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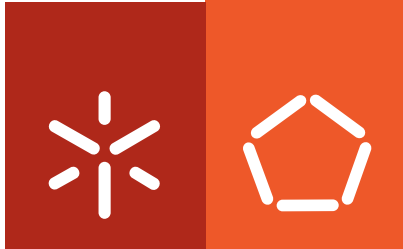
Diana Alexandra Ferreira Rodrigues

Listeria monocytogenes and
Salmonella enterica adhesion,
biofilm formation and control

Diana Alexandra Ferreira Rodrigues *Listeria monocytogenes* and *Salmonella enterica*
adhesion, biofilm formation and control

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Universidade do Minho
Escola de Engenharia

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***Listeria monocytogenes* and
Salmonella enterica adhesion,
biofilm formation and control**

Doctoral dissertation for PhD degree in
Chemical and Biological Engineering

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University of Minho, 3rd September 2010

Whether our efforts are, or not, favored by life, let us be able to say, when we come near the great goal, "I have done what I could".

Louis Pasteur

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Abstract

Food contamination leads to wide economic loss and has a strong impact on public health worldwide. *Listeria monocytogenes* and *Salmonella enterica* Enteritidis are two of the most sight threatening and frequent foodborne pathogens, being responsible for listeriosis and salmonellosis foodborne outbreaks, respectively. The work presented in this thesis aimed at investigating adhesion and biofilm formation ability of these two bacteria regarding yet unexplored growth conditions and exposure to antimicrobials, as well as study possible repercussions of chemical disinfection on the genetic expression of virulence factors and stress response by surviving biofilm cells.

L. monocytogenes has been a polemic bacterium as far as its biofilm formation capability is concerned, with different, and sometimes controversial, conclusions being stated by several authors. After testing this biological process under batch and fed-batch growth modes, both previously used by several authors but never compared simultaneously before, the results herein presented showed that the different growth modes influenced biofilm formation by *L. monocytogenes* on polystyrene, both in terms of biofilms' total biomass and cellular viability. Temperature also played an important role on *L. monocytogenes* biofilm formation since refrigeration temperatures led to biofilms with less biomass but highly metabolic active, while at 37°C biofilms had higher amount of biomass but were metabolically weaker.

Surface coatings and antimicrobial incorporated materials have been two of the most promising attempts to produce new and improve already existing materials to be applied in food processing environments, in order to prevent microbial contaminations. A nitrogen-doped titanium dioxide coating on glass and on stainless steel was tested and showed to have bactericidal effect upon *L. monocytogenes* after only 30 minutes irradiation with visible light (fluorescent and/or incandescent light), when compared to non-coated surfaces. This fact indicated that such coated materials are likely to be applied on food contact surfaces as a means to reduce the risk of bacterial colonization and, thus, to improve food safety. The action of incorporated triclosan was assessed through *S. enterica* adhesion and biofilm formation on yet poorly studied food contact materials - stones. In this way, silestones (artificial stones mainly

made of quartz, with triclosan incorporated) were tested and their performance compared with regular bench cover stones (granite and marble, without any antimicrobial compound) and stainless steel (one of the most commonly found surfaces in food processing environments). Similar levels of bacterial colonization and biofilm formation were observed on all materials, and lower numbers of *S. enterica* viable-culturable cells were found within biofilms formed on silestones. This indicates that, despite having shown some bactericidal effect upon biofilm cells, triclosan incorporated in silestones did not prevent bacterial colonization or biofilm formation.

Once means to prevent contamination have failed and biofilms had already colonized the food contact surfaces, or in those cases where it is practically impossible to avoid microbial colonization during food processing, the greater concern becomes the surface cleaning through disinfection. In this work, susceptibility of *L. monocytogenes* and *S. enterica* monoculture-biofilms to disinfection was evaluated by determining the minimum biofilm eradication concentration (MBEC) of four distinct disinfectants commonly used in food industry – sodium hypochlorite, benzalkonium chloride, hydrogen peroxide and triclosan. Biofilm from both bacterial species were more susceptible to sodium hypochlorite than to any other disinfectant, whereas *S. enterica* biofilms were found to resist to triclosan's action. Moreover, these assays revealed *L. monocytogenes* biofilms to be more susceptible to disinfection than *S. enterica* biofilms, which MBEC mean values concerning each disinfectant were higher than those found by the former bacterium. In order to investigate if disinfection had genetic repercussions on these biofilms, more specifically regarding stress-response and virulence genes expression by the surviving cells, quantitative real-time polymerase chain reaction was performed. Significant up-regulations were observed for *L. monocytogenes* and *S. enterica* stress-response genes *cplC* and *ropS*, respectively, as well as for *S. enterica* virulence gene *avrA*. These findings bring to discussion the fact that, even at concentrations that are able to significantly reduce biofilms biomass, chemical disinfectants seem to induce genetic alterations on the surviving cells that might not only lead to a stress response but, and even more worrying, may also increase their virulence.

Sumário

A contaminação de alimentos não só leva a grandes perdas a nível económico como tem também um forte impacto negativo na saúde pública em todo o mundo. *Listeria monocytogenes* e *Salmonella enterica* Enteritidis são dois dos patogénicos alimentares mais perigosos e frequentes, sendo responsáveis por surtos de listeriose e salmonelose alimentar, respectivamente. O trabalho apresentado nesta tese teve como objectivo estudar a capacidade de adesão e de formação de biofilme por parte de ambas as espécies mencionadas tendo em consideração condições de crescimento e exposição a agentes antimicrobianos, até então não investigados, assim como analisar possíveis repercussões que a desinfecção química possa ter a nível de expressão de genes de resposta ao stresse e de virulência por parte de células de biofilme sobreviventes.

Tem havido alguma controvérsia no que respeita à capacidade de formação de biofilme da espécie *L. monocytogenes*, com vários autores a apresentar conclusões diferentes, e por vezes contraditórias, sobre esta matéria. Após testar o efeito de dois modos de crescimento – em sistema fechado e com alimentação escalonada (ambos usados previamente por vários autores mas que nunca tinham sido comparados simultaneamente) -, os resultados aqui apresentados mostraram que os diferentes modos de crescimento influenciaram a formação de biofilme de *L. monocytogenes* em poliestireno, quer em termos de biomassa total como também a nível da viabilidade celular dos biofilmes. A temperatura também desempenhou um papel importante na formação de biofilmes de *L. monocytogenes*, dado que à temperatura de refrigeração formou-se biofilmes com menos biomassa mas metabolicamente muito activos, enquanto que a 37°C formou-se biofilmes com mais biomassa mas metabolicamente mais fracos.

O revestimento de superfícies e a incorporação de antimicrobianos em materiais têm sido duas das tentativas mais promissoras para produção de novos materiais, e melhoria dos já existentes, para aplicação em meios de processamento de alimentos. Neste contexto, foi testado um revestimento de dióxido de titânio com azoto em vidro e em aço inoxidável, o qual mostrou ter efeito bactericida sobre a *L. monocytogenes* após apenas 30 minutos de irradiação com luz visível (fluorescente e/ou incandescente) quando comparado com superfícies não-revestidas.

Este facto indica que tais materiais são passíveis de serem aplicados em superfícies de contacto com os alimentos como forma de reduzir o risco de colonização bacteriana e, assim, melhorar a segurança alimentar. A acção do triclosano incorporado foi avaliada através da capacidade de adesão e de formação de biofilme de *S. enterica* em materiais de contacto com alimentos ainda pouco estudados – as pedras. Para tal, testou-se o desempenho de silestones (pedras artificiais constituídas maioritariamente por quartzo, com triclosan incorporado) comparando-o com pedras comuns usadas em bancadas de cozinha (granito e mármore, sem qualquer composto antimicrobiano) e aço inoxidável (uma das superfícies mais frequentemente encontradas em meios de processamento de alimentos). Verificaram-se níveis semelhantes de colonização bacteriana e formação de biofilme em todos os materiais e que o número de células viáveis-cultiváveis de *S. enterica* foi mais baixo nos biofilmes formados nos silestones. Isto indica que, embora tendo algum efeito bactericida sobre as células do biofilme, o triclosan incorporado nos silestones não preveniu a colonização bacteriana nem a formação de biofilme.

