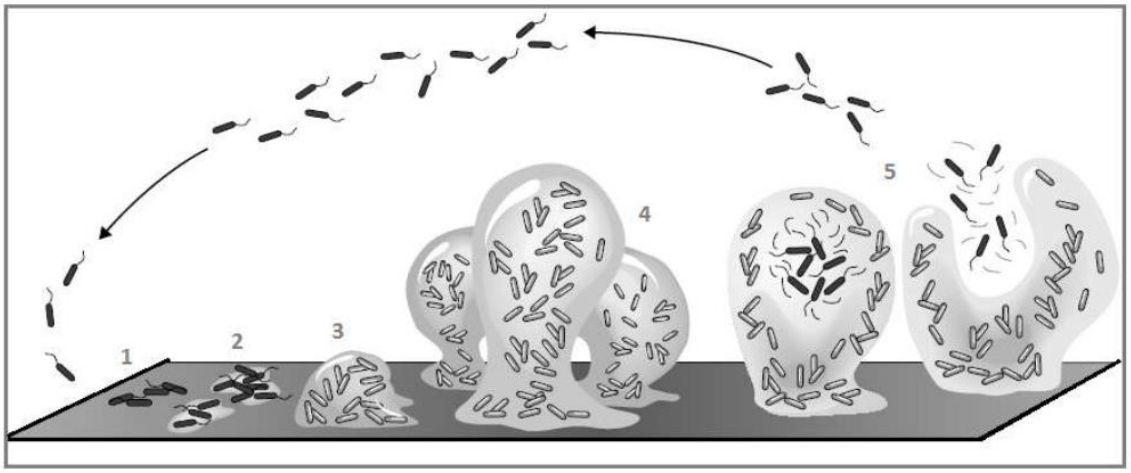

Figure 1.3 Representation of the five main stages of biofilm formation Stage 1 - Initial and reversible attachment; Stage 2 - Irreversible attachment; Stage 3 - Development of biofilm architecture; Stage 4 - Biofilm maturation; Stage 5 - Dispersion of biofilm cells allowing motile cells colonize others surfaces. Adapted from Stoodley *et al.*, 2002.

Initial attachment of cells (stage 1) occur mainly due to electrostatic charges and organic molecules which covering surface. Following, an irreversible attachment happens through the loss of mobility of cell and exopolysaccharides production (stage 2). Division and growth of microorganisms, as well as greater production and excretion of extracellular polymeric substances (EPS) leads to the development of biofilm architecture (stage 3) (Donlan, 2002). However, the development of an organized and defined structure, with quorum sensing playing an important role, contributes to biofilm maturation (stage 4). Quorum sensing is a process by which bacteria sense and respond to their own population density or changes in their environment and is related with cell-to-cell communication, being an important factor in biofilm regulation (Davey and O'Toole, 2000; Skandamis and Nychas, 2012). In addition, quorum sensing is related with the expression of exopolysaccharide biosynthesis genes (Davey and O'Toole, 2000) and it regulates colonization and virulence (Walters and Sperandio, 2006). Finally, release of the cells occurs due to endogenous enzymatic degradation, release of EPS, movement of fluid or mechanical shock (Srey *et al.*, 2013). Dispersion of biofilm cells allows motile cells colonize others surfaces.

Biofilm formation by *Salmonella* is a major problem in the food industry and food processing (Cogan *et al.*, 1999; Moore *et al.*, 2007). Cellulose - which is essential for survival of the bacteria in the environment -, and thin aggregative fimbriae (Tafi) - which is related to adhesion, cell aggregation and biofilm development - are the two components which have been identified as important in the biofilm matrix of *Salmonella* (Gerstel and Römling, 2003). Different studies have reported the *Salmonella* ability of biofilm formation and survival in abiotic surfaces that are nowadays frequently used in food-processing environments, such as plastic, rubber, metal, glass and stainless steel (Joseph *et al.*, 2001; Solano *et al.*, 2002; Stepanovic *et al.*, 2004; Giaouris and Nychas, 2006; Mangalappalli-Illathu and Korber, 2006; Oliveira *et al.*, 2006; Oliveira *et al.*, 2007; Giaouris *et al.*, 2012; Steenackers *et al.*, 2012). Some specific examples include biofilm formation in cooling towers, circuits transport of water, air-conditioning and in heat exchangers. Once that *Salmonella* colonizes these surfaces and has ability of biofilm formation, cells can detach from biofilm structure and quickly spread and continue to contaminate food, utensils and additional surfaces commons in the processing line, thus enabling cross contamination (Cogan *et al.*, 1999; Barker *et al.*, 2003; Moore *et al.*, 2007; Castelijn *et al.*, 2012).

There are several environmental factors with relevance for the food industry that affect biofilm formation, such as nutrient levels, temperature, osmolarity, pH and others conditions (Donlan, 2002;

Stepanovic *et al.*, 2003; Stepanovic *et al.*, 2004; Goeres *et al.*, 2005; Mangalappalli-Illathu *et al.*, 2008a; Castelijn *et al.*, 2012; Abdallah *et al.*, 2014). A study carried by Solano *et al.* (1998) demonstrated that *S.* Enteritidis cells were able to develop a biofilm when incubated under stress conditions, namely in medium with a single source of carbon and energy (glucose) and starvation of several essential elements (nitrogen, phosphorus, calcium, magnesium, sulfur and iron). A study of Bonafonte *et al.* (2000) confirmed the correlation between glycogen accumulation and production of extracellular matrix for biofilm formation, because when glucose concentration was increased the quantity of biofilm was enhanced. The process of bacterial biofilm formation has been an emerging issue, and studies of biofilms molecular physiology have been performed (Ghigo, 2003; Sauer, 2003; Fux *et al.*, 2005). For example, regarding *Salmonella* biofilms, Giaouris and his coworkers (2013) studied the protein expression of *S.* Enteritidis when grown as biofilm and planktonic lifestyle, and observed that only in biofilms cells were expressing proteins involved in global regulation and stress response, nutrient transport, degradation and energy metabolism, detoxification and curli production. However, further investigations have to be done to understand the biological processes involved in the development of surface adherent microbial communities, and also the mechanisms that are involved in antimicrobial resistance, since biofilms demonstrate a high resistance to different chemicals agents and antibiotics compared to planktonic cells.

1.4 Chemical disinfection in food industry

In the food industry, regular cleaning to remove compounds that can promote bacteria proliferation and biofilm formation is required (Simões *et al.,* 2010). However, cleaning is not sufficient to ensure the prevention of contamination because it not allows the total removal of bacteria (Gram *et al.*, 2007). In this way, cells might re-attach to other surfaces and form biofilms (Gram *et al.*, 2007; Srey *et al.,* 2013), which have been identified as a major issue in HACCP programs (Sharma and Anand, 2002). Thus, the application of cleaning and disinfection procedures is essential for maintaining health and safety in food industries. For example, a study conducted by Kuda (2011) to confirm the importance of eliminating food sediment from surfaces in food-related environments demonstrated that egg compounds protect *S.* Typhimurium from drying and from the disinfectant treatments. Nevertheless, the protective effect of egg compounds on bacterial viability disappeared

with a proper washing process (Kuda *et al.*, 2011). Furthermore, it has found that, generally, disinfectants do not penetrate the biofilm matrix after an ineffective cleaning procedure and, thus, do not destroy all the biofilm living cells (Simões *et al.*, 2006), which highlights the importance of a correct cleaning and the need for an association of this process with disinfection.Disinfection is defined as a process that consists in the destruction of microorganisms, except bacterial endospores, on non-living objects or surfaces by physical or chemical agents (disinfectants) (Morello *et al.*, 1998; Dvorak, 2008). Chemical disinfection is influenced by biological factors, pH, exposure concentration, surface characteristics, contact time, temperature, and chemical and physical properties of contaminating substances that may be present (Schmidt, 2003). Studies reported that disinfectants are more effective in the absence of organic material, including fat, carbohydrates, and protein-based materials (Kuda *et al.*, 2008; Simões *et al.*, 2010). The mode of action and disinfectant efficacy against different kinds of organisms are determined by its chemical nature, thus disinfectants can be classified according to their chemical nature and activity (Morello *et al.*, 1998). Table 1.2 demonstrates the main disinfectants used in food industries, their targets and mechanisms of interaction with microorganisms.

Table 1.2 Targets and mechanisms of action of disinfectants commonly used in food industries. Adapted from McDonnell and Russell, 1999; Sheldon, 2005; Rodrigues, 2010; Araújo *et al.*, 2011.

Type / class of disinfectant	Specific examples	Antimicrobial target	Mechanism of interaction
Alcohols	Ethanol Isopropanol	Bacterial membrane	Denaturing of proteins; inhibition of DNA, RNA, protein and peptidoglycan synthesis.
Aldeydes	Glutaraldeyde Formaldehyde	Cell envelope (cell wall, outer membrane) and cross-linking of macromolecules	Cross-linking of proteins, RNA and DNA; inhibition of cellular metabolism and replication.
Bisphenols	Triclosan	Essential enzymes and cell wall	Binding to enoyl-acyl carrier protein reductase, causing inhibition of fatty acid biosynthesis and precipitating cell wall proteins.

Table 1.2 (Cont.). Targets and mechanisms of action of disinfectants commonly used in food industries.

Type / class of disinfectant	Specific examples	Antimicrobial target	Mechanism of interaction
Halogen releasing agents	Iodine and chlorine compounds	DNA and amino groups in proteins	Inhibition of DNA synthesis; disrupt; oxidative phosphorylation and membrane-associated activities
Peroxygens	Hydrogen peroxide Peracetic acid (PAA)	DNA and protein thiol groups	Hydrogen peroxide produces hydroxyl free radicals that function as oxidants, which react with lipids, proteins, and DNA, thus increasing cell permeability; PAA causes disruption of thiol groups in proteins and enzymes.
Phenols and cresols	Lysol Staphene Amphyl	Cytoplasmic membrane	Rupture of cell membranes and denaturation of cellular constituent
Quaternary ammonium compounds	Cetrimide Benzalkonium chloride	Cytoplasmic membrane	Damage cell wall and cytoplasmic membrane mediated by binding to phospholipids, resulting in loss of structural integrity of the cytoplasmic membrane, leakage of intracellular components and cell lysis.

Chlorine compounds, quaternary ammonium compounds (QACs), bis-phenols and peroxygen compounds are among the types of disinfectant more commonly used in food processing areas and, since they were used in this study, a briefly approach of these chemicals agents will be addressed.

Chlorine Compounds – Sodium Hypochlorite

Chlorine compounds may be used as hypochlorite, liquid chlorine, inorganic chloramines and organic chloramines (Schmidt 2003). In spite of chlorine compounds may contribute to equipment corrosion (especially at higher temperatures), environmental contamination and pose health risks (since there is formation of undesirable halogenated compounds and may occur irritation of skin, mucous membranes and eyes, at low pH) (Kennedy *et al.*, 2000), their wide spectrum of action, effectiveness, reduced cost and ease of application provide advantageous characteristics for be used on a wide scale in food industries. Their activity is determined by the amount of the available chlorine of the solution and these compounds have been found to be more effective on Gram-negative bacteria than Gram-positive bacteria (Virto *et al.*, 2005). Chlorine compounds are very reactive and its efficiency can be impacted by organic soling matters, high temperatures, light and high pH levels (Huss 2003; Dvorak, 2008). For food contact surface, the maximum allowable concentration of available chlorine is 200 µg/ml (FDA, 2000). These compounds have ability to damaging the outer cell membrane, producing a loss of permeability control and subsequent lysis of the cell (Virto *et al.*, 2005). Moreover, these compounds inhibit cellular enzymes, such as sulfhydryl enzymes and enzymes involved in glucose metabolism, and destroy deoxyribonucleic acid (DNA) by oxidation of purine and pyrimidine bases (Schmidt, 2003).

Sodium hypochlorite (SH) – NaOCl - is a strong oxidizer (Peng *et al.*, 2002), which bactericidal effect is related to the agent penetration and its oxidative action on essential enzymes in the cell (Lomander *et al.*, 2004). The effectiveness of sodium hypochlorite against *Salmonella enterica* has been evaluated (e.g. Ramesh *et al.*, 2002; Wong *et al.*, 2010; Rodrigues *et al.*, 2011). While in planktonic growth, a SH concentration above 0.5 g/l appears to be sufficient to obtain 5 $\log_{10}$ kill of *Salmonella* after 5 min exposure (Kusumaningrum *et al.*, 2003; Moretro *et al.,* 2009; Wong *et al.*, 2010), on two day old biofilms 0.5 g/l SH leads to 3.2 $\log_{10}$ reduction after 5 min exposure (Vestby *et al.*, 2009a).

Quaternary Ammonium Compounds – Benzalkonium Chloride

Quaternary ammonium compounds (QACs) are disinfectants commonly used for a variety of clinical purposes and also in food production environments, due to several advantageous properties: they have little toxicity and corrosivity, and are nonirritating and odorless. In addition, they form a

residual antimicrobial film after being applied to surfaces and they are effective in the presence of organic matter (McDonnell and Russell, 1999; Schmidt 2003).

QACs are effective against a great variety of microorganisms, and at lower concentrations (0.0005 %) Gram-positive bacteria are more sensitive to QACs than Gram-negative bacteria (Maillard, 2002). They are active in a wide pH and temperature range, but their activity is greater at high temperatures and in alkaline situations (Shirai *et al.*, 2000). QACs may be applied at variable concentrations, however they are commonly applied at maximum 200 μg/ml in food contact surfaces and require a long time exposure (longer than 15 to 60 minutes) (FDA, 2000). In these compounds, one nitrogen atom is bound to four organic groups and their resultant positive charges are attracted to the negatively charged surfaces of microorganisms and bind irreversibly to phospholipids in the cytoplasmic membrane (McBain *et al.*, 2004; Gilbert and Moore, 2005). Therefore, it occurs a loss of structural organization and integrity of the cytoplasmic membrane, impairing permeability, and also cell leakage. Nevertheless, the wide application of QACs in food processing areas may cause the adaptation of the microbial cells (Langsrud *et al.*, 2003).

Benzalkonium chloride (BAC) - alkyl dimethyl benzyl ammonium chloride - is a synthetic derivative of ammonium chloride, with the hydrogen atoms being replaced by organic groups (methyl, ethyl, and/or benzyl groups), and is widely used in food-processing environments (Holah *et al.*, 2002; Ioannou *et al.*, 2007; Kuda *et al.*, 2008; Walton *et al.*, 2008). Whereas at low concentrations BAC acts on membrane permeability, causing damage to the outer membrane and the cytolytic leakage of cytoplasmic materials, at high concentrations it targets the carboxylic groups and cause general coagulation in the bacterial cytoplasm (Russell, 2002a; To *et al.*, 2002).

Peroxides – Hydrogen Peroxide

Peroxides, compounds which contain at least one pair of covalently bonded oxygen atoms, can be divided into the inorganic group (which includes hydrogen peroxide), and the organic group (which includes peracetic acid). They are strong oxidizing agents and, despite being relatively safe in their diluted form, when used in concentrated form (5% and above) they may be eye and skin irritant (Schmidt, 2003). Hydrogen peroxide (HP) is commercially available in a variety of concentrations, ranging from 3 to 90% (McDonnell and Russell, 1999). Whereas in domestic environments it is usually applied in the diluted form (3-10%), in the industrial context it is used at concentrated solutions (30% or greater). At a 5-20% concentration, HP is considered bactericidal and fungicidal

(Dvorak, 2008). HP produces hydroxyl free radicals (•OH) that cause oxidative destruction of cell components, such as lipids, proteins and DNA (McDonnell and Russell, 1999; Block, 2001). HP is a broad spectrum of activity, and it was reported to be more effective against anaerobes because they are incapable of generating catalase, which destroys the peroxide (Block, 2001). Efficacy of HP is strongly affected by temperature, since with the increase of temperature also occurs an increase of the killing effectiveness (Block, 2001). Moreover, HP is approved by the Food and Drug Administration (FDA) to be used in equipment and packages of food products, since it can rapidly degrade into water and oxygen, being considered environmentally friendly (McDonnell and Russell, 1999).

Bis-Phenols – Triclosan

Phenols are broad spectrum disinfectants which alter the cell wall permeability of microorganisms. Phenols are capable of supporting the presence of organic matter, and in order to increase the penetration capacity, phenolic disinfectants are typically formulated in soap solutions (Dvorak, 2008). In spite being generally safe for humans, a prolonged exposure to the skin may cause irritation (Kennedy *et al.*, 2000). Bis-phenols are hydroxy-halogenated derivatives of two phenolic groups connected by various bridges (McDonnell and Russell, 1999). At low concentrations (100 μg/ml), they promote the inactivation of essential membrane-bound enzymes, while and at high concentrations these compounds penetrate and disrupt the cell wall, denaturing cell wall proteins (McDonnell and Russell, 1999; Dvorak, 2008).

Triclosan (TC) - 2,4,4P-trichloro-2P-hydroxydiphenylether - is a synthetic bis-phenol agent with a broad range of activity, including Gram-positive and Gram-negative non-sporulating bacteria, and some fungi (Schweizer, 2001). Whereas at low concentrations, ranging between 0.025 and 100 μg/ml, TC is bacteriostatic, at higher levels it is bactericidal (Suller and Russell, 2000; Russell, 2004a). It is known that this agent inhibits FabI, an NADH-dependent enoyl-acyl carrier protein (ACP) reductase involved in lipid biosynthesis (Heath and Rock, 2000; Heath *et al.*, 2000). Nevertheless, it also acts nonspecifically on the bacterial cytoplasmic (inner) membrane when used at high concentrations (Denyer and Maillard, 2000; Tabak *et al.*, 2007). In fact, it was observed K^+ leakage in bactericidal levels of TC, indicative of membrane damage (Suller and Russell, 2000). TC is accepted by FDA for use as an antimicrobial for fungicide/fungistat and bacteriostat applications (Jones *et al.*, 2000), and it is present in a wide range of products such as cleaning and hygienic

products, plastics, and clothes (Schweizer, 2001; Russel, 2004a). Hence, due to the existence of so many home and personal care products containing TC, the widespread use of this disinfectant has been associated with the development of microbial resistance (Russell, 2004a).

1.5. Microbial resistance

Microbial resistance is defined as the temporary or permanent capacity of microorganisms to survive a certain treatment/compound that would destroy or inhibit other members of the strain. In this thesis, in order to avoid confusion with other interrelated words (such as antimicrobials and antibiotics), "biocidal agent" will be used as more comprehensive word when referring to any compound used to eliminate a microbial population.

Microorganisms adopt different mechanisms of resistance in accordance with their physiology and metabolism (Russel, 2002a; Tenovar, 2006). Currently, there are three types of bacterial resistance described: natural or intrinsic resistance, acquired resistance, and resistance by adaptation. Moreover, a microorganism resistant to a biocidal agent may also acquire resistance to other antimicrobials, a phenomenon known as cross resistance, (McDonnell and Russell*,* 1999; Chapman, 2003; Cloete, 2003). Increasing resistance to biocidal agents, including antibiotics and disinfectants, has been a common problem (Russell, 2004b; Sheldon, 2005; Marin *et al.*, 2009; Krolasik *et al.,* 2010). Concerning antibiotics, the main factors related with the increase of resistance are: the misuse and overuse of these compounds; misdiagnoses; bacteria lacks the target structure of a given antibiotic; and the widespread use and abuse of poorly controlled antibiotics given to cattle as prophylaxis, growth promoters or treatment, as well as in the meat and aquaculture industries (Adetunji and Isola, 2011).

On the other hand, the increasing use of chemical disinfectants in food processing industry, with possible frequent exposure of bacteria to inhibitory or sub-lethal concentrations of disinfectants, may lead to bacterial adaptive resistance towards these compounds (Randall *et al.*, 2007; Condell *et al.*, 2012). In fact, there is evidence that some harmful bacteria found in food are becoming increasingly resistant to disinfectants. Resistance to disinfectants is considered to be mainly intrinsic and, generally, gram-negative bacteria (as *Salmonella*) are more resistant to disinfectants than gram-positive bacteria (McDonnell and Russell, 1999). The main reason for intrinsic resistance in gram

negative bacteria relates to the presence of the outer membrane in cell wall, which LPS-layer acts as a barrier that limits the entry of many chemically unrelated types of biocidal agents (McDonnell and Russell, 1999; Nikaido, 2003; Sheldon, 2005; Araújo *et al.,* 2011). Nevertheless, there are other mechanisms that have been reported and considered important to chemical disinfection resistance, inclusively in *Salmonella*, such as efflux pumps, enzymatic degradation, mutations in specific antimicrobial targets and over-expression of target proteins (Moretro *et al.,* 2012). Concerning biofilms, these microbial communities exhibit a pattern of high resistance towards biocidal agents when compared with the planktonic counterparts (Mah and O'Toole 2001; Joseph *et al.,* 2001; Tabak *et al.,* 2007; Mangalappalli-Illathu *et al.,* 2008b; Marin *et al.*, 2009; Wong *et al.*, 2010). This fact may be due to specific characteristics of the microenvironment formed, as population diversity, interaction of the antimicrobial agents with the biofilm matrix and extracellular polymeric substances, limited diffusion of antimicrobial agents through the biofilm matrix, low metabolic activity of cells inside the biofilm, genetic changes in cells inside the biofilm, and appearance of new species with genetic alterations (Mah and O'Toole, 2001; Donlan and Costerton, 2002; Cloete, 2003; Scher *et al.*, 2005).

1.5.1. *Salmonella* enterica resistance to disinfectants

The ability of *S. enterica* to develop resistance towards disinfectants has been reported (e.g. Mangalapalli-Illathu *et al.*, 2006, 2008b; Araújo *et al.*, 2011; Moretro *et al.*, 2012), as well as the persistence of resistant strains in farm animals and meat-derived products, which represents an increased risk to human health associated with the consumption of contaminated products. Besides inadequate processes of disinfection, extended or repeated intermittent periods of exposure to disinfectants, and consequently development of acquired resistance, may be related to the persistence of *Salmonella* cells in food processing areas (Randall *et al.*, 2007; Condell *et al.*, 2012). The main mechanisms of resistance to chemical disinfectants in *Salmonella enterica*, concerning both planktonic and biofilm lifestyles, are shown in Table 1.3.

Table 1.3 Main mechanisms of resistance to chemical disinfectants in *Salmonella enterica*.

Lifestyle	Mechanisms	Genes	Reference
Planktonic cells	Over expression of efflux pumps	*acr* *tolC* *ramA* *marA*	Braoudaki and Hilton, 2004, 2005; Randall *et al.*, 2007; Bailey *et al.*, 2008; Webber *et al.*, 2008b; Nishino *et al.*, 2009
	Mutations in specific antimicrobial targets	*fabI*	Webber *et al.*, 2008a; Moretro *et al.*, 2012
	Over expression of curli biosynthesis	*csg*	Bailey *et al.*, 2009
	Modifications in membrane fatty acid composition	*cfa*	Kim *et al.*, 2005; Dubois-Brissonnet *et al.*, 2011
Biofilm cells	Over expression of efflux pumps	*acr, marA*	Tabak *et al.*, 2007
	Increase of protein biosynthesis	*wrbA, trxA*	Mangalappalli-Illathu *et al.*, 2006, 2008b
	Up regulation of proteins involved in the stress response	*ynaF, tpx, sodB, cspA*	Mangalappalli-Illathu *et al.*, 2006, 2008b
	Biofilm matrix	*bcsA, bcsE*	Tabak *et al.*, 2007

Several studies have assessed the efficiency of different commonly used disinfectants, both against planktonic and *Salmonella* biofilms (e.g. Ramesh *et al.*, 2002; Gradel *et al.*, 2004; Mangalappalli-Illathu *et al*, 2006; Mangalappalli-Illathu *et al.*, 2008b; Arnold and Yates, 2009; Marin *et al.*, 2009; Moretro *et al.*, 2009; Wong *et al*, 2010). A study performed by Moretro and his coworkers (2009) evaluated the bactericidal activity of nine disinfectants on *Salmonella* isolated from food industry. Although all disinfectants were efficient against *Salmonella* in suspension, disinfectants based on hypochlorite, glutaraldehyde and cationic tensides (as benzalkonium chloride) did not show a sufficient bactericidal effect on *Salmonella* biofilms on stainless steel surfaces when applied at the recommended user concentrations and after 5 min of exposure. In another study, results obtained from Wong *et al.* (2010) show that planktonic *Salmonella* cells were more susceptible to benzalkonium chloride, chlorhexidine gluconate, citric acid, quaternary ammonium compounds,

sodium hypochlorite and ethanol, than 3-day-old *Salmonella* biofilms (Wong *et al.,* 2010). Indeed, disinfection is hampered by bacteria's ability of attachment and biofilm formation, since the effectiveness of most chemical disinfectants commonly used in food processing facilities has been shown to be lower on sessile bacteria than on planktonic cells (Joseph *et al.*, 2001; Scher *et al.*, 2005).

Other studies concerning *S. enterica* biofilms, were performed by Mangalapalli-Illathu and his co-workers (2006, 2008b), who revealed that adaptive resistance plays a role in the endurance of *S.* Enteritidis biofilms against benzalkonium chloride, since biofilms adapted to benzalkonium chloride - by exposure to sub-inhibitory concentrations - acquired the ability to survive a normally lethal exposure of benzalkonium chloride and then resume growth. Additionally, this work analysed the mechanisms involved in resistance, and it was found that adaptation occurred concurrently with the up-regulation of key proteins involved in the stress response, detoxification, and an overall increase in protein biosynthesis. In a different study, Tabak and her co-workers (2007) investigated the effect of triclosan on *Salmonella* planktonic cells, biofilm-associated cells, cells derived from disrupted biofilms, and biofilm-deficient mutant cells. The results demonstrated that the higher resistance of biofilm-associated cells as compared to biofilm-derived cells can be related with matrix, namely with the low diffusion of disinfectant through the extracellular matrix. Indeed, the protective function of cellulose, as the major constituent of biofilm matrix, has been reported (Solano *et al.*, 2002; White *et al.*, 2006). Recently, a study conducted by Corcoran and her co-workers (2014) evaluated the effect of sodium hypochlorite, sodium hydroxide and benzalkonium chloride against early and mature biofilms, demonstrating that none of the agents achieved eradication of mature biofilm. In spite of others authors proposed that *S. enterica* biofilm formation over an extended period of time does not influence the efficacy of disinfection procedures (Wong *et al.*, 2010; Moretro *et al.*, 2009), several studies suggest that biofilms formed over an extensive period of time have increased resistance to biocidal agents (Mangalappalli-Illathu *et al.*, 2008a; Shen *et al.*, 2011). This resistance may be related to increase of extra-polymeric substances and biofilm thickness over time (Korber *et al.*, 1997; Xavier *et al.*, 2005).

In whole, the studies mentioned above, and many other similar to them, have showed that, although there is a wide variety of disinfectants available to combat the persistence of bacteria, there is an increased resistance of *S. enterica* to such compounds and development of new control strategies is important. Electrolyzed water, ultraviolet light, ultrasound and antimicrobial surfaces are

some of novel control strategies to combat the growth of *Salmonella* (Moretro *et al.*, 2012; Mukhopadhyay and Ramaswamy, 2012). Solutions containing enzymes, bacteriophages, microbial derived antimicrobial compounds as quorum-sensing inhibitors, probiotics and natural plant molecules have also been analyzed as possible approaches to control this foodborne pathogen (Burt, 2004; Chorianopoulos *et al.*, 2010; Simões *et al.*, 2010; Bajpai *et al.*, 2012; Oliveira *et al.*, 2012a; Skandamis and Nichas, 2012; Turki *et al.*, 2012; Woo and Ahn, 2013; Tan *et al.*, 2014).

1.5.2. *Salmonella enterica* resistance to antibiotics

The discovery of antibiotics allowed the implementation of new compounds in human and animal health, and agriculture. However, it is now established that microorganisms are able to develop mechanisms of resistance, and the emergence of resistant strains is a serious problem worldwide (Costa *et al.*, 2011). In fact, regardless of the large numbers of new antibiotics that have been developed in the last decades, bacterial resistance to such antimicrobial agents is constantly emerging, which leads to a continuous need to produce new compounds. Nowadays, the intercorrelation of different fields such as microbiology, chemistry and bioinformatics is crucial to the development of new antibiotics in order to fight bacterial resistance (Donadio *et al.*, 2010).

The mechanisms that allow *S. enterica* to survive to antibiotics have been intensively investigated over the years, and the main ones are summarized in Table 1.4. One of these mechanisms consists in biofilm formation, and several authors have been concerned with the problem of antibiotic resistance of such bacterial communities (Tabak *et al.*, 2009; Papavasileiou *et al.* 2010). In fact, a study conducted by Tabak *et al.* (2009) reported that *S.* Typhimurium biofilms formed on microplates are up to a 2000-fold more resistant to ciprofloxacin than planktonic lifestyle. The main identified characteristics that contribute to the high resistance of biofilms towards antibiotics encompass: heterogeneous biofilm growth, the high cell density that provides an efficient horizontal gene transfer, and the prolonged antibiotic selection pressure (phenomenon which alters the behavior and fitness of organisms, due to frequent antibiotic use over long periods of time) (Mah and O'Toole, 2001; Fux *et al.*, 2005). It has also been reported that components of exopolysaccharide matrix may be responsible for hindering diffusion of antibiotics (Mah and O'Toole, 2001). Moreover, it was found that β lactamase-producing organisms increase the production of this enzyme in response to antibiotic exposure. In this way, the enzyme accumulates in biofilm as result of secretion or cell lysis and deactivates β-lactam antibiotics in the surface layers more rapidly than

they diffuse into the biofilm (Fux *et al.*, 2005). Paterson (2006) reported that *S. enterica* cells may be extended-spectrum β-lactamases producers, which may be related to resistance to β-lactam antibiotics such as ampicillin.

Table 1.4 Main mechanisms and genes involved in *S. enterica* resistance to antibiotics.

Mechanisms	Genes	Reference
Mutations in specific antimicrobial targets	*gyrA, gyrB parC, parE*	Griggs *et al.*, 1996; Piddock *et al.*, 1998; Ling *et al.*, 2003; Eaves *et al.*, 2004
Over-expression of efflux pumps	*acr, tolC tet, flor*	Nikaido *et al.*, 1998; Giraud *et al.*, 2000; Piddock *et al.*, 2000; Allen and Poppe, 2002
Down regulation of membrane porins	*ompF ompC*	Karatzas *et al.*, 2008; Birošová and Mikulášová, 2009
Quorum sensing	*luxS*	Surette *et al.*, 1999
Biofilm formation	*flhE, csgD, sirA*	Gerstel and Römling, 2003; Teplitski *et al.*, 2006; Stafford and Hughes, 2007
Production of extended-spectrum β-lactamases	bla_{CTX-M}, bla_{PSE-1}	White *et al.*, 2001; Hur *et al.*, 2011

In relation to salmonellosis treatment, fluoroquinolones, third generation cephalosporins and ampicillin are the main choices for antibiotic therapy (CDC, 2013). On recent findings dated from 2012, EFSA and ECDC (EFSA/ECDC, 2014b) reported that in *Salmonella* from humans, high resistance levels were recorded to ampicillin and tetracyclines, while resistance to third-generation cephalosporins and fluoroquinolones remained low. Similarly, while in *Salmonella* isolates from fowl, pigs, cattle and meat thereof, resistance to ampicillin and tetracyclines was commonly detected, resistance to third-generation cephalosporins was generally low. Moreover, high to very high resistance to fluoroquinolones was observed in *Salmonella* isolates from turkeys, fowl and broiler meat (EFSA/ECDC, 2014b).

Several studies have analyzed antibiotic resistance in different *S. enterica* serotypes (e.g. Papavasileiou *et al.*, 2010; Adzitey *et al.*, 2012; Oliveira *et al.*, 2012b), and many of them

demonstrated the presence of multi-resistance patterns (e.g. Oliveira *et al.*, 2005; Weill *et al.*, 2006; Yan *et al.*, 2010; Ahmed *et al.*, 2012; Habrun *et al.*, 2012; Wang *et al.*, 2013). A study conducted by Suresh *et al.* (2006) demonstrated the presence of seven different resistance patterns in *S.* Enteritidis, one of which included resistance to ampicillin, kanamycin, nalidixic acid, neomycin, polymixin-B, tetracycline and sulphamethoxazole. Surveys have also been performed with the aim of examining *S. enterica* antibiotic resistance profile evolution over the years (e.g. Threlfall *et al.*, 2006; Habing, 2012). Although most studies report an increase, there is no consensus. However, the high rate of multidrug-resistant *S. enterica* isolates is a matter of great concern to global public health.