Uma vez falhadas as medidas de prevenção de contaminação e colonizadas por biofilmes as superfícies de contacto com alimentos, ou nos casos em que é praticamente impossível evitar a colonização microbiana durante o processamento dos alimentos, a maior preocupação torna-se a limpeza de superfícies através da desinfecção. Neste trabalho, avaliou-se a susceptibilidade à desinfecção por parte de biofilmes simples de *L. monocytogenes* e *S. enterica* por meio da determinação da concentração mínima de erradicação de biofilme (CMEB) de quatro desinfectantes diferentes frequentemente usados na indústria alimentar – hipoclorito de sódio, cloreto de benzalcónio, peróxido de hidrogénio e triclosano. Os biofilmes de ambas as espécies bacterianas foram mais susceptíveis ao hipoclorito de sódio do que a qualquer outro desinfectante, tendo-se ainda verificado alguma resistência por parte dos biofilmes de *S. enterica* à acção do triclosano. Além disso, estes ensaios revelaram uma maior susceptibilidade à desinfecção por parte dos biofilmes de *L. monocytogenes* comparativamente com os biofilmes de *S. enterica*, cujos valores médios de CMEB de cada desinfectante foram maiores do que os registados para a primeira bactéria. De modo a investigar-se se a desinfecção teve repercussões genéticas nestes biofilmes, mais especificamente no que respeita à expressão de genes de resposta ao stress e de virulência por parte das células sobreviventes, realizaram-se reacções quantitativas em cadeia da polimerase em tempo-real. Verificou-se a sobre-expressão significativa dos genes de resposta ao stress *cpIC* e *rpoS* de *L. monocytogenes* e *S. enterica*,

respectivamente, assim como do gene de virulência *avrA* de *S. enterica*. Estas descobertas levantam a questão de que, mesmo submetidas a concentrações de desinfetante capazes de reduzir significativamente a biomassa dos biofilmes, as células sobreviventes parecem sofrer alterações genéticas relacionadas não só com a uma reposta ao stresse mas também, e mais preocupante ainda, com um possível aumento da sua virulência.

Outline of the Thesis

The present thesis is organized into five chapters.

Chapter 1 provides an overview of aspects related with foodborne pathogens, their interaction with food contact surfaces by means of bacterial adhesion and biofilm formation, as well as different approaches to control them.

Chapter 2 focuses *L. monocytogenes* biofilm formation capability under different growth modes and temperatures, concerning biomass and cellular viability of the biofilms formed.

Chapter 3 describes the performance of modified food contact surfaces, such as N-TiO₂ coated stainless steel and glass, and triclosan incorporated kitchen bench stones, on affecting *L. monocytogenes* survival and *S. enterica* adhesion and biofilm formation, respectively.

Chapter 4 refers to *L. monocytogenes* and *S. enterica* biofilms susceptibility to disinfection by different compounds commonly used in food industries sanitation, and to the genetic analysis of the surviving cells in terms of stress-response and virulence genes expression.

Chapter 5 provides general conclusions of the present thesis and proposes suggestions for future work.

Contents

Acknowledgements.....	v
Abstract.....	vii
Sumário	viii
Outline of the Thesis	xiii
Contents.....	xiiiiv
List of Tables and Figures	xix
Glossary Abbreviations	xxiii
Scientific Output	xxv
Chapter 1 Introduction	29
1.1 Microbial food contamination	31
1.2 Foodborne diseases and pathogens	31
1.2.1 <i>Listeria monocytogenes</i>	34
1.2.1.1 <i>Listeria monocytogenes</i> and listeriosis history.....	34
1.2.1.2 <i>Listeria monocytogenes</i> characteristics.....	35
1.2.1.3 <i>Listeria monocytogenes</i> as foodborne pathogen.....	37
1.2.1.4 Listeriosis	38
1.2.2 <i>Salmonella enterica</i> Enteritidis.....	40
1.2.2.1 <i>Salmonella enterica</i> and salmonellosis history	40
1.2.2.2 <i>Salmonella enterica</i> characteristics.....	40
1.2.2.3 <i>Salmonella enterica</i> as foodborne pathogen.....	42

1.2.2.4 Salmonellosis	43
1.3 Microbial contamination of food contact surfaces	44
1.3.1 Bacterial adhesion	44
1.3.1.1 <i>Listeria</i> and <i>Salmonella</i> adhesion to food contact surfaces	47
1.3.2 Biofilm formation	48
1.3.2.1 <i>Listeria</i> and <i>Salmonella</i> biofilms on food contact surfaces	51
1.4 Control of foodborne pathogens	53
1.4.1 Surface coatings	54
1.4.1.1 Titanium dioxide	54
1.4.2 Antimicrobial incorporated materials	56
1.4.2.1 Microban®	57
1.4.3 Disinfectants in food industry	59
1.4.3.1 Bacterial biofilms and disinfectants interaction	65
1.5 Stress-response and virulence of bacterial foodborne pathogens	68
1.6 Scope and aims of this thesis	70
1.7 Reference list	71
Chapter 2 Effect of batch and fed-batch growth modes on biofilm formation by <i>Listeria monocytogenes</i> at different temperatures	97
2.1 Introduction	99
2.2 Materials and methods	99
2.3 Results	101
2.4 Discussion	106

2.5 General conclusions	108
2.6 Reference List	109
Chapter 3 Bacterial adhesion and biofilm formation on materials with antimicrobial properties.....	113
Section 3.1 Food contact surfaces coated with nitrogen-doped titanium dioxide: effect on <i>Listeria monocytogenes</i> survival under different light sources.....	115
3.1.1 Introduction	117
3.1.2 Materials and methods.....	118
3.1.3 Results	120
3.1.4 Discussion	124
3.1.5 General conclusions	127
3.1.6 Reference List.....	128
Section 3.2 <i>Salmonella enterica</i> Enteritidis biofilm formation and viability on regular and triclosan incorporated bench cover materials	131
3.2.1 Introduction	133
3.2.2 Materials and methods	134
3.2.3 Results	136
3.2.4 Discussion	141
3.2.5 General conclusions.....	143
3.2.6 Reference list.....	144
Chapter 4 <i>Listeria monocytogenes</i> and <i>Salmonella enterica</i> Enteritidis biofilms susceptibility to different disinfectants and genetic expression analysis of surviving cells.....	151
4.1 Introduction.....	153
4.2 Materials and methods	154

4.3 Results.....	159
4.4 Discussion.....	162
4.5 General conclusions	166
4.6 Reference list	168
Chapter 5 Main conclusions & Suggestions for future work.....	179
5.1 Main conclusions.....	181
5.2 Suggestions for future work	183

List of Tables and Figures

Tables

Table 1.1 Microorganisms responsible for common foodborne illness.....	32
Table 1.2 Antimicrobial targets, mechanism of interactions and antimicrobial effects of selected biocides	60
Table 3.2.1 Total biomass and viability of <i>Salmonella</i> Enteritidis biofilms.....	140
Table 4.1 Primers used for the assesement of gene expression by qPCR	157
Table 4.2 MBEC values of each disinfectant agent	159

Figures

Figure 1.1 <i>Listeria monocytogenes</i> scanning electron microscopy image showing flagella.....	35
Figure 1.2 Listeriosis incidence in European Union countries, with statistically significant increases between 1999–2006.....	39
Figure 1.3 Electron microscope picture of a <i>Salmonella</i> bacterium with several flagella.....	41
Figure 1.4 Incidence of <i>Salmonella</i> Enteritidis, as a percentage of the total number of <i>Salmonella</i> cases in Europe, 2004.....	43
Figure 1.5 Mechanisms of bacterial adhesion.....	45
Figure 1.6 Processes governing biofilm formation.....	50
Figure 1.7 Titanium dioxide photocatalysis reaction.....	55
Figure 1.8 The general structure of quaternary ammonium compounds.....	62

Figure 1.9 Micrographs of biofilm cross-sections composed of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* with progressive exposure to chloramines showing **(a)** untreated control biofilm, which is predominantly composed of respiring bacteria, and **(b)** biofilm which is predominantly composed of respiring bacteria, after 30 min. exposure to disinfectant.....67

Figure 2.1 Biofilm formation measured by crystal violet destaining on (■) batch mode and (□) fed-batch mode at **(a)** 4 °C, **(b)** 25 °C and **(c)** 37 °C. Bars represent average CV-OD₅₇₀ values and standard errors. Each pair of bars represents one strain, from left to right: 747, 925, 930, 994 and 1562. Symbols indicate statistically different values ($p < 0.05$) within each strain considering different growth modes (*) and between strains considering the same growth mode (†).....103

Figure 2.2 Biofilms cellular activity estimated by (OD_{490nm} / OD_{570nm}) ratio on (■) batch mode and (□) fed-batch mode at **(a)** 4 °C, **(b)** 25 °C and **(c)** 37 °C. Bars represent average (OD_{490nm} / OD_{570nm}) values and standard errors. Each pair of bars represents one strain, from left to right: 747, 925, 930, 994 and 1562. Symbol * indicates significantly different values ($p < 0.05$) within each strain considering different growth modes.....105