1.5.3. Cross resistance in *Salmonella enterica*

Another problem raised together with bacteria's resistance is the cross-resistance phenomenon, which is related with the fact that a pre-exposure or adaptation to a biocidal agent can affect the bacterial susceptibility to another different biocide, leading to similar resistance responses by bacteria (Cogan and Humphrey, 2003; SCENIHR, 2009). This can occur due to different situations, such as: a) both biocidal agents act on the same cellular target; b) both biocidal agents have the same transport mechanism; or c) both biocidal agents can be accommodated by the same resistance mechanism (Gilbert and McBain, 2003; SCENIHR, 2009; Condell *et al.*, 2012). Although disinfectants and antibiotics present several distinct aspects, there are also similarities in the action of these two kinds of biocidal compounds. The uptake through bacterial envelope by passive diffusion, the effect on the membrane integrity and morphology, and the effect on diverse key steps of bacterial metabolism are mechanisms that occur either by action of disinfectants and antibiotics (SCENIHR, 2009). In fact, various mechanisms of resistance to antibiotics and disinfectants were found to be common, as can be seen in Figure 1.4.

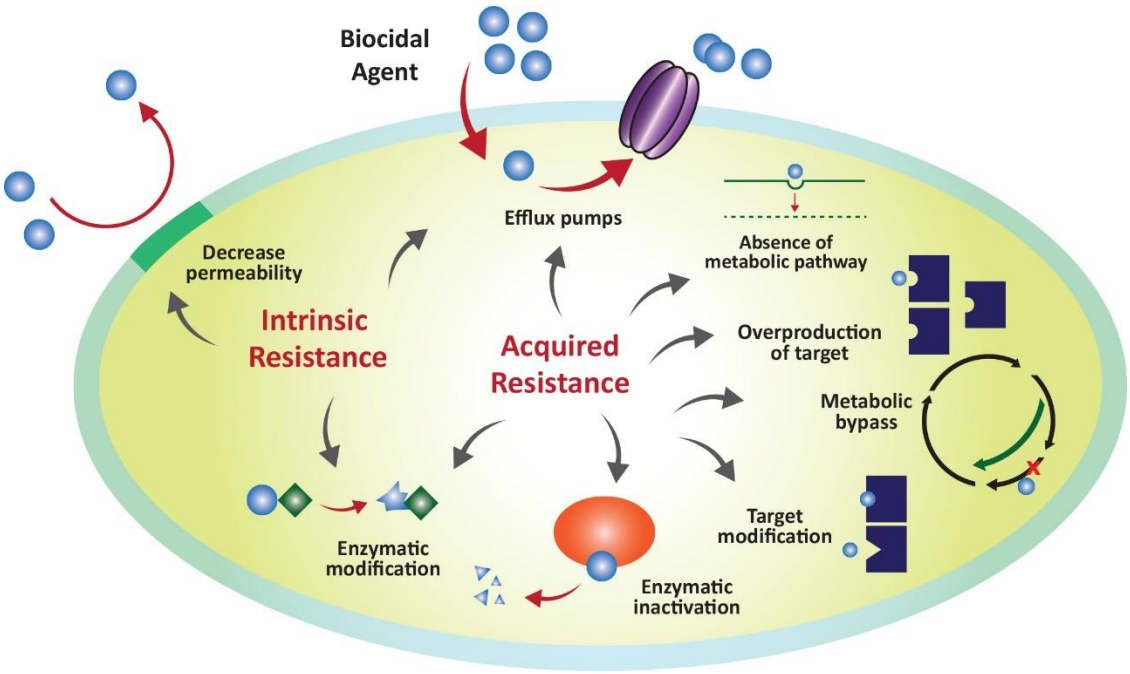

Figure 1.4 Common bacterial resistance mechanisms against disinfectants and antibiotics.

Several studies have been reported concerning the possibility of existence of cross-resistance to disinfectants and antibiotics among members of the family *Enterobacteriaceae*, which comprise *Salmonella*. A study conducted by Levy (2002) confirmed that some of the mechanisms that play a major role in resistance to disinfectants and antibiotics in *E. coli* and *S. enterica*, (namely concerning ampicillin, nalidixic acid, chloramphenicol, tetracycline, triclosan, QACs and chlorhexidine) are controlled by cascade regulations that share common gene regulators, such as *soxS* and *marA* (Levy, 2002). These data are supported by Bailey *et al.* (2009), whom showed that *E. coli* and *S. enterica* exposure to triclosan generates a modification in the expression of regulator genes (*soxS*) that are involved in the genetic control of antibiotic resistance. As previously mentioned, the use of cleaning and hygiene products containing triclosan in industrial settings has increased the environmental exposure to this disinfectant. Consequently, there are several reports that specifically address the relation between the use of triclosan and cross resistance to antibiotics (Levy, 2001; Aiello and Larson, 2003; Karatzas *et al.*, 2007; Birosova and Mikulasova, 2009; Condell *et al.*, 2012).

Randall and coworkers (2007) reported that growth of *S. enterica* serotype Typhymurium in presence sub-inhibitory concentrations of disinfectants favored the emergence of strains resistant to antibiotics. In this same study it was found that, following exposure to an aldehyde-based disinfectant, isolated mutants resistant to ciprofloxacin demonstrated either mutation in GyrA enzyme (subunit A of active

DNA gyrase) or some type of efflux mechanism. Moreover, it is known that the duration of the exposure time to disinfectants is a factor that can influence the cross-resistance to antibiotics (Sullivan *et al.*, 2003; Aiello *et al.*, 2005). In fact, a study of Aiello *et al.* (2005) showed that the use of antibacterial cleaning and hygiene products did not lead to a significant increase in antibiotics resistance after 1 year exposure. However, more extensive and longer term use might provide a suitable environment for emergence of resistant species. It is also important to notice that studies that analyze the relation between bacterial exposure to disinfectants and the emergence of cross resistance to certain antibiotics are usually done with planktonic cells. However, these studies must also be conducted in biofilms, because it has been shown that these microbial communities provide mechanisms that allow bacterial cells to be less susceptible to antimicrobial compounds (Hall-Stoodley *et al*, 2004; Marin *et al*, 2009; Van Houdt and Michiels, 2010). Moreover, although several studies have been performed regarding *S. enterica* cross-resistance to disinfectants and antibiotics, most of them were focused on *S. enterica* serotype Typhymurium, and just a few were focused on *S.* Enteritidis serotype (e.g. Potenski *et al.*, 2003; Braoudaki *et al.*, 2004; Randall *et al.*, 2004), the most common serotype reported among the confirmed *Salmonella* outbreaks. In the whole, the studies mentioned above demonstrate that exposure to sub-inhibitory concentrations of disinfectants can lead to the selection of *S. enterica* strains with reduced susceptibility or resistance to antibiotics. Although several authors have been concerned with the issue of cross-resistance to disinfectants and antibiotics (Poole, 2002; Aiello and Larson, 2003; Russell, 2004b; Aiello *et al.*, 2005; Davin-Regli and Pagès, 2012), further evaluations are needed concerning the risks and benefits of using disinfectants in food environments, as well as the possible role of biocidal resistance mechanisms in virulence.

1.6. Resistance and virulence of *Salmonella enterica*

Salmonella is one of the best studied and characterized pathogenic bacteria in terms of genetics, molecular mechanisms and virulence. Virulence can be defined as the degree of pathogenicity of an organism, and it is often correlated with pathogen's ability to survive in the external environment (Prescott *et al.,* 2002). Virulence factors help pathogenic bacteria to survive adverse environmental conditions, but are also required to facilitate colonization and to allow multiplication inside the host (Archer, 1996; Spector and Kenyon, 2012). In the specific case of

S. enterica, a series of adaptive mechanisms enables it to survive during infection against a variety of hostile conditions, such as acidic pH of the stomach, reduced oxygenation, increased osmolarity, nutrient starvation and competition with other microorganisms for space (Rychlik and Barrow, 2005). The virulence of this bacterium is quite complex since it involves numerous different virulence factors and, although various studies have been performed, this is a subject matter that needs continuous investigation and analysis, due to the emergence of new strategies that enhance its survival and contributes to pathogenicity. Virulence genes may be located on mobile genetic elements (plasmids, bacteriophages or transposons), within the chromosome as units of a few of virulence genes (islets), or in the designated pathogenicity islands (Prescott *et al.*, 2002; Schmidt and Hensel, 2004). Different virulence phenotypes of *S. enterica* are encoded by genes on *Salmonella* Pathogenicity Islands (SPIs). There are 12 SPI described, which present different size, structure, function and distribution in *Salmonella* subspecies and serotypes (Marcus *et al.,* 2000). On the other hand, mobile genetic elements can transfer virulence factors between members of the same species or different species by horizontal gene transfer, and this acquisition enables bacteria to gain virulence determinants from other species. The *Salmonella* virulence plasmid, that contains the *spv* operon, was found in eight serotypes of subspecies *enterica*, including Enteritidis (Gulig *et al.*, 1993). Some of virulence and stress-response genes identified in *S. enterica* are described in Table 1.5.

The use of omic tools, such as genomics, transcriptomics and proteomics, has been widely used to characterize bacterial virulence factors. Besides being helpful to understand the molecular determinants involved in pathogenesis, these approaches also allow evaluating antimicrobial resistance mechanisms (Di Cagno *et al.*, 2011; Radhouani *et al.*, 2012). Recent studies have been identifying *S. enterica* virulence genes associated with resistance to disinfectants and antibiotics.

Table 1.5 Virulence and stress-response genes of *Salmonella*.

Gene	Function	Reference
spiA	Cellular invasion; Biofilm formation;Possibly post-invasion stages of the disease	Raffatellu *et al.,* 2005; Giacomodonato *et al.,* 2007; Dong *et al.,* 2011
invA	Cellular invasion	Galán *et al.*, 1992
***spv* genes**	Survival and intracellular multiplication	Libby *et al.,* 1997; Libby *et al.,* 2000
rpoS, stiA, stiB, stiC	Starvation survival	Spector and Cubitt, 1992, O'Neal *et al.*, 1994; Archer, 1996
avrA	Cellular invasion; Inflammatory response of hosts against infection	Hardt and Galán, 1997; Ben-Barak *et al.*, 2006
sopA, sopB, sopD, sopE2	Cellular invasion; Possibly post-invasion stages of the disease	Raffatellu *et al.,* 2005; Giacomodonato *et al.,* 2007
hilA	Cellular invasion	Lostroh and Lee, 2001
astA	Coding EAST1 toxin (enteroaggregative thermostable enterotoxin)	de Sousa and Dubreuil, 2001
agfB	Encoding the minor fimbrial subunits of Tafi	White *et al.*, 2001
agfC , agfE	Facilitating Tafi synthesis	Gibson *et al.*, 2007
csgD [1]	Triggers the biosynthesis of the major extracellular matrix components	Romling *et al.*, 1998, 2000; Latasa *et al.*, 2005
fliC, fliD, motA, motB	Motility (flagella-associated genes)	Apel and Surette, 2008
***bcs* genes**	Cellulose biosynthesis	Solano *et al.,* 2002
sefA, sefD	Genes encoding major fimbrial proteins (SEF14 and SEF18)	Collinson *et al.*, 1996
sirA, ycfR	Biofilm formation	Zhang *et al.*, 2007; Wang *et al.,* 2010
ostA, ostB	Osmotic stress	Archer, 1996

[1] *csgD* (curli subunit gene D) has previously been referred to as *agfD* (aggregative fimbriae D)

1.6.1. Expression of virulence genes related with resistance to disinfectants

As previously mentioned, the different disinfectants commonly used in the food industry are not always effective concerning the complete elimination of microorganisms, especially when these have grown as biofilm. Microorganisms that survive exposure to these agents can present altered gene expression profile, with the possibility of occur up-regulation of virulence genes (Rodrigues *et al.*, 2011). Accordingly, surveys have been showing that changes in genes and proteins expression profile take place when *S. enterica* is exposed to common disinfectants (Webber *et al.*, 2008a; Webber *et al.*, 2008b; Bailey *et al.*, 2009; Wang *et al.* 2010; Rodrigues *et al.* 2011, Condell *et al.*, 2012; Salazar *et al.*, 2013). A study conducted by Tabak and her coworkers (2007), which aim was to investigate the susceptibility of planktonic and biofilm associated *S.* Typhimurium to triclosan and identify potential mechanisms of adaptation, showed that although triclosan did not induce the bcs genes in planktonic cells, it did up-regulate the transcription of *bcsA* and *bcsE* within the biofilms matrix. This is a worrying finding because, since these genes are associated with cellulose synthesis (Solano *et al.*, 2002) - one of the major exopolysaccharides in biofilms matrix (Zogaj *et al.*, 2001) -, it suggests that triclosan may enhance the potential of biofilm formation. Another work, performed by Wang *et al.* (2010), studied transcriptomic responses of *S.* Enteritidis and *S.* Typhimurium strains after chlorine treatments, and the findings suggest that oxidative-stress response may render *S. enterica* resistant or susceptible to certain types of environmental stresses. For example, genes involved in bacterial biofilm formation, such as *ycfR*, were up-regulated under chlorine oxidation. Moreover, a work performed by Rodrigues *et al.* (2011) showed that exposure of *S.* Enteritidis biofilms to four disinfectants commonly used in the food industry - sodium hypochlorite, benzalkonium chloride, hydrogen peroxide or triclosan - had influenced the expression of stress response and virulence genes by the surviving cells. More precisely, it was found a significant overexpression of the stress response gene *rpoS*, as well as of the virulence gene *avrA*, which are involved in starvation survival, and invasion and interaction with hosts, respectively (Archer, 1996; Hardt and Galán, 1997).

Though studies regarding this subject are yet very scarce, the up-expression of virulence genes on biofilms that were treated with disinfectants is an area that deserves special attention, because surviving cells may compromise food safety and potentiate public health risk (Rodrigues *et al.*, 2011). On the other hand, besides resistance to disinfectants, resistance to antibiotics commonly used to treat salmonellosis may also be related with different regulation patterns of virulence genes.

1.6.2. Expression of virulence genes related with resistance to antibiotics

As mentioned previously, fluoroquinolones, third-generation cephalosporins and ampicillin are the antibiotics used to treat salmonellosis (CDC, 2013). However, an increasing resistance to these and other antibiotics has been detected. Recent studies have demonstrated the association between resistance patterns to antibiotics with virulence genes in the same strain of *Salmonella* (Bolton *et al.,* 2013; de Toro *et al.,* 2013), which may have consequences at the level of pathogenicity of these strains. Moreover, surveys also suggest that exposure to subinhibitory concentrations of antibiotic may increase the expression of genes related to virulence and pathogenicity (e.g. Down *et al.,* 2007; Weir *et al*, 2008). A study conducted by Weir and her coworkers (2008) analyzed the effect of subinhibitory tetracycline treatment on multi-drug resistant *S.* Typhimurium, and on the expression of factors involved in virulence and host colonization. In this study it was observed that *hilD, hilA, fliC, fliD, motA, motB* and *fur* genes were up-regulated, which have important functions in pathogenicity and virulence. In fact, *hilA* gene is involved in type III secretion system expression, which is essential for *Salmonella* to initial penetration of intestinal epithelial cells (Lostroh *et al*., 2001), while *fliC*, *fliD*, *motA*, and *motB* are flagellar genes that play an important role in mobility (Apel and Surette, 2008). By its turn, *fur* gene is required to iron acquisition and acid tolerance allowing these bacteria to survive within the host, when there are iron-limiting conditions, and within the gastro-intestinal tract (Litwin and Calderwood, 1993; Hall and Foster, 1996). Wang and his coworkers (2009) have also investigated SPI1 gene expression and the pathogenicity of quinolone-resistant *Salmonella.* Messenger ribonucleic acid (mRNA) expression of two SPI1 genes – *invA*, associated with cellular invasion (Galán *et al*., 1992); and *avrA*, associated with invasion and inflammatory response of hosts against infection (Hardt and Galán, 1997; Ben-Barak *et al.,* 2006) – was decreased in the quinolone-resistant *Salmonella* strains compared to quinolone-susceptible strains. Furthermore, the invasiveness of and intracellular replication in epithelial cells and macrophages of quinolone-resistant strains were markedly reduced comparing to quinolone-susceptible strains, which may be associated with the decreased expression of *invA* and *avrA.* These results suggest that quinolone-resistance may be associated with lower virulence and pathogenicity into mammalian cells, which is a satisfactory conclusion since quinolones are the antibiotics commonly used to salmonellosis treatment.

In view of the examples given here, and although not all of them verified this problem, the significant association between some virulence genes and antibiotic resistance may have important implications regarding the spread and persistence of antibiotics resistant *Salmonella*. Hence, like to all pathogenic microorganisms in general, it should be ensured a prudent use of antibiotics in humans and animals in order not to restrain the increase of *Salmonella* virulence and pathogenicity.