Figure 2.3 Visualization of metabolically active cells by epifluorescence microscopy on five days old *L. monocytogenes* biofilms formed on polystyrene coupons under fed-batch **(a)** and batch mode **(b)** at 4 °C, and under fed-batch **(c)** and batch mode **(d)** at 37°C. Pictures were taken under a 40x objective after L/D staining.....106

Figure 3.1.1 *L. monocytogenes* survival on uncoated and N-TiO₂ coated glass and stainless steel surfaces after 30 min exposure to fluorescent, incandescent and UV light. Symbols indicate statistically different values ($p < 0.05$) between control and coated surfaces of the same material considering the same light irradiation (*) and between the same surface considering different light irradiation (†).....121

Figure 3.1.2 Light spectra of **(a)** fluorescent, **(b)** incandescent and **(c)** UV lamps.....122

- Figure 3.1.3** Diffuse reflectance of N-TiO₂ coated glass and stainless steel.....123
- Figure 3.1.4** Water contact angles of uncoated and N-TiO₂ coated glass and stainless steel surfaces at dark and after different exposure times to UV-light. Symbol * indicates statistically different values ($p < 0.05$) between control and coated surfaces of the same material.....124
- Figure 3.2.1** Number of *Salmonella enterica* Enteritidis adhered cells per square centimeter of the different materials after 2 hours incubation. Symbols indicate statistically different values ($p < 0.05$) concerning the adhesion of different strains to the same material (*) and concerning the adhesion of the same strain to different materials (†).....137
- Figure 4.1** Genetic expression analysis of *L. monocytogenes* and *S. enterica* biofilm cells. The relative expression of stress-response (■) and virulence (■) genes was assessed by qPCR using biofilm cells of the most resistant strains to each disinfectant, namely (a) *L. monocytogenes* strains 994 and (b) 1562, and *S. enterica* strains (c) 355, (d) CC and (e) NCTC 13349. Abbreviations BAC, SH and HP stand for benzalkonium chloride, sodium hypochlorite and hydrogen peroxide, respectively. Symbol * indicates significantly different values ($p < 0.05$) when comparing the relative expression of control (cont) and surviving biofilm cells.....161

Glossary of Abbreviations

BAC	Benzalkonium chloride
bST	Beige silestone
CBD	Calgary Biofilm Device
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming units
CV	Crystal violet
DLC	Diamond-like carbon
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetracetic acid
EPS	Extracellular polymeric substances
FDA	Food and Drug Administration
GRAS	Generally recognized as safe
HP	Hydrogen peroxide
LPS	Lipopolysaccharides
LB	Luria Bertani Broth Miller
LBA	Luria Bertani Broth Miller agar
MBEC	Minimum biofilm eradication concentration
MH	Mueller-Hinton II Broth
MRD	Maximum Recovery Diluent

N-TiO ₂	Nitrogen-doped titanium dioxide
OD	Optical density
PBS	Phosphate buffer saline
PMS	Phenazine methosulphate
QACs	Quaternary ammonium compounds
qPCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RTE	Ready-to-eat
SH	Sodium hypochlorite
SPSS	Statistical Package for the Social Sciences
SS	Stainless steel
TSA	Trypticase soy agar
TSB	Tryptic soy broth
UV	Ultraviolet
WHO	World Health Organization
wST	White silestone
XTT	2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt

Scientific Output

Papers in peer reviewed journals:

Rodrigues D, Almeida M, Teixeira P, Oliveira R, Azeredo J. Effect of batch and fed-batch growth modes on biofilm formation by *Listeria monocytogenes* at different temperatures. *Curr Microbiol.* 2009;59(4):457-62.

Rodrigues D, Teixeira P, Oliveira R, Azeredo J. *Salmonella enterica* Enteritidis biofilm formation and viability on regular and triclosan incorporated bench cover materials. *J Food Prot.* In press.

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Abstracts in conferences:

Rodrigues D., Cerqueira B., Teixeira P., Oliveira R. and Azeredo J. Monoculture and mixed biofilms of *Listeria monocytogenes* and *Pseudomonas fluorescens* – effect of different culture media and temperature. *Biofilms4 International Conference*, 1-3 September, 2010 Winchester, UK.

Rodrigues D., Teixeira P., Oliveira R., Ceri H. and Azeredo J. Minimum Biofilm Eradication Concentration (MBEC) of different antimicrobials on *Listeria monocytogenes* and *Salmonella enterica* biofilms. *ISOPOL XVII 2010 – International Symposium on Problems of Listeriosis*, 5 – 8 May, 2010 Porto, Portugal.

Rodrigues D., Cerqueira B., Teixeira P., Oliveira R. and Azeredo J. Monoculture and mixed biofilms of *Listeria monocytogenes* and *Pseudomonas fluorescens* – evidences of antagonism and self-repression. *BioMicroWorld 2009 – III International Conference on Environmental, Industrial and Applied Microbiology*, 2 - 4 December, 2009 Lisbon, Portugal.

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Rodrigues D., Teixeira P., Oliveira R., Tavares C.J. and Azeredo J. Bactericidal activity of surfaced coated with nitrogen-doped titanium dioxide under different light sources. *Photocatalytic Products and Technologies Conference*, 11-13 May, 2009 Guimarães, Portugal.

Rodrigues D., Almeida M., Teixeira P., Oliveira R. and Azeredo J. Biofilms formation by *Listeria monocytogenes* isolates under different growth conditions at refrigeration temperature". // *International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld2007)*, 28 November – 1 December, 2007 Seville, Spain.

Chapter 1

Introduction

This chapter encloses the literature review, presenting in the first sections a brief introduction to microbial food contamination, foodborne diseases and pathogens. Then follows a presentation of *Listeria monocytogenes* and *Salmonella enterica* general characteristics, an overview of their relevance as two of the major foodborne pathogens responsible for severe outbreaks worldwide, and the main aspects related with their adhesion and biofilm formation. Different approaches to control foodborne microorganisms, such as antimicrobial surfaces and chemical disinfectants, are also addressed as well as bacterial foodborne pathogens stress-response and virulence.

In the last section of this chapter the scope and aims of this thesis are described.

1.1 Microbial food contamination

Food contamination is an ongoing public concern. There are three main types of food contaminants: microbiological, chemical and physical (1) but the vast majority of outbreaks of food-related illness are due to microbial pathogens rather than chemical or physical contaminants. Because the same nutrients in foods are also the same nutrients that microbes need for their growth, food spoilage is inevitable. Uncontrolled and unwanted microbial growth destroys vast quantities of food, causing significant losses both economically and with respect to nutrient content. Moreover, the consumption of food contaminated with particular microorganisms or microbial products can also cause serious illness, such as food-mediated infections and food poisoning. Every minute, there are over 50,000 cases of gastrointestinal illnesses, and many individuals, especially children, die from these infections (2). The increasing number and severity of food poisoning outbreaks worldwide has significantly increased public awareness about food safety, which is gaining much attention in recent years and Governments all over the world are intensifying their efforts to improve it.

Microbial contamination of foods can occur during any stage of the manufacturing or processing phase. Despite the difficulty and uncertainty in identifying the source of contamination in foodborne disease outbreaks, several surveillance reports have shown that post-process contamination of foods has been a major cause in many of the outbreaks. The sources of recontamination identified are unprocessed raw materials added to finished processed foods, food contact surfaces and environments, defective packaging and food handling personnel (3). The review by Reij and Den Aantrekker (2) provides a comprehensive list of outbreaks that have been caused due to post-process contamination of foods by various pathogens.

1.2 Foodborne diseases and pathogens

More than 40 different foodborne pathogens are known to cause human illness (4). Over 90% of confirmed foodborne human illness cases and deaths caused by foodborne pathogens reported to the Center for Disease Control and Prevention have been attributed to bacteria, while the rest is being due to fungi, parasites and viruses (5). In consequence, microbiological quality control programs are being increasingly applied throughout the food production chain in order to minimize the risk of infection for the consumer.

Table 1.1 shows the major foodborne pathogens and summarizes the main characteristics of the diseases they cause.

Table 1.1 Microorganisms responsible for common foodborne illness.

Adapted from: <http://www.faqs.org/nutrition/Ome-Pop/Organisms-Food-Borne.html>.