1.7. Scope and aims

The main goal of this research was to investigate the effect of exposure to chemical disinfectants in the virulence of *Salmonella enterica* serotype Enteritidis biofilm cells. The fact that microbial cells that survive chemical disinfection may express resistance to antibiotics and changes in gene expression, associated with virulence and pathogenicity, is a matter of concern that has been reported. However there is still a lack of knowledge about this phenomenon when biofilms are concerned, which is becoming truly alarming since these microbial communities constitute a potential threat due to their increased disinfection resistance. Nevertheless, by using the transcriptomic tools available nowadays, it is possible to characterize the expression of virulence genes on biofilm cells that have survived the exposure to different disinfectants. Moreover, by screening different gene expression profiles caused by disinfection, it can be elucidated the possible role of antimicrobial resistance mechanisms in virulence. Therefore, in order to complete the surveys that have been performed, the purpose of this study was to characterize *S. enterica* biofilm-derived cells after exposure to chemical disinfectants, regarding their resistance and virulence, in order to have some insights about what may occur in case cells released from these biofilms come in contact with a host.

In an initial stage, biofilms susceptibility to four disinfectants commonly used in food industry was evaluated in order to determine the concentrations to be used later, in the prolonged exposure of biofilms to the disinfectant agents. In order to give an insight into the response of surviving biofilm cells, they were phenotypically characterized after exposure to the disinfectants, in terms of antibiotics resistance and biofilm formation ability. At the final stage of this study, analysis of virulence genes expression by biofilm-derived cells after exposure to chemical disinfectants was also performed. With all the results obtained, it is expected to get further elucidation about chemical disinfection effects on *S.* Enteritidis biofilm cells and, thus, contribute to the evaluation of the approaches that are currently being used to combat pathogens in food processing areas, and their

possible consequences for human health in the case of a possible infection occur by contact of these biofilm-derived cells with a host.

Chapter 2 – Theoretical foundations of the methodology

2.1 Biofilm formation

Currently, there are available several devices that are widely used to biofilm formation (Azevedo *et al.*, 2009). These *in vitro* systems are usually divided into flow (Modified Robbins Device and flow cell biofilm system) and static models (microtiter plate, Calgary Biofilm Device, perfused biofilm fermentor and Constant Depth Film Fermentors). Static models are preferable, since they are easier to handle and versatile, allowing to study the effect of different conditions of biofilm formation as well as different behaviors of these bacterial communities (Merritt *et al.*, 2005; Abdallah *et al.*, 2014). The main disadvantages of these methods are associated to their static nature that may lead to a change in environment in the wells during the experiment, because there is no flow into or out of the device (Coenye and Nelis, 2010).

The microtiter plate assay is a static device model, initially derived from a protocol published by Christensen and his coworkers (Christensen *et al.*, 1985), which consists in a large number of reactors in small scale with the same conditions of space and fluid dynamics (Stepanovic *et al.*, 2000). A microtiter plate may have 6, 24, 96, 384 or even 1536 sample wells arranged in a rectangular matrix. In this system, biofilms may grow on the bottom and the walls of the wells (Coenye and Nelis, 2010). The main drawback of this method is the use of batch-growth conditions. Nevertheless, a regular renewal of liquid phase allows avoiding the limitation of nutrients and accumulation of potentially toxic metabolites. Nowadays, due to its low cost, flexibility and speed (it allows the processing of multiple samples simultaneously with simplicity), requirement of common laboratory equipment, and need to use smaller quantities of reagents and culture media, the microtiter plate assay remains among the most frequently used method to biofilm formation (Agarwal *et al.*, 2011; O'Toole, 2011; Machado *et al.*, 2012). In addition to these features, this simple high throughput platform allows to assess vary multiple parameters in biofilm formation (Stepanovic *et al.*, 2003; Coenye and Nelis, 2010), including differences in biofilm formation between strains (Djordjenvic *et al.*, 2002), and to use a wide variety of abiotic surfaces, by just incorporating coupons of the different materials inside the wells of the microtiter plates - however, these assays are usually performed only on 6 or 24-wells flat-bottom polystyrene microtiter plates - (Merritt *et al.,* 2005; Coenye and Nelis, 2010). In spite of microtiter plates may consist of different materials, polystyrene has been widely used for *in vitro* assays since it proves to be an excellent material for promoting adherence of cells (Stepanovic *et al.,* 2004; Giaouris *et al.*, 2012), and there is a wide commercial availability of polystyrene plates which are relatively inexpensive. Moreover, there are various

techniques of analysis which can be performed in microtiter plates, thereby contributing to their widespread use. Assays for quantification of biofilm formation (Stepanovic *et al.*, 2000; Solomon *et al.*, 2005; Peeters *et al.*, 2008a; Vestby *et al.*, 2009b) and susceptibility testing of compounds (Andrews, 2001; Pitts *et al.*, 2003; Peeters *et al.*, 2008b; Peeters *et al.*, 2008c; Wiegand *et al.*, 2008) are some of procedures which have been used in these static models.

2.2 Quantification of biofilm biomass

In order to compare biofilm formation capacity, it is critical to have an efficient and highly reproducible method for quantification, with little random error. Biomass can be measured by distinct methods, such as microscopy, molecular probes, biochemical analysis of biomass components, and, the most usual, staining of biofilms with specific compounds and subsequent determination of optical density (Stepanovic *et al.*, 2000; Li *et al.*, 2003; Peeters *et al.*, 2008a; Azevedo *et al.*, 2009). These different approaches vary in their sensitivity and specificity, however a major criterion when selecting an appropriate method is the amount of biomass present (Demain and Davies, 1999).

Crystal violet (CV) staining was first described by Christensen and his coworkers (1985) and, since then, has been modified and enhanced to become a more accurate and reliable technique. In this staining assay, the dye binds to negatively charged surface molecules and polysaccharides located in the extracellular matrix (Peeters *et al.*, 2008a), allowing to determine biofilm biomass without disrupting the biofilm. Once total biomass (cells and matrix) is stained in purple, the dye can be easily dissolved in acetic acid (Stepanovic *et al.*, 2000) and, finally, the absorbance is read at 570nm. CV staining is not a suitable method to assess biofilm cells' viability, because this method does not depend on the integrity of the cells, staining both living and dead cells together with the extracellular matrix (Pitts *et al.*, 2003; Romanova *et al.*, 2007). Besides this limitation, this technique requires successive washing steps, which can result in loss of part of the biomass present (Peeters *et al.*, 2008a). Nevertheless, CV is a straightforward, quick, and low cost technique to indirect quantification of microbial adhesion and amount of biofilm formed on inert surfaces by a broad range of microorganisms (Stepanovic *et al.*, 2000; Djordjenvic *et al.*, 2002; Li *et al.*, 2003; Romanova *et al.*, 2007; Negri *et al.*, 2010; O'Toole, 2011), including *Salmonella* species (Agarwal *et al.*, 2011; Tang *et al.*, 2012).

2.3 Biocidal action sensitivity testing

Sensitivity to biocides may be determined by different methods. EFSA reports the use of disk diffusion and broth dilution for antibiotic resistance testing in *Salmonella* (EFSA and ECDC, 2014b). Although currently there is not a standardized method that reproduces growing conditions *in vivo* to evaluate biocidal action susceptibility, dilutions methods have been reported as a reference testing (Andrews, 2001; Wiegand *et al.*, 2008; Jorgensen and Ferraro, 2009). In a dilution test is evaluated the ability of a bacteria to grow in a range of concentrations of a given biocidal agent (Wiegand *et al.*, 2008). Currently, microdilution is a method that entails several advantages. Briefly, in this broth dilution method, dilutions of biocidal compounds solutions are prepared in a liquid bacterial growth medium, which is then are inoculated with the standardized cell suspension. Microtiter plates are then incubated overnight at the incubation conditions recommended described for the bacteria under study - for *Enterobactereriaceae* is 35-37°C in air for 18-20h (Andrews, 2001) -, period after which the plates are examined for macroscopically visible evidence of bacterial growth in the form of turbidity. The biocidal activity of a compound can be quantified by determining the minimum concentration of the compound capable of inhibiting the visible growth of a microorganism, a value called Minimum Inhibitory Concentration (MIC) (mg/L) (Andrews, 2001; Wiegand *et al.*, 2008; Jorgensen and Ferraro, 2009). Posteriorly, MICs values may be translated into clinical categories, namely sensitive (S), intermediary (I) or resistant (R). Concerning antibiotics, these clinical categories and correlated MICs are provided by several committees, including the European Committee on Antimicrobial Susceptibility Testing - EUCAST - (EUCAST, 2014). This micromethod, performed in 96-well microtiter plates, allows analyzing a large number of compounds in a rapid, economical, and equally effective way as compared to methods that use a larger volume (Jorgensen and Ferraro, 2009).

Nevertheless, determining MIC is a standard procedure to quantify planktonic cells susceptibility to biocidal agents, and is not directly applicable to biofilms. Therefore, Minimum Biofilm Eradication Concentration (MBEC) testing should be considered when analyzing biofilms (Ceri *et al.*, 1999; Girard *et al.*, 2010; Allan *et al.*, 2011). MBEC is usually assessed using the Calgary Biofilm Device (CBD) (Ceri *et al.*, 1999), in which biofilms are exposed to the biocidal compounds for a specific period of time, and posteriorly submerged in fresh medium and incubated overnight. The lowest biocidal concentration that eradicates the biofilm is identified as the MBEC value (Ceri *et al.*, 1999). However, CBD is expensive and not readily available, being necessary to adopt new methods

to determine MBEC. Indeed, some modifications to CBD assay have been performed. One of these alternatives consists in, after biocidal exposure, the biocidal compound is removed from the wells and biofilms are then scraped thoroughly. Posteriorly, samples correspondent to each concentration of biocidal agent are plated on solid medium and incubated from a specific period of time. In order to assess MBEC, the presence of colonies is evaluated and MBEC value is determined, as indicated above (Mataraci and Dosler, 2012). Besides these, several others methods have been used to assess MBEC (Bueno *et al.*, 2014), such as Biofilm Eradication Surface Test (BEST) Assay™ (Harding *et al.*, 2011) and BioTimer Assay (De Giusti *et al.*, 2011).

2.4 Gene expression analysis

The study of gene expression provides the analysis of specific genes in a given organism under a particular condition. Northern blot and reverse transcriptase polymerase chain reaction (PCR) were the classic molecular techniques initially used to analyze gene expression. However, advances in bioinstrumentation and molecular biology have led to the development of several new techniques with different range of sensitivities, throughputs and quantitative capabilities such as quantitative real-time PCR (qPCR), DNA microarrays and ribonucleic acid (RNA) sequencing analysis (Roth, 2002).

qPCR is a widely used methodology to study gene expression due to its high sensitivity, accuracy and reliability. This technique allows measuring little amounts of DNA molecules from a small initial sample and produces rapid quantification results. Moreover, it is considered the standard technique for analysis of gene expression (Botteldoorn *et al.*, 2006; Tabak *et al.*, 2007; Weir *et al.*, 2008; Wang *et al.*, 2009; Wang *et al.*, 2010; Rodrigues *et al.*, 2011). qPCR allows amplification and simultaneous quantification of target DNA molecules at each PCR cycle through fluorescent dyes. Template quantification is based on the fluorescence signal during the exponential phase of amplification, before limiting reagents or accumulation of inhibitors. The point at which fluorescence is first detected as statistically significant above the background, is called the quantification cycle (Cq). Cq is directly correlated to the starting target concentration of the sample. The greater the amount of initial DNA template in the sample, the earlier a significant increase in fluorescent is observed and the Cq value is reached (Bustin and Mueller, 2005). In order to validate the results obtained by qPCR, negative and positives controls should be performed. While negative controls are

performed to detect possible contamination, positive controls are used to assess quality of sample or reagent and the presence of inhibitors (D'haene and Hellemans, 2010).

qPCR involves different experimental steps, which ensure success of gene expression analysis (Gibson *et al.*, 1996), and will be addressed in the following subtopics.

RNA extraction and gDNA degradation

Cell lysis and RNA extraction are the first mandatory steps to perform gene expression analysis. There are different approaches to cell lysis procedure (enzymatic, chemical, mechanical, or a combination between these) and RNA isolation (isolation columns or organic). To ensure genomic DNA (gDNA) degradation, which can lead to false positive results, DNase treatment or organic methods are used prior to reverse transcription reaction (Pfaffl, 2004). Since qPCR is a RNA-based analysis, it is important to note that it depends on quality of RNA extracted (Fleige and Pfaffl, 2006). RNA quality, which is related to quantity, purity and integrity of the RNA extracted, can be influenced by handling and RNA storage (Schoor *et al.*, 2003; Bustin and Nolan, 2004; Fleige and Pfaffl, 2006; Fleige *et al.*, 2006), nature of the sample (Cury *et al.*, 2008), cell lyses procedure, and RNA isolation method (Rump *et al.*, 2010; França *et al.*, 2011; França *et al.*, 2012). Hence, in this initial step it is crucial the use of products that stabilize the samples during the RNA procedures, both to avoid the induction of the transcription and the RNA degradation by specific enzymes called RNases (Arraiano *et al.*, 2013).

RNA concentration and purity

In order to ensure reproducible and accurate results, RNA quality indicators must be assessed. Due to the low sample consumption and easiness, RNA concentration and purity are conventionally measured using ultraviolet spectroscopy, such as using a Nanodrop™ device (Sieber *et al.*, 2010; Carvalhais *et al.*, 2013). The concentration is calculated using the Beer-Lambert law, knowing that an absorbance reading at 260 nm wavelength of 1.0 is approximately 40 µg/mL of RNA. Absorbance ratios A260/A280 and A260/A230, also provided by the same device, are used as indicators of contamination by proteins and polysaccharide, or by phenol and chaotropic salts, respectively (Nolan *et al.*, 2006; Tavares *et al.*, 2011).

Complementary DNA synthesis

After all protocols and analysis related to RNA sample, these molecules are used to perform complementary DNA (cDNA) synthesis. In reverse transcription reaction, catalyzed by reverse

transcriptase enzyme, a double stranded hybrid molecule (mRNA:cDNA) is constructed. Since RNA is a quite unstable molecule that may be easily and rapidly degraded (Arraiano *et al*. 2010), cDNA storage to further analysis presents an important advantage (Wacker and Godard 2005). Moreover, for each reverse transcription reaction it is important to incorporate a No Reverse Transcriptase (NRT) control, in order to identify erroneous signals due to genomic DNA contamination.

Quantitative PCR run

As in cDNA synthesis, in order to validate the results obtained by qPCR it is essential to include a negative control - the No Template Control (NTC). During qPCR setup, and for every different gene analyzed, NTC is incorporated to detect possible primers dimers formation and/or reagents contamination.

Relative quantification is one of the approaches to analyze gene expression through qPCR, and allows assessing the changes in mRNA levels of a gene by comparison with a control sample. In this approach, a normalization method is performed to reduce technical variation (Pfaffl, 2004; Fleige and Pfaffl, 2006). Due to its relatively low cost and simplicity (Dheda *et al*., 2004), normalization by reference genes is the most used methodology (Fleige and Pfaffl, 2006). Besides being essential for cell survival, these genes are expressed in all nucleated cells under study, and their mRNAs must be subjected to the same conditions during qPCR procedures as the target genes (Bustin *et al*., 2009). It is required that reference genes have a constitutive expression in all cell types and tissues, and that such expression remains unaltered regardless of experimental treatments that cells were subject, otherwise quantification can be misleading (Bustin, 2000; Hocquettea and Brandstetter, 2002). Some authors suggest that more than one reference gene should be used in order to obtain more accurate results (Bustin *et al*., 2009).

To fluorescent detection during qPCR, several techniques are available including DNA-binding dyes, and dye-labeled sequence-specific oligonucleotide primers/probes. The main advantage of DNA-binding dyes methods, as SYBR Green I and EvaGreen, is their reduced economic cost (Bustin, 2000; Bustin and Nolan, 2004) because they are non-specific. EvaGreen has arisen as a dye widely used, since is more stable, can be used at relatively high concentration, and allows using a fast cycling protocol in comparison with SYBR Green I (Monis *et al*., 2005; Wang *et al*., 2006). Moreover, as with SYBR Green I, EvaGreen also supports the performance of a melt curve analysis in order to verify the specificity of the reaction (Bustin, 2000). This is based in the fact that distinct double-stranded DNA (dsDNA) molecules melt at different temperatures. So, when each PCR

product is submitted to a sequential temperature increase, the dsDNA molecules become denatured ("melted") at a specific temperature, leading to dye's dissociation from these molecules and a consequent decrease in the fluorescence levels. This is why melt curves are nowadays a widely used approach to recognize amplified qPCR products (Nolan *et al.*, 2006).

Since qPCR is the most efficient, fast, sensitive, accurate and reliable methodology to assess gene expression, it is regarded as benchmark technology. Nevertheless, since it appears as a multifactorial technique and is influenced by numerous variables, a maximum precision during it is perform is required.

Data analysis

There are distinct methods available to performed data analysis of gene expression (Pfaffl, 2004; Schmittgen and Livak, 2008), and to choose the right one is important to have into consideration the specific presupposes of each method and how the assay was performed (Pfaffl, 2001).

When amplification efficiencies of reference and target gene are close to 100%, the Livak or $2^{-\Delta\Delta CT}$ method is the most commonly used to relative gene expression quantification (Livak and Schmittgen, 2001). In this method, the gene expression quantification and the relative expression ratio are calculated by the following formulas, respectively:

$$\Delta\Delta C_T = \left(C_{T\,target} - C_{T\,reference}\right)control - \left(C_{T\,target} - C_{T\,reference}\right)test$$

$$\text{Normalized expression} = 2^{-\Delta\Delta C_T}$$

Moreover, when amplification efficiencies of reference and target gene are close to each other but not 100%, the "2" in the previous formula must be substituted by the efficiency value determined experimentally, as follows:

$$\text{Normalized expression} = E^{-\Delta\Delta C_T}$$

On the other hand, Pfaffl is a method that should be applied if reference and target genes have different amplification efficiencies, in particular with a difference higher than 5% between them (Pfaffl, 2004). To calculate relative expression of target genes in distinct samples the following formula is used:

$$Normalized\ expression = \frac{(E\ target)^{\Delta\ Cq\ target\ (control-sample)}}{(E\ ref)^{\Delta\ Cq\ ref\ (control-sample)}}$$

Chapter 3 – Methodology

3.1 Microorganisms and growth conditions

In order to evaluate the behavior of different strains from distinct sources, two *Salmonella* Enteritidis were used in the course of this study: 1 reference strain (NCTC 13349) and 1 food isolate (350). Bacteria were preserved at –70 °C in 20% glycerol stocks. For each experiment, strains were subcultured on Tryptic Soy Agar plates (TSA Liofilchem, Italy) for 24 h, at 37 °C.
In order to prepare the bacterial suspension, bacterial cells were collected from TSA plates and inoculated in approximately 35 ml of Luria Bertani Broth Miller (LB; Liofilchem) for 18±2 h at 37 °C, in a horizontal shaker under agitation at 120 rpm (Shaker & Incubator, NB-205Q, N-Biotek). After incubation, the cells were harvested by centrifugation at 9000 rpm for 5 min at 4 °C (5430 R Centrifuge, Eppendorf) and washed twice with 0.9% sodium chloride solution (Panreac Química, Spain). Subsequently, the cellular concentration was adjusted to ≈ $1x10^8$ CFU/ml, which corresponds to an optical density (OD) of ≈ 0.1 at 640 nm, as confirmed by colony forming units (CFU's) count after plating serial dilutions on TSA.

3.2 Biofilm formation

In order to obtain a cell concentration of $1x10^5$ CFU/ml on the well (concentration to initiate biofilm formation), the initial standardized cell suspensions were diluted in 0.9% sodium chloride solution. Biofilm formation was performed on regular microtiter plates under optimized conditions for the bacteria under study and, each assay was performed in triplicate. Briefly, in each well of 96-wells flat-bottom polystyrene microtiter plates (Orange Scientific) the bacterial inoculum was added to fresh LB medium so that a concentration of 1 x 10^5 CFU/ml in a final volume of 250 µl was obtained. Culture plates were incubated at 37 °C in an orbital shaker at 120 rpm during three days, in order to form a consistent biofilm. During this period, at each 24±2 h of incubation, 240 µl of liquid phase was removed and an equal volume of fresh LB medium added to the wells.

3.3 Evaluation of biofilms' susceptibility to chemical disinfectants

The chosen compounds represent different classes of disinfectant agents that are commonly used in the food industry, which also allows studying the effect of disinfectants with different mechanisms of interaction with the bacterial cells. Thus, in this study, four different disinfectants

were tested: sodium hypochlorite (SH) 10-15% available chlorine (Sigma-Aldrich), hydrogen peroxide (HP) 50% wt/v solution in water (Sigma-Aldrich), benzalkonium chloride (BAC) (Sigma-Aldrich), and triclosan (TC) (Sigma-Aldrich).

3.3.1 Disinfectants and neutralizer preparation

Stock solutions of BAC and TC were prepared with a concentration of 100 000 μg/ml, while in the case of SH and HP the own products were the stock solutions. For all disinfectants, work solutions were always fresh, prepared immediately before application. In disinfectants preparation, the purity is a very important point to note, as well as the use of the correct solvent, which corresponded to ultrapure water for BAC, and ethanol 50% for TC. Once dissolved, BAC and TC solutions were sterilized by filtration with 0.2 μm filter (Frilabo, Porto, Portugal). BAC solution was stored at 4 °C, while TC solution was aliquotted and stored at -70 °C in microtubes (BIOplastics BV, Landgraaf, The Netherlands). SH and HP were kept at 4 °C in their original packaging. All disinfectants solutions were protected from light.

A universal neutralizer was used to inactivate disinfectants after incubation with biofilms. This neutralizer was composed for L-histidine (Sigma Aldrich), L-cysteine (Sigma Aldrich) and reduced glutathione (Sigma Aldrich) dissolved in ultrapure water and sterilized by filtration. Aliquots of 1 ml each were prepared and preserved at -20 °C. Prior to each use, a fresh solution of LB medium and neutralizer was prepared with a ratio of 1 volume of universal neutralizer per 40 volumes of LB medium.

3.3.2 Minimum Eradication Biofilm Concentration assay

Once biofilms were formed, their susceptibility to disinfectants was evaluated by determining the Minimum Biofilm Eradication Concentration (MBEC). After 3 days of biofilm formation in 96-wells flat-bottom polystyrene microtiter plates, biofilms were washed once with 0.9% sodium chloride solution in order to remove free cells. Thereafter, 11 different concentrations of disinfectants solutions diluted in LB medium were added to wells. Culture plates were incubated overnight at 37 °C, in an orbital shaker at 120 rpm. After this period, culture medium was discarded, wells were washed once with 250 μl of 0.9% sodium chloride solution, and 250 μl of LB + neutralizer solution

were added. Biofilms were then scraped from the wells with the aid of a sterile micropipette tip, plated on TSA, and incubated for 24 h at 37 °C for subsequently evaluation of MBEC - the minimum concentration at which no cell growth was observed.

3.4 Prolonged exposure of biofilms to disinfectants

Based on the MBEC values previously determined, biofilms were periodically exposed to sublethal concentrations of each disinfection agent, in order to get an approach to what may happen in food processing facilities when insufficient cleaning and disinfection take place. In these assays, biofilm formation was performed as described above (section 3.2), but this time using 24-wells flat-bottom polystyrene microtiter plates (Orange Scientific), in order to obtain a larger amount of biomass. Since the volumetric capacity of these plates is higher than on 96-well plates, each bacterial inoculum was added to fresh LB medium in order to obtain a concentration of 1 x 10^5 CFU/ml in a final volume of 1.5 ml LB medium, and every 24 h 1.4 ml of LB medium were renewed. Subsequently, biofilms exposure was performed in the same plates for 6 additional days (a total of 9-day incubation), with the application of disinfection agents in the first, second, fourth and sixth day, while on the third and fifth day only LB medium was added to the wells. Thus, in the days of exposure were added to each well 1.4 ml of disinfectant solution diluted in LB medium at a final concentration correspondent to half the MBEC, while in the other days 1.4 ml of only fresh LB medium were added to wells. All these liquid phase removals/renewals were performed very carefully to not damage the biofilm. Culture plates were always incubated in an orbital shaker at 120 rpm and 37 °C. At the same time, identical assays were performed but without the exposure to disinfectants, which corresponded to the controls. In these biofilms, during the final six days only LB medium was removed from and added to each well.

After the total 9 days of incubation, the liquid phase was removed and the wells were washed once with 1.5 ml 0.9% sodium chloride solution. A solution composed for LB medium, neutralizer and Tween 1% (Fisher Scientific International, US) was applied in order to neutralize the effect of disinfectants and disrupt biofilms. Culture-plates were then subjected to ultrasonic bath (Sonicor model SC-52, UK) operating at 50 kHz, for 10 min and biofilms were scraped from the bottom and walls of the wells with the aid of a sterile micro spatula. Straight after the scraping of the well, cells were harvested by centrifugation at 9000 rpm for 10 min at 4 °C, and resuspended in 5 ml of 0.9% sodium chloride solution. Serial dilutions of the suspensions obtained were prepared and plated on

TSA, in order to confirm cellular density by CFU's count. All these experiments were performed in triplicate, in at least three independent assays.

3.5 Evaluation of susceptibility to antibiotics

Susceptibility to antibiotics of surviving cells from biofilms exposed to the action of disinfectants, as well as from control biofilms (without chemical treatment), was performed by determination of the Minimum Inhibitory Concentration (MIC). With the aim of comparing the influence of different lifestyles in susceptibility to antibiotics, it was also determined the MIC of planktonic cells. The choice of antibiotics has fallen on those commonly used to treat salmonellosis, in order to have a perspective of what can happen in a real situation when biofilm cells survive or adapt to disinfection treatment and subsequently promote human infection. Since ampicillin, chloramphenicol, quinolones, and third-generation cephalosporins are relevant antibiotics/antibiotics classes, one representative of each was chosen and used in this work. Besides these, an antibiotic with a wide range of activity was also tested - tetracycline.

3.5.1 Preparation of antibiotics

In this study were tested five different antibiotics:

1) Ampicillin (AMP) - a beta-lactam antibiotic able to penetrate Gram-positive and some Gram-negative bacteria, since it acts as an irreversible inhibitor of the transpeptidase enzyme, which is needed to bacterial cell wall synthesis (Sigma Aldrich);
2) Ciprofloxacin (CIP) - a broad-spectrum antibiotic, corresponding to a second-generation fluoroquinolone that inhibits topoisomerase II (DNA gyrase) and topoisomerase IV enzymes which are required for bacterial DNA replication, transcription, repair and recombination (Sigma Aldrich);
3) Cefotaxime (CEF) - third-generation cephalosporin antibiotic, with broad spectrum activity against Gram positive and Gram negative bacteria, and that inhibit bacterial cell wall synthesis via affinity for penicillin-binding proteins (Aplichem, Germany);
4) Chloramphenicol (CLO) - a bacteriostatic antibiotic, which binds to binds to the 50S subunit of bacterial ribosomes, preventing transfer of amino acids and inhibiting protein

synthesis (Sigma Aldrich);

5) Tetracycline (TET) - an antibiotic which exhibited activity against a wide range of microorganisms, and that reversibly binds to the 30S ribosomal subunit, preventing binding of tRNA to the mRNA-ribosome complex, and thus interferes with protein synthesis (Sigma-Aldrich).

All antibiotics' stock solutions were prepared at a 5120 μg/ml concentration. As for disinfectants, in antibiotics preparation is very important to use the correct solvent. According to EUCAST recommendations, ultrapure water was used as solvent for preparing cefotaxime, ciprofloxacin and tetracyclin solution, while phosphate buffered saline (PBS) pH 8, 0.1 M and ethanol 95% was used to dissolve ampicillin and chloramphenicol, respectively. Once prepared, the antibiotics solutions were sterilized by filtration with a 0.2 μm filter and stored at -80 °C as 1 ml aliquots.

3.5.2 Determination of Minimum Inhibitory Concentration

To determine the Minimum Inhibitory Concentration (MIC) of the five selected antibiotics against the *S.* Enteritidis strains under study, it was performed the microdilution assay using 96-wells flat-bottom polystyrene microtiter plates. After confirming the initial concentration of biofilm cells collected after exposure to the chemical disinfectants (as described in section 3.4), these cellular suspensions were adjusted (when needed) in order to obtain a final concentration of $1x10^5$ CFU/ml in LB medium. Antibiotic solutions were tested in 11 different concentrations and in different ranges depending on the antibiotic. At the same time, it was always performed the correspondent control with no addition of antibiotic solution in order to support bacterial growth (positive control). After addition of the cell suspension (containing 1×10^5 CFU/ml) to the wells already containing the antibiotic solutions, the culture plates were incubated at 37 °C, in an orbital shaker at 120 rpm, for 24 h. The MIC was then determined visually and confirmed by optical density reading at OD_{640nm}. The exact same procedures were used to evaluate MIC values concerning planktonic cells, with the exception that these cells were not previously exposed to chemical disinfectants. All these experiments were performed in triplicate, in at least three independent assays.

3.6 Evaluation of biofilm formation ability

In order to complement the phenotypic characterization of the biofilm cells under study, their biofilm formation ability was evaluated by the CV staining method. This allowed assessing if exposure to chemical disinfectants can affect the capacity of biofilm formation by the *S. enterica*.

Cells from biofilms exposed or not-exposed (controls) to the action of disinfectants were allowed to form biofilm on 96-wells flat-bottom polystyrene microtiter plates by following the same protocol described above (section 3.2). After 3 days of biofilm formation, the medium was removed from the wells and biofilms were washed once with 0.9% sodium chloride solution in order to remove free cells. In order to fix the biofilms, 250 µl of 100% methanol were added to each well for 15 min. Afterwards, culture plates were allowed to dry at room temperature until they were completely dehydrated. The fixed biofilm on each well was stained with 250 µl of 1% (v/v) crystal violet solution, for 5 min, and then washed once with 0.9% sodium chloride solution. Once again, culture plates were allowed to dry to ensure that there are no traces of liquid in the wells. At last, in order to solubilize the CV bound to the biofilms, 250 µl of 33% (v/v) acetic acid were added to each well, and the optical density was measured at 570 nm in a microtiter plate reader (Sunrise™, Tecan). All these experiments were performed in triplicate, in at least three independent assays.

3.7 Analysis of gene expression

Analysis of gene expression on both biofilms cells exposed and not-exposed to chemical disinfectants was performed by quantitative Real-Time Polymerase Chain Reaction (qPCR). The cells used in these assays were collected as described in section 3.4, immediately resuspended in 500 µl of RNAlater® solution (Sigma-Aldrich), and stored at - 70 °C.

The genes selected for the study are involved in *S.* Enteritidis virulence, pathogenicity, and stress-response. Based on the literature, it was selected the stress response gene *rpoS* (known to be involved in starvation survival), and the virulence genes *csgD*, *avrA* and *invA*. *csgD* gene is involved in initial adhesion and biofilm formation, while the *avrA* gene is related with the inflammatory response of hosts against infection. By its turn, *invA* gene is associated to cellular invasion.
Prior to genetic expression analysis, the effectiveness of the primers to be used in these assays was tested in order to determine the optimal annealing temperature. The sequences correspondent to each of the primers used are presented in Table 3.1.

Table 3.1 Primers used for the assessment of gene expression by qPCR.

Gene	Sequence (5'-3')
16S rRNA	F: CAGAAGAAGCACCGGCTAAC R: GACTCAAGCCTGCCAGTTTC
avrA	F: GAGCTGCTTTGGTCCTCAAC R: AATGGAAGGCGTTGAATCTG
invA	F: ATCGAGATCGCCAATCAGTC R: CGCTGCCGGTATTTGTTATT
rpoS	F: GAATCTGACGAACACGCTCA R: CCACGCAAGATGACGATATG
csgD	F: GCCTCATATTAACGGCGTGT R: TCGCGATGAGTGAGTAATGC

3.7.1 RNA extraction

Total ribonucleic acid (RNA) of each sample was extracted using the PureLink™ RNA Mini Kit (Invitrogen). The samples specifically prepared for these assays (mentioned in the first paragraph of section 3.7) were thawed and harvested by centrifugation at 12 500 x g, for 15 min at 4 °C. In order to promote bacterial cell wall disruption, 100 µl of prepared lysozyme solution were added to the cell pellet, which was resuspended by vortexing. 0.5 µl of 10 % SDS solution, a denaturing agent, was then added, followed by an incubation period of 10 min at room temperature. After this period, it were added 350 µl of Lysis Buffer (from the kit) with 2-mercaptoethanol and the cell lysis was further potentiated by passing the cells 10 times through a 19-gauge needle (BD Microlance ™3, Ireland), attached to an RNase-free syringe. Samples were then centrifuged at 12 500 x g, for 2 min, at room temperature, and supernatant were transferred to a clean RNase-free micro centrifuge tube. Once the final lysate was thus obtained, the next steps consisted on the RNA binding, wash, DNase treatment (On-column PureLink™ DNase treatment; Invitrogen) and elution, all this by using the specific columns of the kit and following the manufacture's recommended protocols.