Microorganism	Disease	Symptoms	Food sources	Incubation
<i>Bacillus cereus</i>	Intoxication	Watery diarrhoea and cramps, or nausea and vomiting	Cooked product that is left uncovered _milk, meats, vegetables, fish, rice, and starchy foods	0.5–15 hours
<i>Campylobacter jejuni</i>	Infection	Diarrhea, perhaps accompanied by fever, abdominal pain, nausea, headache, and muscle pain	Raw chicken, other foods contaminated by raw chicken, unpasteurized milk, untreated water	2–5 days
<i>Clostridium botulinum</i>	Intoxication	Lethargy, weakness, dizziness, double vision, difficulty speaking, swallowing, and/or breathing; paralysis; possible death	Inadequately processed, home-canned foods; sausages; seafood products; chopped bottled garlic; kapchunka; molona; honey	18–36 hours
<i>Clostridium perfringens</i>	Infection	Intense abdominal cramps, diarrhea	Meats, meat products, gravy, Tex-Mex type foods, other protein-rich foods	8–24 hours
<i>Escherichia coli</i> group	Infection	Watery diarrhea, abdominal cramps, low-grade fever, nausea, malaise	Contaminated water, undercooked ground beef, unpasteurized apple juice and cider, raw milk, alfalfa sprouts, cut melons	12–72 hours

<i>Listeria monocytogenes</i>	Infection	Nausea, vomiting, diarrhea; may progress to headache, confusion, loss of balance and convulsions; may cause spontaneous abortion	RTE foods contaminated with bacteria, including raw milk, cheeses, ice cream, raw vegetables, fermented raw sausages, raw and cooked poultry, raw meats, and raw and smoked fish	Unknown; may range from a few days to 3 weeks
<i>Salmonella</i> species	Infection	Abdominal cramps, diarrhea, fever, headache	Foods of animal origin; other foods contaminated through contact with feces, raw animal products, or infected food handlers. Poultry, eggs, raw milk, meats are frequently contaminated.	12–72 hours
<i>Shigella</i>	Infection	Fever, abdominal pain and cramps, diarrhea	Fecally contaminated foods	12–48 hours
<i>Staphylococcus aureus</i>	Intoxication	Nausea, vomiting, abdominal cramping	Foods contaminated by improper handling and holding temperatures—meats and meat products, poultry and egg products, protein-based salads, sandwich fillings, cream-based bakery products	1–12 hours
<i>Hepatitis A</i>	Infection	Jaundice, fatigue, abdominal pain, anorexia, intermittent nausea, diarrhea	Raw or undercooked molluscan shellfish or foods prepared by infected handlers	15–50 days
<i>Giardia lamblia</i>	Infection	Diarrhea, abdominal cramps, nausea	Water and foods that have come into contact with contaminated water	1–2 weeks

Identification of agents involved in foodborne diseases began at the end of the 19th century with the clarification of the aetiology of botulism in humans (reviewed by Notermans and Powell) (3). Later milestones include the recognition of *Clostridium perfringens* as a foodborne pathogen in 1943, and *Bacillus cereus* in the 1950s. Awareness of human infections with *Listeria monocytogenes* spread throughout Europe and North America in the 1950s and foodborne transmission was suspected (6), but it was not until the occurrence of an outbreak in Canada in 1981 that proper evidence was obtained for its foodborne transmission (7). Nowadays, *Salmonella* spp., *L. monocytogenes*, *Escherichia coli* and *Campylobacter* spp. can be considered the major foodborne pathogens, although the impact of the foodborne pathogens has important geographical- and seasonal-dependent aspects. For instance, in USA noroviruses cause the largest number of illness, followed by *Salmonella* spp., *Campylobacter* spp., *Giardia lamblia*, staphylococci, *E. coli* and *Toxoplasma gondii*, respectively (8). In developing countries, the principal causes of diarrhoea are enterotoxigenic *E. coli* and *Entamoeba enterocolytica* (9). On average, only three pathogens - *Salmonella*, *Listeria* and *Toxoplasma* - are responsible for more than 1,500 deaths each year (6), and foodborne illness accounts for around 1% of USA hospitalisations cases and 0.2% of deaths (10). In England and Wales, foodborne pathogens produce 1.3 million illnesses, 20,759 hospitalisations and 480 deaths each year (11).

1.2.1 *Listeria monocytogenes*

1.2.1.1 *Listeria monocytogenes* and listeriosis history

L. monocytogenes was discovered by EGD Murray in 1924 following an epidemic affecting rabbits and guinea pigs in animal care houses in Cambridge (9). This organism, originally named *Bacterium monocytogenes*, was reported to be a human pathogen a few years later by Nyfeldt (11). At the end of the 1970s and the start of the 1980s the number of reports on *Listeria* isolations began to increase, and in 1983 the first human listeriosis outbreak directly linked to the consumption of *Listeria* contaminated foodstuffs was reported (5). After that, several reports have been made of foodborne listeriosis, both epidemics and sporadic cases, due to all kinds of foods (12, 13, 14, 15, 16, 17, 18) clearly establishing listeriosis as a severe foodborne infection (19), and thereby *L. monocytogenes* as a foodborne pathogen.

Today, the disease listeriosis caused by *L. monocytogenes* is diagnosed regularly. The incidence of listeriosis in developed countries is about 0.2 to 0.8 cases per 100,000 persons annually (20, 21, 22, 23). The incidence is not high, but as the mortality is high (24), the disease is a public health concern. Listeriosis usually manifests in the elderly, in foetuses or newborns and in individuals with severe underlying diseases. The growing number of people with predisposing factors has increased the size of the population at risk (25).

1.2.1.2 *Listeria monocytogenes* characteristics

The genus *Listeria* currently contains six species: *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria welshimeri*, *Listeria innocua*, *Listeria seeligeri* and *Listeria grayi* (26, 27). *L. monocytogenes* and *L. ivanovii* are pathogenic, the former causing disease in humans and animals, and the latter in animals (sheep cattle, etc.), while the other species are non-pathogenic (16, 17, 18). The *Listeria* species are regular Gram-positive non-sporing rods with a diameter of about 0.5 μm and a length of 0.5-2.0 μm (Figure 1.1). They are facultative anaerobes with no capsule, catalase-positive, oxidase-negative and motile at 20-25°C due to peritrichous flagella but non-motile at 37°C (24).

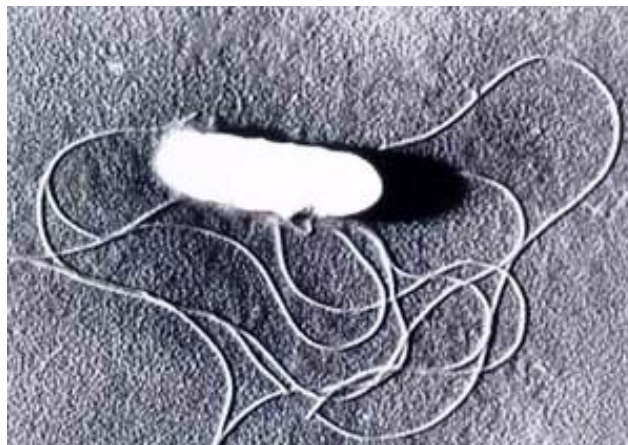


Figure 1.1 *Listeria monocytogenes* scanning electron microscopy image showing flagella.

Adapted from: http://www.textbookofbacteriology.net/Listeria_2.html.

L. monocytogenes can grow over the temperature range of 1 - 45°C with optimum between 30°C and 37°C (4, 20, 21). This bacterium can grow in laboratory media with a pH ranging from 4.3 (22) to 9.6 (19), and the minimum water activity (a_w) for growth in a laboratory medium containing glycerol has been reported to be 0.90 (23). The effects of temperature, pH, water activity, oxygen availability, and antimicrobial agents on the growth of *L. monocytogenes* have been widely studied in both model systems and foods, and there are several mathematical models available for describing the effects of these factors on the growth rate (24). Since it is a facultative anaerobic organism (19), it can grow in aerobic modified atmosphere also with competitive organisms (25). Temperature, pH, NaCl and oxygen content are parameters often adjusted to control bacterial growth in food products but, since *L. monocytogenes* can grow at low temperatures and oxygen content and with high NaCl, this bacterium is very well equipped to survive these hurdles. This ability to rapidly adapt to sudden changes in the environment is achieved by synthesising a group of proteins that act as chaperones and proteases. The chaperones assist the proper folding and refolding (assembly) of proteins while the proteases process those that cannot be refolded. This group of proteins allows *L. monocytogenes* to survive adverse conditions such as adverse temperatures (-2°C to 44°C), starvation, variations in pH and osmolarity, chemical stress and competition with other microorganisms (26, 27, 28). The adaptive response of *L. monocytogenes* to acidic conditions, such as encountered in the stomach, macrophage phagosome (29) and certain foods, may increase its virulence. Acid adapted bacteria are more likely to survive digestion in the stomach with increased internalisation by Caco-2 cells (derived from human colon adenocarcinoma that display characteristics similar to intestinal enterocytes) and are thus more likely to cause disease (30, 31).