Purified RNA was analyzed as regard as RNA yield and quality. The concentration (ng/µl) and purity of the total RNA was spectrometrically assessed using a NanoDrop device (NanoDrop

1000 Spectrophotometer, V3.6.0, Thermo Fisher Scientific, Inc.), and the absorbance ratios A260/A280 and A260/A230 were used as indicators of contamination.

3.7.2 Synthesis of cDNA

In order to ensure a reliable comparison of gene expression between different samples, it is necessary to have equivalent starting amounts of RNA to be converted on complementary DNA (cDNA). Taking this into account and using the concentration values obtained in the NanoDrop device, the necessary dilutions of samples were prepared in RNase-free water. cDNA synthesis from purified RNA was performed using the iScript™cDNA Synthesis Kit (BioRad). A final reaction volume of 20 µl contained 4 µl of 5x iScript Reaction Mix, 1 µl of iScript Reverse Transcriptase, and 15 µl of RNA template, according to the proportions recommended by the kit manufacturer. Finally, complete reaction mix was incubated in a thermocycler (MyCycler™ Thermal Cycler, BioRad) with a specific reaction protocol: 5 min at 25 °C, 30 min at 42 °C, and 5 min at 85°C.

3.7.3 Quantitative Real-Time PCR

qPCR reactions were performed on a CFX96™ Real-Time PCR Detection System Bio-Rad system (Bio-Rad Laboratories, Inc.), and each sample was run in triplicate.
A total of 20 µl of reaction mixture contained 2 µl of diluted cDNA, 1 µl of each primer, 10 µl of 2x SSoFastTM EvaGreen® Supermix (Bio-Rad Laboratories, Inc.), and 6 µl of nuclease-free water. Optimized thermal cycling conditions were performed, namely: 3 min of initial denaturation at 95 °C, followed by 40 cycles of 10 s denaturation at 95 °C, 10 s annealing at 57 °C (concerning primers efficiency previously determined), and a 15 s extension at 72 °C. At the end of each run, a melt curve was performed with readings from 65 °C to 95 °C every 1 °C for 5 s, in order to confirm that only the desired products were amplified.

3.7.4 Gene expression analysis

Expression of *avrA*, *invA*, *csgD* and *rpoS* genes was analyzed using a relative quantification method, the Pfaffl analysis method, which describes the change in expression of the target genes relative to the reference gene in distinct samples. Moreover, this method accounts with reaction efficiency of both target and reference genes. In the present study, the housekeeping gene used to normalize the data was the 16S ribosomal RNA (rRNA). Each reaction was performed in triplicate

and the mean values of relative expression were analyzed for each of the target genes. Two negative controls, No Reverse Transcriptase control (NRT) and No Template Control (NTC), were performed in order to validate the reactions. All Cq values of these controls were 10 or more cycles apart from the correspondent test sample, confirming the absence of contamination from gDNA or from the qPCR reaction components.

3.8 Statistical Analysis

Data analysis was performed using the statistical program GraphPad Prism® for Windows, version 6.01 (GraphPad Software, Inc., San Diego, CA, USA). Biofilm formation results were compared using Kruskal-Wallis test and Dunn's multiple comparisons test. qPCR results were compared using multiple t-tests. All tests were performed with a confidence level of 95%.

Chapter 4 – Results

4.1 Minimum Biofilm Eradication Concentration

In this study, biofilms susceptibility to benzalkonium chloride (BAC), triclosan (TC), hydrogen peroxide (HP), and sodium hypochlorite (SH) was assessed as described above (section 3.3.2). The Minimum Biofilm Eradication Concentration (MBEC) values of each disinfectant and for each strain tested are presented in Table 4.1.

Table 4.1 MBEC value and recommended use concentration of each disinfectant, (µg/ml).

Salmonella enterica Enteritidis	BAC	TC	HP	SH
NCTC 13349	800	400	8 000	6 000
350	1 600	200	8 000	12 000
Recommended use concentration in food contact surfaces	200	NA	200 - 300	200

NA - Concentration not available

An intraspecies variability was observed, since it was not possible to establish a susceptibility trend concerning the strains tested. For both strains, TC was the disinfectant with the lowest MBEC values. However, concerning the highest MBEC values, has been observed differences between strains. While in *S.* Enteritidis NCTC 13349 the highest value of MBEC corresponded to HP, in food isolate 350 it was related to SH. Moreover, the reference strain was the most susceptible to BAC and SH, while the *S.* Enteritidis food isolate was the most susceptible to TC. HP disinfection agent was the only exception to the intraspecific variability, since both strains presented the same MBEC value. With the exception of triclosan, for which there is no concentration value defined, all MBEC values were way above the in use recommended concentration for food surfaces' disinfection procedures.

Based on the MBEC values obtained, biofilms were periodically exposed to half the MBEC concentration of each disinfection agent, following the protocol previously mentioned in Chapter 3 (section 3.4). In the specific case of *S.* Enteritidis 350 exposure to BAC, it was necessary to apply a disinfectant concentration lower than half the MBEC value, because the amount of survival cells after the prolonged exposure was not sufficient to perform the subsequent assays (< 5 Log). Hence, for *S.* Enteritidis NCTC 13349, 400 µg/ml BAC, 200 µg/ml TC, 4000 µg/ml HP, and 3000 µg/ml SH were the concentrations applied during biofilms exposure to disinfectant. On the other hand, for the

food isolate were used the following exposure concentrations: 400 µg/ml BAC, 100 µg/ml TC, 4000 µg/ml HP and 6000 µg/ml SH.

4.2 Evaluation of susceptibility to antibiotics

Biofilm cells exposed to a disinfectants were tested for their susceptibility to ampicillin (AMP), ciprofloxacin (CIP), cefotaxime (CEF), chloramphenicol (CLO), and tetracycline (TET) by determination of the MIC through the microdilution method. Likewise, susceptibility of planktonic cells was also determined. The MIC results obtained for the different antibiotics are presented in Table 4.2 and Table 4.3, concerning *S.* Enteritidis NCTC 13349 and *S.* Enteritidis 350, respectively.

Table 4.2 Susceptibility of *S.* Enteritidis 13349 to antibiotics (µg/ml).

		AMP (S ≤ 8 R > 8)*	CIP (S ≤ 0.06 R > 0.06)*	CEF (S ≤ 1 R > 2)*	CLO (S ≤ 8 R > 8)*	TET (S ≤ 4 R > 16)*
	Planktonic	0.5 – 1	1 – 2	0.0625 – 0.125	1 – 2	0.5 – 1
Biofilm	Control	0.5 – 1	8 – 16	0.125 – 0.25	1 – 2	0.5 – 1
	BAC	1 – 2	8 – 16	0.0625 – 0.125	2 – 4	0.5 – 1
	TC	0.5 – 1	8 – 16	0.0625 – 0.125	1 – 2	0.5 – 1
	HP	1 – 2	8 – 16	0.0625 – 0.125	1 – 2	0.5 – 1
	SH	2 – 4	16 – 32	0.0625 – 0.125	2 – 4	1 – 2

* Breakpoints were adopted from EUCAST (2014) except for tetracycline, which breakpoint was adopted from CLSI (2009).

For the reference strain used, exposure to BAC led to a decrease of susceptibility to AMP and CLO, and a susceptibility increase to CEF, comparing to controls. Besides, after exposure to TC no decrease of susceptibility was observed, and HP exposure only led a decreased susceptibility to AMP. Finally, biofilms cells exposed to SH shown a decrease of susceptibility to all antibiotics tested except for CEF. Concerning the results obtained with different lifestyles of *S.* Enteritidis reference strain, it was observed that MIC ranges obtained with control biofilm cells were equal or higher than those obtained with planktonic cells. Moreover, a considerable decrease of susceptibility was observed concerning CIP.

Table 4.3 Susceptibility of *S.* Enteritidis 350 to antibiotics (μg/ml).

		AMP (S ≤ 8 R > 8)*	CIP (S ≤ 0.06 R > 0.06)*	CEF (S ≤ 1 R > 2)*	CLO (S ≤ 8 R > 8)*	TET (S ≤ 4 R > 16)*
	Planktonic	1 – 2	8 – 16	0.0625 – 0.125	4 – 8	0.5 – 1
Biofilm	Control	1 – 2	8 – 16	0.125 – 0.25	4 – 8	1 – 2
	BAC	1 – 2	8 – 16	0.0625 – 0.125	2 – 4	0.5 – 1
	TC	2 – 4	32 – 64	0.5 – 1	4 – 8	2 – 4
	HP	1 – 2	16 – 32	0.125 – 0.25	2 – 4	0.5 – 1
	SH	1 – 2	16 – 32	0.125 – 0.25	4 – 8	1 – 2

* Breakpoints were adopted from EUCAST (2014) except for tetracycline, which breakpoint was adopted from CLSI (2009).

For food isolate, biofilm cells exposed to BAC had no decrease in susceptibility. Conversely, exposure to TC led to susceptibility decrease to all antibiotics tested, except for CLO. While biofilm cells exposed to HP showed a decreased susceptibility for CIP and an increase for CLO and TET, exposure to SH only led to decreased susceptibility for CIP. Concerning the results obtained with different lifestyles of *S.* Enteritidis 350, it was observed that MIC ranges obtained with control biofilm cells were equal or higher than those obtained with planktonic cells.

Comparing the effect of the four disinfectants tested on the susceptibility to antibiotics, it was observed a considerable intraspecies variability. Accordingly, TC was the disinfectant that caused less variations in the MIC ranges concerning *S.* Enteritidis NCTC 13349, but exposure of *S.* Enteritidis 350 to this same disinfectant has caused an increase of MIC range of most antibiotics (80% of cases). Moreover, while exposure of reference strain to SH showed a decreased susceptibility of most antibiotics, exposure of food isolate to this disinfecting agent only led to decreased susceptibility for CIP.

Concerning MIC breakpoints, in none of the cases *S.* Enteritidis strains became resistant to antibiotics after exposure to disinfectants. Moreover, there was no resistance reported on planktonic and biofilm control cells except for CIP, towards which all planktonic, control and exposed biofilm cells were found to be considered resistant.

4.3 Evaluation of biofilm formation ability

Biofilm formation ability of biofilm cells after exposure to each disinfectant was assessed by crystal violet staining method, as previously described in Chapter 3 (section 3.6). The results obtained to both strains are represented in Figure 4.1.

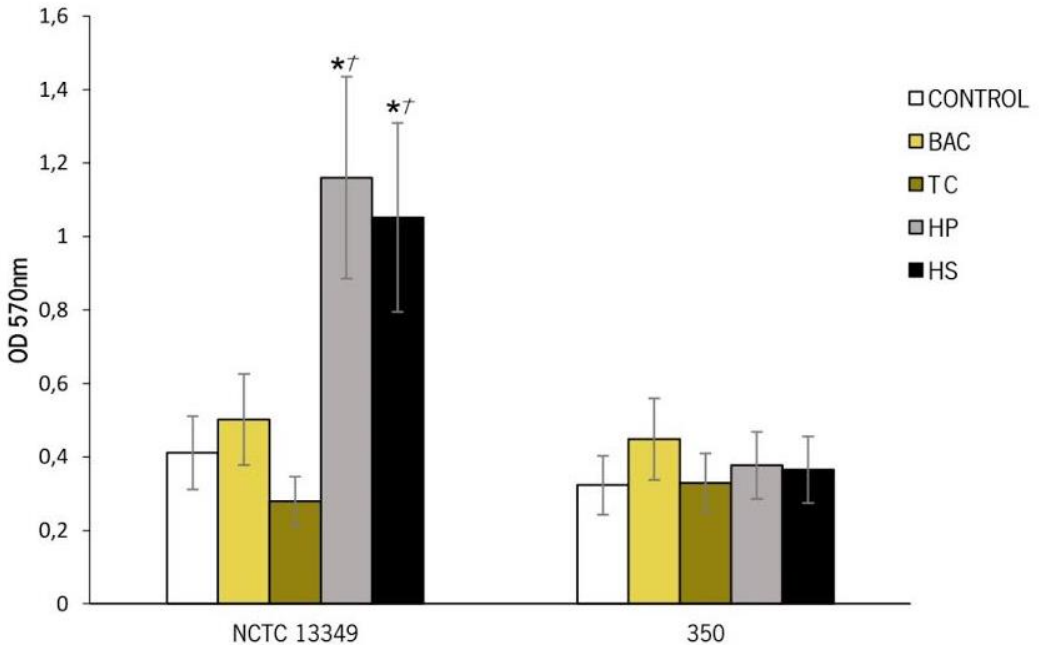

Figure 4.1 Biofilm formation by biofilm cells after exposure to different disinfectants. Bars represent average CV-OD_{570nm} values and standard deviations. Symbols indicate statistically different values ($p \leq 0.001$) within each strain comparing to the respective control (*), and between strains considering the same experimental condition (*†*).

In most cases, biofilm formation ability was very similar for all the conditions tested, both within the same strain and between strains. In fact, for food isolate 350 no significant differences were observed, and the only changes worth of note were those observed for the reference strain, which HP and SH exposed biofilm cells showed a higher biofilm formation ability in comparison with all other cells of this strain, as well as in comparison with the same conditions tested with food isolate 350 ($p \leq 0.001$).

4.4 Analysis of gene expression

Analysis of gene expression on both biofilms cells exposed and not-exposed to chemical disinfectants was performed by qPCR. As described in Chapter 3 (section 3.7), the stress response gene *rpoS* and the virulence genes *csgD*, *avrA* and *invA* were the genes considered in this study. Results obtained from genetic expression analysis are presented in Figure 4.2 concerning biofilm cells of *S.* Enteritidis reference strain NCTC 13349 (A) and food isolate 350 (B).

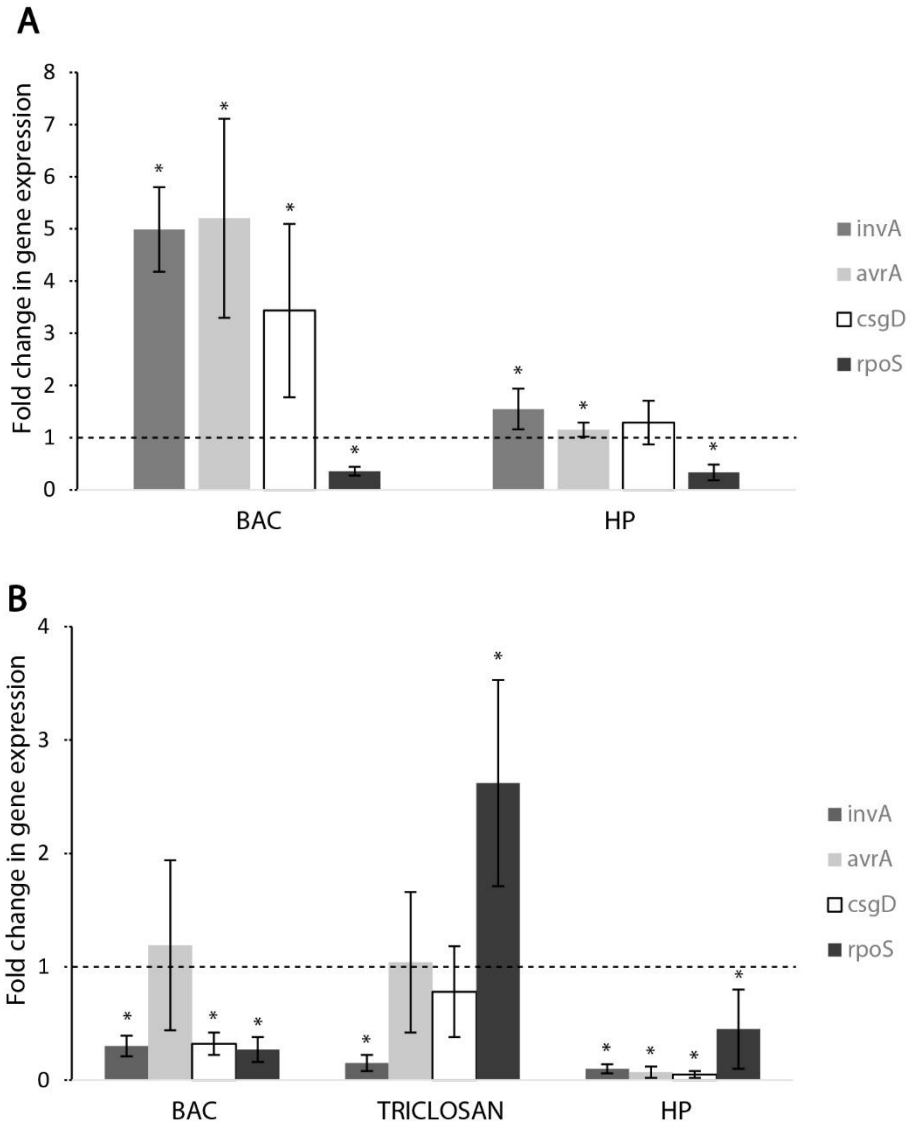

Figure 4.2 Genetic expression analysis of *S.* Enteritidis NCTC 13349 (A) and food isolate 350 (B) biofilm cells. Results are shown as the fold-change in expression comparing to that of control biofilm cells (represented by the dotted line). Symbol * indicates significant differences ($p < 0.05$) on gene expression of biofilm cells exposed to chemical disinfectants compared to control.