L. monocytogenes strains are divided into three divisions, designated lineages I, II and III, as shown by molecular subtyping methods. These methods include ribotyping, multilocus enzyme electrophoresis, pulsed-field gel electrophoresis, and virulence gene sequencing (32). Strains of serotypes 1/2b, 3b, 3c, and 4b are in lineage I, serotypes 1/2a, 1/2c, and 3a strains are in lineage II, while 4a and 4c are in lineage III. Several studies reported that *L. monocytogenes* subtypes and lineages differ in their association with specific host and other environments (33, 34, 35). Although human listeriosis may be caused by all 13 serovars of *L. monocytogenes*, serovars 1/2a, 1/2b, 1/2c and 4b cause at least 95% of the cases (36, 37). Among the outbreaks of invasive listeriosis, serovar 4b strains caused the majority of the

outbreaks worldwide from 1980-2005, whereas strains of serovar 1/2 caused the majority of the non-invasive, gastrointestinal listeriosis outbreaks worldwide from 1993-2001 (37). Among food isolates, serotype 1/2 is the most frequently found (38, 39).

1.2.1.3 *Listeria monocytogenes* as foodborne pathogen

L. monocytogenes has been recognized as an important foodborne pathogen ever since an outbreak of listeriosis in Canada was linked to the consumption of contaminated coleslaw (5). Many food hygienists consider this bacterium a major food safety challenge in the food industry. The psychrotrophic nature of *L. monocytogenes* allows replication in refrigerated ready-to-eat (RTE) food products that were contaminated during processing and packaging. Consequently, *L. monocytogenes* is frequently associated with foodborne disease outbreaks that are characterized by widespread distribution and relatively high mortality rates (40).

Foods of different product categories have been implicated in outbreaks of listeriosis. These include meat products like pork tongue in jelly, sausage, paté, sliced cold meat and rillettes; dairy products like different types of cheeses, soft, semi-soft and mould-ripened including cheeses of raw milk, butter and ice cream; seafood products like gravad trout, cold-smoked rainbow trout, vacuum-packed fish products and shellfish; vegetables products like rice and corn salad, and coleslaw (38, 41). Most of these are RTE products that are eaten without further cooking or reheating. Furthermore, these products are kept refrigerated, have a long shelf-life, and contain concentrations of salt and oxygen that *L. monocytogenes* benefits by. This gives *L. monocytogenes* the ability to grow in the products during storage. Poultry also seems to be often contaminated with *L. monocytogenes*, the prevalence being as high as 50%, with beef and pork also being highly contaminated (42, 43, 44). Although *L. monocytogenes* is also found in raw fish and milk, the prevalence is usually lower than for meat or poultry (45, 46, 47, 48).

The prevalence of *L. monocytogenes* in processed products varies greatly depending on the product and the study at hand. The RTE foods represent a large variety of foods in which the prevalence of *L. monocytogenes* can range from high to low. Products that are manipulated (e.g. sliced) are at higher risk for contamination (49). Cold-smoked and gravad fish have been shown to have a particularly high prevalence (50, 51), since *L. monocytogenes* is not destroyed in the processing of these products. The prevalence is higher in vacuum-packed fish products than in products that are not vacuum-packed (51). Among processed milk products, soft cheeses are

especially susceptible, but *L. monocytogenes* can also be found in other cheeses and processed milk products.

L. monocytogenes exists widely in food production environments (52), and can survive for a long time in foods, processing plants, households, or in the environment, particularly at refrigeration temperatures. Although it commonly exists in raw foods of both plant and animal origin, it is also present in cooked foods due to post-processing contamination, if the cooked food is improperly handled after cooking. *L. monocytogenes* has been often isolated from food processing environments; especially those that are cool and wet (53). Even though *L. monocytogenes* is present at a low level in contaminated foods (< 10 CFU/gram or ml), its ability to grow at refrigeration temperature indicates that cell numbers are likely to increase during delivery and storage of those foods that can support the growth of this bacterium. Under the Federal Meat Inspection Act and the Poultry Products Inspection Act (both from USA), a RTE product is considered to be adulterated if it contains *L. monocytogenes* or if it comes into direct contact with a food contact surface that is contaminated with this bacterium (54).

The prevalence of *L. monocytogenes* in RTE foods in the US was generally determined to be 1.82% in 31,705 tested samples. The highest rates of positive samples were from seafood salads (4.7%) and smoked seafood (4.3%) (55). The majority of positive samples had a contamination level of < 10 CFU/g. However, a few samples had a contamination level of > 100 CFU/g, which exceeds to EU guidelines, and were from luncheon meats and smoked seafood. In a European survey of RTE products, the highest prevalence (18.2%) was found in smoked fish. Also, fishery products had the highest proportion of samples exceeding 100 CFU/g (2.2%) (56). The minimal number of pathogenic *L. monocytogenes* cells which must be ingested to cause illness in either normal or susceptible individuals is not known. However, it has generally estimated to be $>10^3$ CFU/g (57).

1.2.1.4 Listeriosis

L. monocytogenes causes listeriosis, which can be a non-invasive disease but primarily occurs in an invasive form. The non-invasive form is a self-limiting acute gastroenteritis in immunocompetent persons, whereas the invasive form generally affects those with a severe underlying disease or condition, e.g. immunosuppression and HIV/AIDS, pregnant women, unborn or newly delivered infants, and the elderly. The clinical signs of the invasive form are flu-

like illness, septicaemia, infection of the central nervous system including meningitis, and abortion in pregnant women (37, 58).

Ingestion of *L. monocytogenes* is likely to be a very common event, given the ubiquitous distribution of these bacteria, but the incidence of human listeriosis is low (56). Nevertheless, a general increase in human cases of listeriosis has been seen in Europe from 2003 to 2006 (Figure 1.2) affecting mainly the elderly, but reasons for this increase are unknown. One may speculate that it could be due to an overall increase in the number of elderly. Also, the general changes in eating habits to consumption of more RTE products could contribute to the increased incidence.

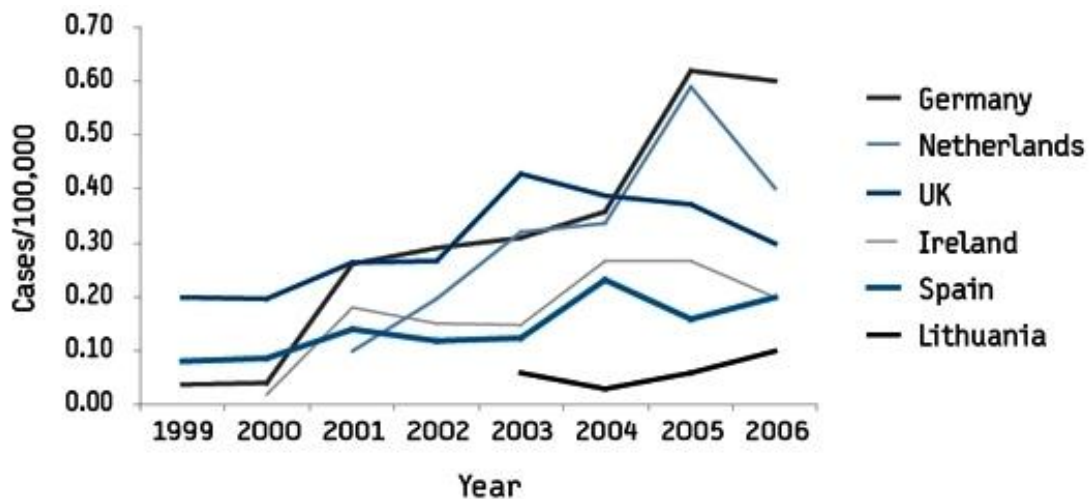


Figure 1.2 Listeriosis incidence in European Union countries, with statistically significant increases between 1999–2006. Adapted from: Denny and McLauchlin, 2008 (59).

Although infrequent as compared to other foodborne pathogenic bacteria, listeriosis is a severe infection and has an average case-fatality rate around 30% (6, 56, 60). In the Canadian outbreak in summer 2008, the case-fatality rate was as high as 39% (61). This is a markedly higher fatality rate than seen for other foodborne pathogens, which makes the control of *L. monocytogenes* very important.

1.2.2 *Salmonella enterica* Enteritidis

1.2.2.1 *Salmonella enterica* and salmonellosis history

A. A. Gärtner, in 1888, isolated from meat incriminated in a large food-poisoning outbreak a bacterium subsequently named *Salmonella enteritidis*. The genus *Salmonella* was named in 1900 after a U.S. Department of Agriculture bacteriologist, Dr. Salmon, who first described a member of the group, *Salmonella choleraesuis* (62). *Salmonella* spp. are well known pathogens and human salmonellosis is an important zoonotic infection that causes widespread morbidity and economic loss (63, 64). One of the worst food poisoning incidents in the history of the United States occurred in 1985 when 16,284 cases and 7 deaths were documented when pasteurized milk somehow became contaminated with *Salmonella* serovar Typhimurium. In 1994, this was exceeded by a national outbreak of *Salmonella* serovar Enteritidis affecting 225,000 people who consumed ice cream products (62, 65).

1.2.2.2 *Salmonella enterica* characteristics

Salmonella spp. (Figure 1.3) are typical members of the family *Enterobacteriaceae*; facultative anaerobic Gram-negative bacilli able to grow on a wide range of relatively simple media and distinguished from other members of the family by their biochemical characteristics and antigenic structure. Their normal habitat is the animal intestine (66, 67). There are over 2,500 different antigenic types (serovars or serotypes) of genus *Salmonella*, as determined based on their somatic (O) and flagellar (H) antigens (67, 68). Many serovars are host-specific; those causing infections in man might not cause disease in animals and vice versa. Certain serovars are major causes of foodborne infection worldwide. Most infections are relatively benign and restricted to the intestinal tract, causing gastroenteritis and short-lived diarrhoea, but some *Salmonella* spp. cause life-threatening systemic disease (e.g., typhoid fever) (69).

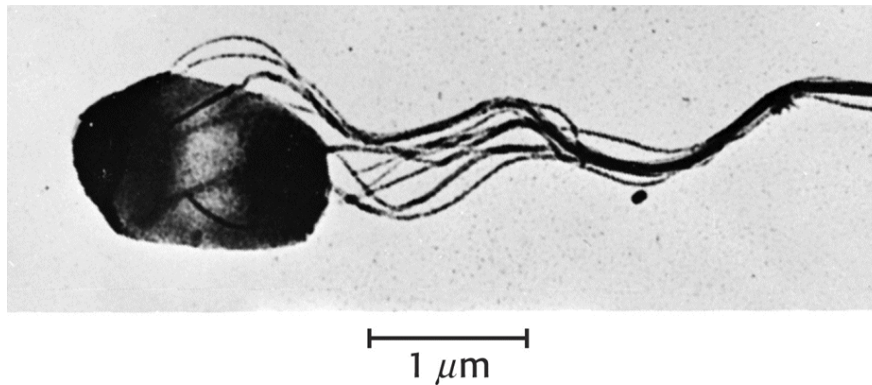


Figure 1.3 Electron microscope picture of a *Salmonella* bacterium with several flagella.

Adapted from: www.bmb.leeds.ac.uk/illingworth/6form/index.htm.

Currently, the genus is divided into two species, *Salmonella enterica* and *Salmonella bongori* (70). The genus *Salmonella* has a large number of named serovars, but most belong to *S. enterica*, which can be divided into a number of subspecies and these can be divided into serovars that might display different phage types. *S. enterica* subspecies are: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) (64). *S. bongori* is listed as subspecies V, even though this is a separate species (64, 71). The complete correct designation is, for example: *S. enterica* subspecies *enterica* serovar Enteritidis, but this is usually abbreviated to *S. serovar* Enteritidis (*S. serovar* Enteritidis) or simply *S. Enteritidis* (64, 67). Subspecies I (*enterica*) includes nearly 1,400 serovars, some of which are commonly isolated from infected birds and mammals, including humans, and are responsible for most *Salmonella* infections in humans; the other subspecies mainly colonize cold-blooded vertebrates (66, 72). Isolates, which are pathogenic to man belong to subspecies I, but not all serovars, subspecies, or species are pathogenic. A variety of virulence factors have been described for *Salmonella*, some of which appear to have a broad distribution, whereas others appear to be present in a limited number of serovars or even strains (64). Certain serotypes are a major cause of foodborne infection worldwide. Most infections are relatively benign and restricted to the intestinal tract, causing a short-lived diarrhea, but some *Salmonella* spp. cause life-threatening systemic diseases, such as typhoid fever and paratyphoid fever (67).

Salmonella strains have enhanced adaptability and survival in the external environment (soil, water, and on a variety of surfaces) relative to *E. coli*, which promotes its transmission and

infection to a new host (69). The ability of *Salmonella* to respond effectively to the environmental changes by mounting a stress response is important in their survival in the food chain just like any other foodborne pathogen (73). *S. Enteritidis* resembles *S. Typhimurium* with respect to known virulence mechanisms central to mammalian cell invasion, survival, and multiplication in the host. Both pathogens share the highly conserved pathogenicity island-encoded type III secretion systems and virulence effector proteins, both harbour a large virulence plasmid, both are motile, and have a galactose-rhamnose-mannose repeating subunit of the lipopolysaccharide (LPS) O-chain backbone connected with dideoxyhexose that determines serovar specificity (74, 75, 76, 77, 78, 79, 80). However, it is unclear as to how *S. Enteritidis* specially follows the human infection route, while it is also possible for this pathogen to successfully contaminate and grow in egg contents (80). *S. Enteritidis* has been shown to generate a remarkable degree of strain heterogeneity, suggesting that a complex network of characteristics might underlie its diverse behaviour (80).

1.2.2.3 *Salmonella enterica* Enteritidis as foodborne pathogen

Salmonella can be isolated from poultry processing equipment, especially in the slaughter and evisceration area, and several authors showed that *Salmonella* can attach and form biofilms on surfaces found in food processing plants, including plastic, cement, and stainless steel (81, 82, 83, 84, 85).

Although primarily intestinal bacteria, *Salmonella* are widespread in the environment and commonly found in farm effluents, human sewage, and in any material subject to faecal contamination. Salmonellosis has been recognized in all countries but appears to be most prevalent in areas with intensive animal husbandry, especially poultry and swine production. The disease can affect all species of domestic animals; however, young animals and pregnant animals are most susceptible. Many animals might also be infected without showing signs of illness (63). There are reports of various *Salmonellae* being extensively isolated from wild-living avian species such as passerines, gulls, owls, and waterfowl (86). In the UK, annual isolations of selected serotypes from man almost tripled between 1981 and 1988. This dramatic increase was due largely to the emergence of strains belonging to *S. Enteritidis*, which peaked in 1997–98 and continues to be the most isolated serovar, as can be observed in Figure 1.4. In developing

countries in which large-scale farming and processing of food animals has not been established, *Salmonella* is not as important cause of community-acquired diarrhoea. However, infections with *S. Typhi* and *Paratyphi*, which are mainly encountered as imported infections in developed countries, remain prevalent in other parts of the world (87).

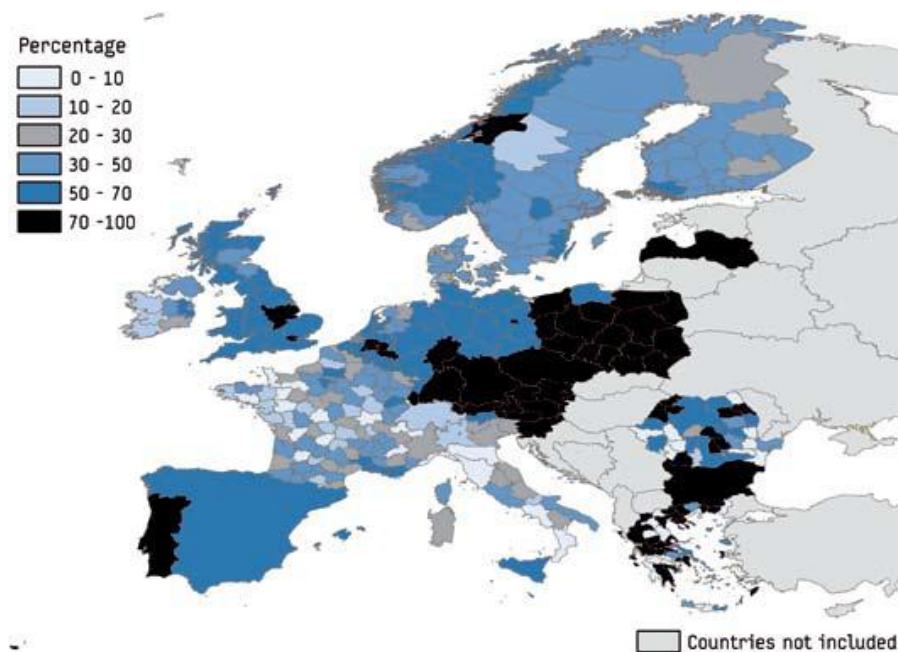


Figure 1.4 Incidence of *Salmonella* Enteritidis, as a percentage of the total number of *Salmonella* cases in Europe, 2004. Adapted from: Jepsen *et al.* (87).