According to results presented in Figure 4.2, is possible to evaluate how each disinfectant has affected the expression of the selected genes in biofilm cells. It was not possible to obtain data regarding gene expression after exposure to HS, for both strains, and after exposure of *S.* Enteritidis reference strain to TC, because the amounts of RNA extracted from these biofilm cells were too low to proceed with gene expression analysis. Only genes overexpressed were interpreted, since they may have a greater influence than under expressed genes in the case an eventual infection caused by these cells. Furthermore, despite the statistically significant differences ($p < 0.05$), only differences above 2-fold were interpreted as biologically significant.

The overall results showed that BAC was the disinfectant with the highest influence on the expression of all the virulence genes tested. In fact, exposure to this disinfectant has promoted the highest increment on *avrA* and *invA* expression (a 5-fold change in *S.* Enteritidis reference strain) compared to control biofilm cells (Figure 4.2 A). On the other hand, biofilm cells of the food isolate exposed to TC did not suffer significant alterations of *avrA* and *csgD* gene expression. However, in this same experimental condition, it was observed an up-regulation of *rpoS* gene (Figure 4.2 B). By its turn, exposure to HP did not lead to biologically significant alterations of gene expression on *S.* Enteritidis biofilms cells (Figure 4.2 A, B).

Concerning BAC and HP results, even though significant biologically differences were not always detected, intraspecies variability on gene expression was observed to all virulence genes analyzed, while the stress response gene *rpoS*, suffered a down-regulation in all the conditions tested.

Chapter 5 – Discussion

Salmonella's biofilm formation capacity in abiotic surfaces is one of the biggest problems in food processing areas (Cogan *et al.*, 1999; Moore *et al.*, 2007). Despite of the a great variety of commercially available disinfectants, it has been reported that bacterial resistance to these agents is increasing (Randall *et al.*, 2007; Moretro *et al.*, 2009, 2012; Araújo *et al.*, 2011; Condell *et al.*, 2012). Moreover, although it has been already reported that microbial cells that survive chemical disinfection may express resistance to antibiotics and changes in gene expression (Bailey *et al.* 2009; Condell *et al.*, 2012; Rodrigues *et al.*, 2011; Salazar *et al.*, 2013), there is still a lack of knowledge when biofilms are concerned. In this context, the main goal of the present research was to investigate the effect of exposure to chemical disinfectants common used in food processing areas in the resistance and virulence of *S.* Enteritidis biofilm cells. In order to give an insight into the response of surviving biofilm cells, they were phenotypically characterized after exposure to the disinfectants, in terms of resistance to antibiotics, biofilm formation ability, and expression of virulence genes. With the purpose of developing an appropriate and structured evaluation of the results obtained in this research, the exposure to each of the disinfecting agents studied - benzalkonium chloride (BAC); triclosan (TC); hydrogen peroxide (HP) and sodium hypochlorite (SH) - will be analyzed separately concerning their effect on biofilm cells. Following, a general analysis will be performed based on the disinfection procedures that are used in the food industry and their potential impact on human health, in the case of an eventual infection caused by these biofilm-derived cells.

Benzalkonium chloride (BAC) is a quaternary ammonium compound (QAC) that binds to phospholipids in the cytoplasmic membrane of bacterial cells, causing its loss of structural integrity as well as impairing permeability (McBain *et al.*, 2004; Gilbert and Moore, 2005). MBEC values obtained of BAC (Table 4.1) were higher than the in use recommended concentration for QACs – 200 μg/ml. As mentioned in Chapter 3 (section 3.3.2), biofilms were exposed to half the MBEC obtained. However, concerning this disinfectant and *S.* Enteriditis food isolate, the amount of survival cells after the prolonged exposure to the correspondent sublethal concentration (800 μg/ml) was not sufficient to perform the subsequent assays (< 5 Log). This occurrence could be related with the fact that determination of MBEC value was performed in 96-wells microtiter plates, while prolonged exposure of biofilms to disinfectants was performed in 24-wells microtiter plates. Hence, in order to evaluate the influence of using different well-plates, MBEC was also evaluated in 24-wells microtiter plates (data not shown). These assays confirmed that biofilms formed in 24-wells plates corresponded to a lower MBEC of BAC - 400 μg/ml. Hence, in order to obtain a sufficient amount of biofilm cells for subsequent trials, this was the concentration used for biofilms exposure to BAC.

Although this situation only occurred for BAC, it still emphasizes how different experimental conditions can influence the final results and conclusions of a study.

S. Enteritidis food isolate biofilms cells exposed to BAC did not present phenotypic changes that would contribute to the selection of harmful and virulent bacteria. In fact, no decreases in antibiotic susceptibility were observed (Table 4.3), as well as no change on biofilm formation ability comparing with control group (Figure 4.1), which also complies with the result obtained for expression of *csgD* since no alteration in expression was observed (Figure 4.2 B). Moreover, no biologically significant differences were observed regarding the expression of the other genes tested. Nevertheless, *S.* Enteritidis reference strain showed a very different response to BAC exposure, which demonstrates the variability found within the same species. Indeed, after BAC exposure these biofilms cells showed a decreased susceptibility to AMP and CLO (Table 4.2), besides an up-regulation of *invA* and *avrA* genes (Figure 4.2 A). In spite of no significant alteration on biofilm formation ability has been observed with crystal violet (CV) staining method (Figure 4.1), gene expression analysis revealed an up-regulation of *csgD* gene, which is involved in initial adhesion and biofilm formation (Romling *et al.*, 1998, 2000; Latasa *et al.*, 2005). This apparent disparity between the results obtained by CV staining and qPCR analysis may be due to the large standard deviation associated with the fold-change of the expression of this gene, which minimum value is below 2 and thus do not correspond to a biologically significant difference (Figure 4.2 A). Moreover, in order to supplement this result and clarify the actual effect of exposure to BAC, it would be important to conduct further analysis regarding *Salmonella* biofilm formation, namely concerning the involvement of biofilm associated protein BapA, flagella, cellulose and fatty acids (Gerstel and Römling, 2003; Solano *et al.*, 2002; White *et al.*, 2006). The evidence that, after BAC exposure, surviving biofilm cells may show an up-regulation of genes involved in cellular invasion *(invA)* and inflammatory response of hosts against infection *(avrA)* is a concerning finding. In fact, an over-expression of such genes may present an increased ability to invade intestinal epithelial cell and inhibit inflammation, in case these cells are transmitted and ingested by humans, which would enhance the survival of this pathogen inside the host.

QACs have been used for a very long time, however several findings have indicated resistance against QAC in the food industry. The ability of adapted *Salmonella enterica* strains to rapidly develop resistance to BAC and cross-resistance to antibiotics is reported in several studies (Braoudaki and Hilton, 2004; 2005, Karatazas *et al.*, 2007). Various resistance mechanisms

contribute to adaptive resistance to QACs, such as changes in the phospholipid content of the membrane, presence of multi-drug efflux pumps, and degradation of the biocides (Langsrud *et al.*, 2003). In spite of, in the present study, BAC exposure did not lead to resistance towards the antibiotics tested, the decreased susceptibility of *S.* Enteritidis NCTC 13349 to some antibiotics (AMP and CLO) after exposure to this disinfectant (Table 4.2) is in agreement with previous findings. Accordingly, although concerning planktonic cells study, a high degree of cross-resistance between BAC and several biocidal compounds, including CLO and β-lactam antibiotics, was previously detected on *S.* Virchow (Braoudaki and Hilton, 2004). Despite less information is available when considering biofilm cells, in the present study the observed decreased susceptibility to the antibiotics tested after exposure to BAC can be related with the expression of efflux pumps systems. Indeed, a study by Mangalappalli-Illathu and Korber (2006) showed that adaptation of *S.* Enteritidis biofilms to BAC occurred with the up-regulation of key specific proteins involved in energy metabolism, protein biosynthesis, adaptation, and detoxification, including proteins which might act as efflux pumps. These are integral membrane proteins that use cellular energy to extrude biocidal compounds actively out of the cell, and nine drug efflux pump genes are known to exist in *S. enterica* (Nishino *et al.*, 2009). The importance of multidrug efflux systems in intrinsic and acquired multi-resistance in *Enterobacteriaceae* and others gram-negative bacteria has been reported (Poole, 2004), and a study of Randall *et al.* (2007), on planktonic cells, showed that AcrAB–TolC efflux system was required for resistance of *S.* Typhimurium to a disinfectant based on a QAC. Moreover, AcrAB–TolC appears to direct efflux-mediated resistance to antibiotics as quinolones, CLO and TET (Baucheron *et al.*, 2004). Hence, through the results obtained in this study, and as on planktonic cells, also on biofilms cells an efflux pump system could be involved in reduction of susceptibility to antibiotics after exposure to BAC.

Triclosan (TC) is a bis-phenol compound which can inhibit FabI enzyme, involved in lipid biosynthesis, and act nonspecifically on the cytoplasmic membrane (Heath *et al.*, 2000; Tabak *et al.*, 2007). Based on the MBEC values obtained in this study (Table 4.1), biofilm exposure to TC was performed with 100 μg/ml (food isolate strain) or 200 μg/ml (reference strain). For *S.* Enteritidis reference strain, exposure to TC only led to a susceptibility increase to CEF, no other change in susceptibility to antibiotics was observed (Table 4.2). Moreover, this disinfectant did not trigger changes on the biofilm formation ability of NCTC 13349 biofilm cells. This last observation could not be confirmed with the analysis of *csgD* gene expression, because the amount and/or quality of RNA extracted from these biofilm cells were insufficient to proceed with gene expression analysis. It is

likely that the low RNA concentration of these samples can be related with the low cell concentration of biofilms cells recovered. Indeed, for the reference strain, after TC exposure only 5 log cfu/ml was obtained, while different manufacturers' protocols recommended a concentration between 10^7-10^9 cfu/ml for optimal RNA extraction (PureZOL™ RNA isolation reagent, Bio-Rad; PureLink™ RNA Mini Kit, Invitrogen; FastRNA® Pro Blue, MPBiomedicals). Accordingly, Sirsat *et al.* (2011) tested the minimum number of cells required for quantifiable bacterial RNA yield, showing that 8 log cfu/g of *Salmonella* is ideal to obtain optimal RNA amount and purity, since lower dilutions of cells exhibited a lower RNA concentration with poor purity.

Although the results obtained with the reference strain suggest that exposure to TC did not lead to a significant alteration on those biofilm cells, exposure of food isolate led to considerable alterations. Indeed, it was observed a susceptibility decrease to all antibiotics tested, except for CLO (Table 4.3). Moreover, despite these biofilm cells exposed to TC did not suffer alterations on their ability to form biofilm (which was observed with CV staining results– Figure 4.1 -, and confirmed by *csgD* gene expression analysis – Figure 4.2 B), an up-regulation of *rpoS* gene was observed. This last result is a worry finding, since the *rpoS*-encoded sigma factor (σ^s) is the master regulator of the general stress response in gram-negative bacteria (Hengge-Aronis, 1996). General stress response is accompanied by a significantly reduced growth rate, which allows cells to survive long periods of starvation and different environmental stresses (Cohen *et al.,* 2013). Moreover, this reduced growth rate is associated with the appearing of persister cells, which have been proposed to arise primarily in biofilms and in stationary-phase cultures (Lewis, 2008). It is, thus, probable that the dramatically reduced growth rates of persister cells is the major reason for the reduced susceptibility of biofilms to several compounds. Indeed, in the present study a decrease on antibiotic susceptibility was observed accompanied with an overexpression of *rpoS* gene. Hence, exposure to TC may lead to a several additional changes in virulence of *S.* Enteritidis.

Various mechanisms have been proposed to explain TC resistance on *Salmonella enterica.* It has been reported that multidrug efflux pumps, overexpression or mutations on specific target (FabI), and increased fatty acid synthesis may act synergistically and contribute to *Salmonella* resistance against TC (Tabak *et al.*, 2007; Webber *et al.*, 2008a, 2008b). In spite of low diffusion through biofilm matrix has been considered as a factor that may contribute to TC resistance on biofilm cells (Tabak *et al.*, 2007), mechanism of resistance or decreased susceptibility reported for TC on this lifestyle are still scarce. Findings obtained in present study were similar to results of a

study performed on *S.* Typhimurium planktonic cells, where exposure to TC lead to reduced susceptibility to several antibiotics and reduced invasiveness on intestinal epithelial cells (Karatzas *et al.,* 2007). Moreover, these results were associated with the overexpression of a multidrug efflux pump, which allows inferring that this same mechanism could be also occurring on *S.* Enteritidis biofilms cells, besides the occurrence of persister cells.

Hydrogen peroxide (HP) is a compound that produces hydroxyl free radicals, which act as oxidants and react with lipids, proteins and DNA, increasing cell permeability. MBEC values obtained with HP (Table 4.1) were higher than the in use recommended concentration for this agent, 200 - 300 µg/ml. In general, exposure of *S.* Enteritidis reference strain biofilms to HP did not lead to significant antibiotic susceptibility alterations (Table 4.2). Moreover, although no overexpression on *csgD* gene has been observed (Figure 4.2 A), HP exposure seems to enhance biofilm formation ability of these cells, as observed by CV staining results (Figure 4.1). Besides *csgD*, other *Salmonella* genes could have been involved in this biofilm formation promotion. Indeed, *spiA*, *sirA and ycfR* are some other genes that were not tested in this study but that can also influence *Salmonella*'s biofilm formation (Zhang *et al*., 2007; Wang *et al.,* 2010; Dong *et al.,* 2011). Anyway, the enhanced biofilm formation ability is a concerning finding due to the increased biocidal resistance associated with biofilms.

Exposure of food isolate biofilms cells to HP had similar results to those of NCTC 13349, except regarding biofilm formation ability, which not was enhanced comparing with control group (Figure 4.1). These results show that, although it is considered a strong disinfecting agent due to its antioxidant properties, HP does not seem to trigger virulence patterns on *S.* Enteritidis biofilm cells, at least concerning virulence factors analyzed in this study. In fact, except a possible increase in biofilm formation capacity (observed in only one of the strains tested), no additional virulence evidence was detected after HP exposure. Peroxides-based disinfectants have been reported to be effective for removal bacterial biofilms and are widely used in the food industry (Stopforth *et al*., 2002; Trachoo and Frank, 2002). However, researches on evaluation of possible resistance mechanism have been performed. A study by Randall *et al.* (2007) with *S.* Typhimurium planktonic cells showed that exposure to an oxidizing compound based disinfectant did not activate efflux systems, and this was not considered to be a resistance mechanism against this type of chemical agent. In spite of exposure of gram-negative planktonic cells to sub inhibitory concentrations of HP has been shown to induce catalase and glutathione reductase (both preventing oxidative stress enzymes) (Storz and Altuvia, 1994; Mukhopadhyay and Schellhorn, 1997), remarkable resistance of

biofilms to HP cannot be attributed to abnormally high initial or induced levels of catalase activity (Elkins *et al.*, 1999). In fact, studies with *Pseudomonas aeruginosa* suggest that, unlike planktonic cells, biofilm cells that actually had lower levels of catalase were effectively protected to hydrogen peroxide (Elkins *et al.*, 1999; Hassett *et al.*, 1999). Concerning biofilms, the constituents of extracellular matrix may play a key role in neutralizing this type of agents, which consequently results in increased resistance to them. In the present study, these evidences are also supported by the high MBEC obtained to this disinfecting agent (Table 4.1), as well as by the increased biofilm formation ability observed on *S.* Enteritidis reference strain after biofilms exposure to HP (Figure 4.1).