1.2.2.4 Salmonellosis

It has been reported that more than 1.3 billion cases of human salmonellosis occur worldwide annually, resulting in three million deaths (73, 88). That is why salmonellosis remains a major problem (89, 90), with *S. enterica* ranking as the leading cause of foodborne outbreaks worldwide (73, 89, 91). Historically, *S. Typhimurium* is the most common agent of human foodborne disease, although in the last few decades *S. Enteritidis* has become more common (92, 93). This bacterium causes gastroenteritis associated with a high mortality rate in the absence of appropriate antibiotic treatment (94), which is mainly because of its unique ability to

contaminate eggs without causing any discernible illness in the infected birds. In fact, *S. Enteritidis* is currently the only *Salmonella* serovar that causes frequent human illness associated with egg contamination, which determines its unique threat to food safety (80). The infection route to humans involves colonization, survival, and multiplication of the pathogen in the hen-house environment, the bird, and finally, the egg. The altered growth patterns and specific cell surface characteristics contribute to the adaptation of *S. Enteritidis* to these diverse environments (80).

1.3 Microbial colonization of food contact surfaces

The adherence and biofilm formation of bacteria on food contact surfaces have great implications on hygiene because adhered and biofilm cells show increased resistance against stress factors commonly used in the decontamination of food contact surfaces (95, 96, 97, 98). A significant number of reports have appeared on the persistence of some foodborne pathogens on food contact surfaces and biofilms, affecting the quality and safety of the food products. Outbreaks of pathogens associated with biofilms have been related to the presence of *L. monocytogenes*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Salmonella* spp. *Staphylococcus* spp. and *E. coli* O157:H7 (99, 100, 101, 102, 103, 104, 105, 106).

1.3.1 Bacterial adhesion

The attachment of microorganisms to surfaces and the subsequent biofilm development are very complex processes, affected by several variables. Various mechanisms have been proposed to explain the adherence process and biofilm formation on food contact surfaces. Initially, the surface is conditioned by the presence of food residues, and microorganisms have access to the conditioned surfaces. Attractive and repulsive forces are involved in the adhesion of bacteria to surfaces. These include van der Waals forces at a distance of 50 nm and electrostatic forces at a distance of 20 nm between the surface and the microorganisms; at this point, microorganisms are reversibly adhered to a surface. At a distance of 1.5 nm, ionic links and hydrophobic forces are present (107, 108). When attractive forces are greater than repulsive forces, irreversible adhesion begins to take place. In the transition from reversible attachment to

irreversible attachment, various short-range forces are involved, including covalent and hydrogen bonding, as well as hydrophobic interactions (Figure 1.5).

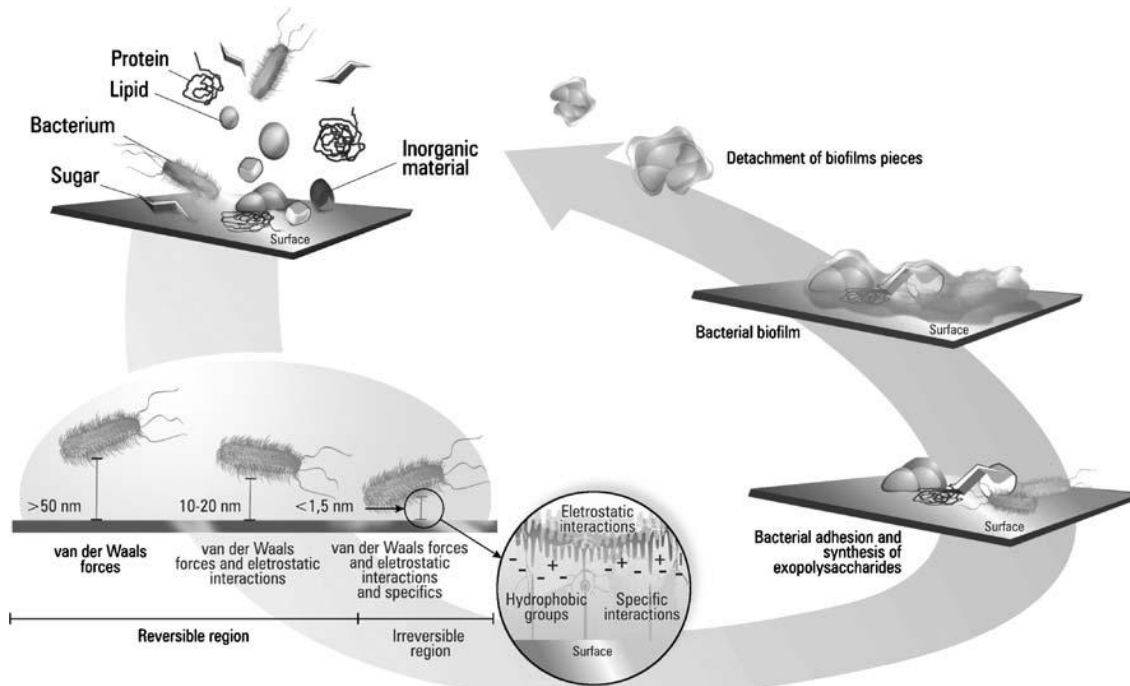


Figure 1.5 Mechanisms of bacterial adhesion. Adapted from: Araújo *et al.* (109) .

Researchers have shown that the physical and chemical properties of the cell surface and food contact surfaces contribute to the adhesion process. These properties include hydrophobicity, electrical charge, and roughness. Several studies have demonstrated the importance of surface hydrophobicity in the adhesion process. This property may be the primary driving force for the adhesion of most pathogens (110). The microorganisms have many different ways of using the hydrophobic effect in order to adhere to substrata (111). Sende and Carballo (112) reported the effect of hydrophobicity in the adhesion of *Salmonella* spp. and *L. monocytogenes* to typical surfaces in the food industry, such as stainless steel, rubber, and polytetrafluoroethylene. *Salmonella* strains showed higher hydrophobicity than *L. monocytogenes*. Polytetrafluoroethylene was the most hydrophobic material, followed by rubber and stainless steel. Bacteria attached in higher numbers to the more hydrophobic materials. However, it is well

known that bacteria change their surface composition in response to the environment. Therefore, cell surface hydrophobicity is not necessarily constant for bacteria, and there is no clear trend in cell adhesion based solely on hydrophobicity effects (113). Flint *et al* (114) evaluated the hydrophobicity of the cell surface of 12 strains of streptococci and correlated those properties with the ability of the cell to attach to stainless steel surfaces. They observed that in this case, there was no relationship between hydrophobicity and attachment to stainless steel. Evidence shows that the presence of LPS on a cell surface tends to make a bacterial cell more hydrophilic in nature and that the loss of LPS from a cell surface results in the cell surface becoming more hydrophobic in nature. There are reports that show a reduction in oxygen levels of the medium induced structural modifications in the LPS of some bacteria, resulting in an increase in surface hydrophobicity of the cell. This tends to indicate that the bacterial cell is quite capable of sensing changes in its external environment and in turn changing a major cell surface characteristic such as surface hydrophobicity (115).

Bacteria acquire a surface electric charge in aqueous suspensions due to the ionization of their surface groups, such as phosphoryl, carboxyl, and amino groups. The bacteria are almost always negatively charged. Since the cell surface is in direct contact with the environment, the charged groups within the surface layers are able to interact with ions or charged molecules present in the external medium (116, 117). Most studies show that in the bacterial cell wall, the anionic groups dominate over the cationic groups. This statement is a general phenomenon, and it is in agreement with the observation that most bacterial cells have isoelectric points below pH 4 (118). The surface charge of bacteria changes according to bacterial species and is also influenced by the growth medium, the pH, and the ionic strength of the suspending buffer, bacterial age, and bacterial surface structure (119). The correlation between surface charge and adhesion is not simple. This difficulty in relating cell surface characteristics to adhesion performance for different bacterial strains is due to the heterogeneity of the cell surface, in which many components will differ between various strains.

A relevant factor to physicochemical effects on bacterial attachment is the influence of surface topographical properties. The substrate is important in the biofilm formation process and an understanding of how substrate properties affect adhesion of bacterial cells may help in designing or modifying substrates to inhibit bacterial adhesion (119). Different food contact surfaces, such as glass, stainless steel, and granite, show distinct patterns of microtopography

and can have fissures, cracks, and crevices that can be large enough to hold bacteria. The surface roughness is typically considered as a possible cause for the large discrepancies observed between the theoretical predictions and experimental observations of bacteria at surfaces (120). In the literature, there are contradictory opinions about the effect of surface properties on the bacterial adhesion process. Several studies have shown that there is a positive correlation between adhesion and increased surface roughness while others report no correlation between surface irregularities and the ability of bacteria to adhere. This conflict of opinion may be due to the degree of surface roughness studied, the bacterial species tested, the physicochemical parameters of the surface, and the technique utilized to determine the presence of the cell on the surface (119). It has been hypothesized that bacteria preferentially stick to rougher surfaces for three reasons: a higher surface area available for attachment, protection from shear forces, and chemical changes that cause preferential physicochemical interactions (121).