As a chlorine compound, sodium hypochlorite (SH) may damage the outer cell membrane, producing a loss of permeability control, as well as inhibit cellular enzymes or destroy DNA (Schmidt 2003; Virto *et al.*, 2005). Having determined the MBEC of SH (Table 4.1), prolonged exposure to this agent was performed at a concentration higher than the in use recommended one – 200 µg/ml. Exposure of *S.* Enteritidis reference strain biofilms to SH cause a decrease of susceptibility to all antibiotics tested except to CEF, to which an increase of susceptibility was detected (Table 4.2). Although there was no data regarding *csgD* gene expression, CV staining revealed an increase of ability to form biofilm after exposure to SH (Figure 4.1). Results regarding this strain are worrying and relevant for food industries, since SH exposure enhanced biofilm formation accompanied by susceptibility decrease of antibiotics commonly used to treat salmonellosis infections. However, concerning food isolate, it only was observed a decrease of susceptibility to CIP (Table 4.3). Moreover, no changes in ability to form biofilm were detected (Figure 4.1). In this case, the difference between strains analyzed is notorious. This fact could be related to variation between strains within the same serotype, which may differ by the presence or absence of several genes. In fact, groups of strains that share a distinct profile of gene content may be referred to as genovars to distinguish them from serotypes (Porwollik *et al.*, 2004). Moreover, gene contents sometimes differed more within a serotype than between serotypes (Porwollik *et al.*, 2004). The fact that genetic differences within a serotype can have profound consequences for the pathogenicity of the isolate has already been noted (Heithoff *et al.*, 2012). Hence, is probable that SH affects and triggers specific genes that differ within the serotype tested and, consequently, it was observed the disparity of between the *S.* Enteritidis strains tested.

The amount of RNA extracted from both *S.* Enteritidis strains after biofilms exposure to SH did not allow to proceed with gene expression analysis. As stated above concerning TC, this should be related

with the low concentration of biofilm cells collected after SH exposure since, once again, only 5 log cfu/ml were achieved in these samples. In spite of gene expression analysis has not been performed, a previous work has shown that expression of a putative stress regulatory gene, *ycfR*, was significantly induced in *S. enterica* upon exposure to chlorine treatment, which conferred chlorine resistance and contributed to attachment upon (Salazar *et al.*, 2013). Moreover, as mentioned above, this gene is also related with biofilm formation. The decreased antibiotic susceptibility observed for reference strain's biofilm cells is in accordance with previous studies concerning planktonic cells. Capita *et al.* (2007) verified, in different serotypes of *Salmonella,* that the use of acidified sodium chlorite may induce the selection of resistance against this biocidal agent, and cross-resistance to various antibiotics. Potenski and her coworkers (2003) described mutants of *S. Enteritidis* selected after exposure to chlorine showing resistance to multiple antibiotics, including most of those tested in this present study as tetracycline, chloramphenicol, and ciprofloxacin. Moreover, they also suggested that the *mar* operon mutation was responsible for resistance, which has also been assumed to have a crucial role on the possible association between *Salmonella* persistence in poultry houses and resistance to commonly used disinfectants (Gradel *et al.*, 2005).

Making an overall appreciation of the results obtained with all the different disinfectants used in this study, it is noticed that, with the exception of triclosan, for which there is no concentration value defined, all MBEC values were way above the in use recommended concentration for food surfaces' disinfection procedures (Table 4.1). In general, *S.* Enteritidis biofilm cells were more susceptible to TC than to any other disinfectant tested, since this was the agent that presented the lowest MBEC value. On the other hand, SH and HP, both oxidizing agents, were the disinfecting agents with the highest MBEC values (Table 4.1), which correlates with a lower susceptibility of *Salmonella* biofilms to these agents. The lower susceptibility of *S.* Enteritidis biofilms to SH may be related to cellulose, which is one of two components that have been identified as important in the *Salmonella*'s biofilm matrix (Gerstel and Römling, 2003). In fact, a study by Solano *et al.* (2002) shown that cellulose is directly responsible for the chlorine resistance of *Salmonellae* in the biofilm, since cellulose-minus mutants did not survive under low concentrations of sodium chloride. Moreover, the poorly biofilm penetration of this compound may be due to the neutralization of the active chlorine as it reacts with organic materials on the surface of biofilms (Stewart *et. al.* 2001). In fact, the efficiency of this compounds can be impacted by organic soling matters (Huss, 2003). Similarly, biofilm constitution

could have acted as a diffusion barrier to HP, with matrix polymers reacting chemically with this agent and contributing to a decreased susceptibility.

Concerning antibiotic susceptibility of different lifestyles, although no resistance was found, there was a considerable difference between the results obtained for the conditions tested. *S.* Enteritidis biofilm cells demonstrated equal or higher MIC values than planktonic counterparts (Tables 4.2 and 4.3), which demonstrate a lower susceptibility in these microbial communities. It is nowadays very well known that, when grown as a biofilm, most bacteria become more resistant to antibiotics (Tabak *et al.*, 2009; Papavasileiou *et al.* 2010). Several reasons to support this phenomenon have been reported: delayed penetration of the antimicrobial through the biofilm matrix; modified nutrient environments, which leads to slower growth rate within the biofilm and physiological changes, such as oxygen limitation; and up-regulation of efflux pumps (Mah and O'Toole, 2001; Donlan, 2002; Fux *et al.*, 2005). Previous evidences that only during biofilm growing mode *S.* Enteritidis cells express proteins involved in global regulation, stress response, detoxification, degradation and energy metabolism, also explains the lower susceptibility observed in this lifestyle (Giaouris *et al.*, 2013). Moreover, since *S. enterica* is a β-lactamases producer, lower susceptibility observed on biofilms to β-lactam antibiotics can be related to the accumulation of these enzymes within biofilm and with their ability to deactivate those antibiotics more rapidly than they can diffuse into the biofilm and act on *Salmonella* cells (Fux *et al.*, 2005; Paterson *et al.*, 2006). Finally, the resistance pattern observed against CIP on planktonic and biofilm cells (before and after exposure to disinfectants) is particularly worrying, since this is an antibiotic commonly used to treat salmonellosis. This is in agreement with previous reports that showed that resistance to ciprofloxacin in *Salmonella enterica* has become a global alarm (Raveendran *et al.*, 2008; Westrell *et al.*, 2014), being active efflux the primary mechanism of resistance in *Salmonella* Typhimurium (Giraud *et al.*, 2000). Hence, in view of such previous findings as well as those obtained in the present study, ciprofloxacin should no longer be considered as antibiotic of choice in treating *Salmonella enterica* infections.

Regarding biofilm formation ability, it was just observed an increase after exposure to oxidizing compounds - SH and HP - and only in one of the strains tested (Figure 4.1). However, this improved biofilm formation ability after adaptation to disinfectants are in agreement with similar observations in previous studies, performed with different bacterial species and chemical compounds (Machado *et al.*, 2012; Pagedar *et al.*, 2012). In fact, a study by Capita *et al.*, (2014) showed that

cells adapted to sodium nitrite and sodium hypochlorite had a greater ability to produce biofilm than non-adapted *E. coli* cells. These findings suggest that the use of disinfecting agents at sublethal concentrations in the food processing areas may increase the ability of bacteria to produce biofilm, thus imposing a food-safety threat. Indeed, it has been reported that approximately 80% of bacterial infections are associated with biofilms (Steenackers *et al.*, 2012), which highlights the importance of biofilm formation as a virulence factor in human infections.

Concerning gene expression analysis, although the results have shown different expression patterns, even between strains, it was observed that BAC was the disinfectant with the highest influence on the overexpression of the virulence genes tested (Figure 4.2 A and B). Although studies have been performed regarding altered gene expression profile of microorganisms that survived to exposure to disinfectants (Wang *et al.*, 2010; Salazar *et al.*, 2013), research on surviving biofilm cells are yet very scarce (Tabak *et al.*, 2007; Rodrigues *et al.*, 2011). However, and in view of the results of this study, the possibility of occur an up-regulation of virulence genes is a matter of concern. Besides these cells may compromise food safety, they also potentiate public health risk. In fact, virulence factors encoded by these genes help pathogenic bacteria to survive adverse environmental conditions and are also required to enhance their pathogenicity (Archer, 1996; Spector and Kenyon, 2012). A general view of the results obtained in this study also allows concluding that the use of transcriptomic approaches to assess impact of disinfecting agents' exposure is a pertinent tool since, together with other kinds of analysis, it gives an important contribution towards a thorough study. Nevertheless, it was also confirmed by this study that a good RNA samples is a critical feature in order to get a successful qPCR analysis.

In summary, an overview on the impact that disinfecting agents commonly used in food processing facilities may present on surviving biofilm cells highlights a potential risk for public health. In fact, the phenotypic characterization performed in this study demonstrated that *S.* Enteritidis biofilm cells exposed to disinfectants can lead to a decreased susceptibility to antibiotics commonly used to treat salmonellosis, enhanced biofilm formation ability, and/or an overexpression of virulence and stress response genes.

Chapter 6 – Conclusions

The aim of the present study was to improve the knowledge about the phenomena involved in foodborne contaminations caused by *Salmonella enterica* Enteritidis, particularly regarding exposure of biofilm cells to chemical disinfectants, and its impact on the virulence and resistance of these bacteria. In order to achieve this, several aspects were studied, including biofilms susceptibility to disinfecting agents, biofilm formation ability, susceptibility to antibiotics, and virulence and stress response-related genes expression.

Besides biofilm formation on food processing areas allows the microorganism to persist in these environments, it also leads to an increased resistance to cleaning and disinfection procedures. In the present study, *S.* Enteritidis biofilm cells were more susceptible to TC and less susceptible to oxidizing agents (SH and HP), which must be predominately related with the importance of biofilm matrix on resistance to biocidal compounds. In view of this conclusion, and transposing to the reality of food processing areas, the application of SH and HP on these areas can present a matter of concern, since in this study they only eradicated *S.* Enteritidis biofilms at concentrations above the recommended for use in food industries. Hence, even when the maximum concentration allowed is applied, those cells may, in fact, being exposed to a sublethal concentration and, thus, can be triggered to develop resistance against the chemical agent.

In spite of antibiotic therapy is not recommended for the treatment of infections caused by *Salmonella enterica* (Gill and Hamer, 2001; Kit *et al.*, 2011), the most severe cases require the use of these agents. The results here obtained showed that, even without exposure to disinfectants, *S.* Enteritidis biofilm cells had a lower susceptibility to antibiotics than planktonic cells. This finding constitutes an aggravating problem to human health, since biofilms are the most common lifestyle of microorganisms, mainly due to the set of advantages that it comprises comparing to the planktonic form. Hence, even though in this study it was not found a pattern of true resistance (values obtained were below the respective breakpoints), it points out that is possible to an infection caused by *Salmonella* biofilm cells to be associated with a decreased susceptibility to antibiotic therapy. Furthermore, a resistance pattern was observed to CIP on planktonic and biofilm cells, both before and after exposure to disinfectants, which demonstrates that the use of this antibiotic should be re-evaluated. In fact, by failing to kill the pathogen, a therapy with this antibiotic might contribute to the retention of *Salmonella* within the host, allowing the infection to develop for salmonellosis' hazardous stages, which can lead to dehydration, bacteremia and, ultimately, death.

Although further studies are required, the analysis of the effect of disinfectants on biofilm formation showed that oxidizing compounds (such as SH and HP) may contribute to enhance this *S*. Enteritidis ability. This is actually a relevant subject, since the goal of the using disinfectants is to eliminate possible pathogens, not to contribute to their increased virulence and persistence on food environments, as can be the case when enhancing biofilm formation ability.

Concerning impact on gene expression, it was observed that BAC was the disinfectant with the highest influence on the overexpression of virulence genes. Hence, the use of this disinfectant should be reconsidered, since it may improve virulence and pathogenicity of *S*. Enteritidis biofilm cells. The finding that cells exposed to disinfectants can also show an overexpression of virulence genes is a matter of concern. Despite virulence gene expression contributes to pathogenicity, it is also related with the capacity of bacteria to survive to adverse environmental conditions, as those found within the host, and thus become harder to control.

Although the increased virulence observed has to be further analysed and confirmed with *in vivo* studies, the results obtained in this study suggest that biofilm cells that survived chemical disinfection may represent an increased public health risk. In fact, these cells can present decreased susceptibility to antibiotics, enhanced biofilm formation ability, and overexpression of virulence and stress response genes, which may lead to an increase in *Salmonella* pathogenicity in the case of an eventual infection.

Finally, this works allows concluding that it is important to assess and understand the phenotypic characteristics of pathogenic biofilm cells after exposure to chemical treatment since, besides enabling access to the mechanisms involved in biocidal resistance, this approach may allow the development of additional treatments that do not lead to cross resistance and/or induction of virulence and pathogenicity.

Chapter 7 – Future approaches

The present study was performed in order to have a perspective of what can happen on food processing areas when biofilm cells survive or adapt to disinfection treatment. These assays were performed with pure cultures but it has been proved that are several conditions that may affect chemical disinfection process and biofilm formation, such as nutrient and organic matter levels, temperature, osmolarity, and pH (Solano *et al.*, 1998; Bonafonte *et al.*, 2000; Stepanovic *et al.*, 2003; Goeres *et al.*, 2005; Mangalappalli-Illathu *et al.*, 2008a; Castelijn *et al.*, 2012; Abdallah *et al.*, 2014). Hence, further studies should be performed in order to get a better approximation between the laboratorial conditions and those existent in food processing environments. Moreover, different materials used as food contact surfaces can also affect disinfectants' performance and biofilm formation ability (Joseph *et al.*, 2001; Schmidt, 2003; Stepanovic *et al.*, 2004; Oliveira *et al.*, 2006; Giaouris *et al.*, 2012; Steenackers *et al.*, 2012), and should also be assayed. For comparative purposes, it would also be interesting to test exposure to disinfecting agents using the concentrations and the periods of time at which they are actually applied in food processing areas.

The concern about *S.* Enteritidis biofilm occurrence on food processing facilities was the main subject of the present study. As mentioned in Chapter 1 (1.2.1), this serotype has been the most significant regarding *Salmonella* infections reported in recent years, and that is way it was chosen to perform a characterization of biofilms after exposure to disinfectants. However, it is known that most natural biofilms exist as multispecies consortia (Manuzon and Wang, 2007; Moons *et al.*, 2009). Hence, it would be important for future studies to be performed with multispecies biofilms, involving other important foodborne pathogens such *E. coli*, *Campylobacter* and *Listeria* (EFSA and ECDC, 2014a). Moreover, in order to evaluate the behavior of a higher variety of strains, it would be important that future studies include a higher number of strains and isolates from different sources.

To complete this study, additional analysis on biofilm cells exposed to disinfectants may be performed in the future. Namely, in order to verify whether exposure to disinfecting agents could change the MBEC value and infer on its resistance, biofilm cells should be re-exposed to them. It would be also relevant to further analyze cell viability on biofilms, since the method applied – CV - stains both living and dead cells together with the extracellular matrix and, thus, only allows to assess total biomass (Pitts *et al.*, 2003; Romanova *et al.*, 2007). Moreover, additional omic tools, as proteomics, could help to elucidate the responses and mechanisms involved after exposure to disinfecting agents, and flow cytometry assays would also be very interesting in order to detect possible sub-populations of dormant cells (persister cells) after disinfection procedures. Finally,

studies using animal cells would be a very motivating approach to collect data about pathogenicity, virulence and resistance, and could be conducted by using human epithelial cell or mouse models.

Concerning qPCR assay, several features could be improved. In a first approach, more than one reference gene would be used in order to ensure a more robust analysis. Ideally, a large number of genes should be analyzed, either related with the same virulence factors investigated in this study or with other additional factors related to virulence and stress-response of *Salmonella*. In order to overcome limiting steps found in this study, namely the low RNA concentration of some samples, it becomes crucial to optimize all the procedures in order to ensure the collection of sufficient cell concentration before performing qPCR assays.

Since this study suggests that, after exposure to disinfectants, microorganisms can acquire virulence and resistance patterns considered harmful to human health, development of new chemical-free control strategies involving enzymes solutions, bacteriophages, or microbial derived antimicrobial compounds continues to be a relevant and attractive research challenge.

Chapter 8 – References

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