Microbiological properties must also be taking into account, since all aspects of the biology of bacteria, the cell wall and surface properties of bacteria play important roles in bacterial adhesion and in the formation of biofilms. For both Gram-positive and Gram-negative bacteria, it is essentially the biomolecules decorating the cell wall that determine the surface properties of the bacteria and thus the interaction of the bacterium with the environment (122). The adhesion process depends on the bacterial species and strains since they have different physicochemical characteristics. Some parameters in the general environment, such as temperature, time of exposure, bacterial concentration, electrolyte concentrations, pH value, and the associated flow conditions, can affect the bacterial adhesion process. Several studies have shown that cellular appendages, such as flagella, fimbriae, pili, and extracellular polymers, are also involved in the bacterial adhesion process (112, 123, 124).

1.3.1.1 *Listeria* and *Salmonella* adhesion to food contact surfaces

L. monocytogenes has been shown to adhere to several different food contact materials such as stainless steel, polypropylene and glass (95, 125, 126, 127), and the adhered cells show increased resistance to cleaning agents, disinfectants and heat (95, 96, 97, 98), all of which are used in the sanitation of the food processing plants. Differences in adherence of *L. monocytogenes* between food contact materials have been observed, although these differences are small (128), with lower adherence to stainless steel surfaces than to rubber or

polytetrafluorethylene (112), but higher than to nylon (129). *L. monocytogenes* has been demonstrated to adhere to stainless steel, rubber, glass and polypropylene in as little as 20 minutes (127), and this organism has also been observed to produce extracellular material (130) within a one-hour period (127) and a biofilm consisting of cells in two layers on a glass surface within 24 hours (125). Differences in the number of adhered cells have been observed between *L. monocytogenes* strains (96, 125, 126, 131), with the highest differences in adherence levels between strains achieving approximately 100-fold (125, 131). Differences in the formation of micro-colonies and cell aggregates have also been observed (126). Differences in the rate of attachment of certain bacterial strains are thought to be a contributing factor in the composition of the initial microbial flora, for example, *Pseudomonas* spp. have been reported to attach more rapidly to meat surfaces than several other types of spoilage bacteria (132, 133, 134).

Salmonella spp. is able to colonize different inert food contact surfaces, however with different extents of adhesion (82, 135, 136, 137). Joseph *et al* (82) studied the ability of biofilm formation of two poultry *Salmonella* isolates to plastic, cement, and stainless steel and observed that the biofilm formation of both isolates was very similar, with the highest density being on plastic, followed by cement and stainless steel. As for other bacteria, several studies have shown that adhesion of *Salmonella* partly depends upon the nature of the inert surfaces and partly upon the bacterial surface properties (112, 138, 139), with hydrophobicity and surface charge being the most important surface properties in the adhesion process, as demonstrated by numerous studies (140, 141, 142, 143). Moreover, the adhesion of this bacterium has also been shown to be strongly strain dependent (144).

1.3.2 Biofilm formation

More than 60 years after the first report on biofilms (145), they are still a concern in a broad range of areas, and specifically in the food, environmental and biomedical fields (114) (146, 147, 148). Biofilms are defined as cells irreversibly adhered to a surface, i.e. cells that are not removed by gentle rinsing, and enclosed in a matrix consisting mainly of extracellular polymeric substances (EPS) (149). It is a natural tendency of microorganisms to attach to wet surfaces, to multiply and to embed themselves in a slimy matrix composed of EPS that they produce, forming a biofilm. Biofilms are problematic in particular food industry sectors such as brewing, dairy processing, fresh produce, poultry processing and red meat processing (150, 151,

152, 153), but they are capable of being formed equally well on biotic (living tissue or cells) as well as abiotic surfaces (metal, concrete, biomedical implants etc.) as long as the surfaces are immersed in aqueous environments (125, 126, 127, 154). Moreover, the bacterial populations within the biofilms can either be single species or derived from multiple microbial species.

Properties of the cell surface, particularly the presence of extracellular appendages, the interactions involved in cell–cell communication and EPS production are important for biofilm formation and development (149, 155, 156, 157, 158). An increase in flow velocity or nutrient concentration may also equate to increased attachment, if these factors do not exceed critical levels (159, 160, 161). At present, processes governing biofilm formation that have been identified include (Figure 1.6): 1. pre-conditioning of the adhesion surface either by macromolecules present in the bulk liquid or intentionally coated on the surface; 2. Transport of planktonic cells from the bulk liquid to the surface; 3. Adsorption of cells at the surface; 4. Desorption of reversibly adsorbed cells; 5. Irreversible adsorption of bacterial cells at a surface; 6. Production of cell–cell signalling molecules; 7. Transport of substrates to and within the biofilm; 8. Substrate metabolism by the biofilm-bound cells and transport of products out of the biofilm. These processes are accompanied by cell growth, replication, and EPS production; 9. Biofilm removal by detachment or sloughing (162). Shedding of planktonic cells is part of the biofilm cycle and is of importance in the dissemination of the infection in the host or contamination in the food processing plant (163), making these microbial communities responsible for serious problems in chronic bacterial infections, as well as food contamination in food processing environments, as they are a continuous source of contamination (106, 149).

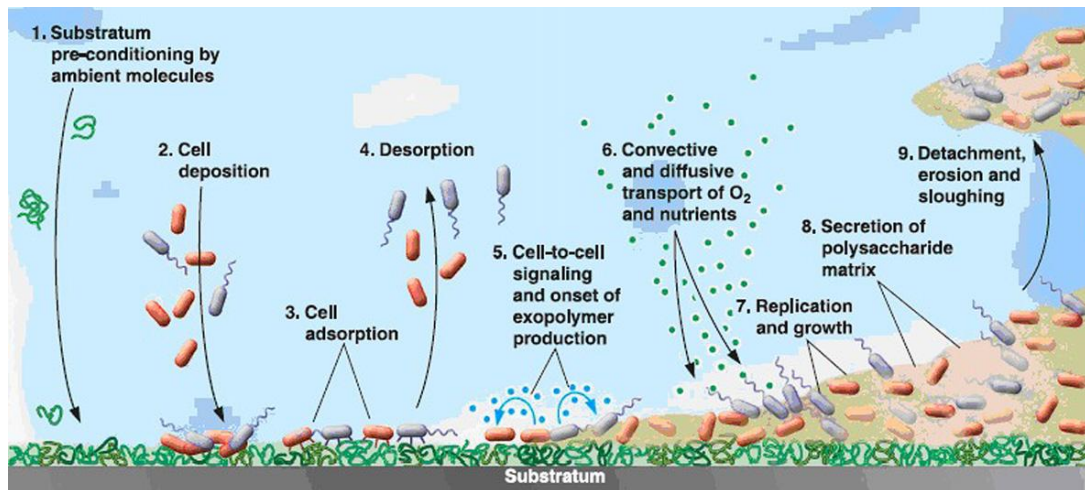


Figure 1.6 Processes governing biofilm formation. Adapted from Breyers and Ratner (162).

Formation of biofilms on surfaces can be regarded as a survival strategy whereby the inhabitants are protected from predators, dehydration, biocides and other environmental threats while regulating bacterial growth and diversity (164). Observation of a wide variety of natural habitats has shown that the majority of organisms prefer to exist attached to surfaces in biofilms and not in the planktonic state (164, 165). However, the extent to which the adherent bacteria will form biofilms is dictated by the availability of nutrients in their particular micro-niche (166). In flowing systems such as industrial and natural aquatic systems, there is generally a continual source of nutrients being carried past the bacteria thus rapid biofilm formation will occur on available surfaces. Bacteria that are unable to locate sufficient nutrients will merely survive in a starved state (167, 168).

Another factor affecting biofilm formation is a conditioning film covering on a hard surface in a solution. When a material surface is exposed in an aqueous medium, it will inevitably and almost immediately become conditioned or coated by polymers from that medium, and the resulting chemical modification will influence the rate and extent of microbial attachment onto a surface (169). The conditioning film on the surface was thought to be organic in nature and it is able to form within minutes of exposure and continue to grow for several hours (170). The properties of the film are determined by the aqueous medium to which the surface is exposed (171, 172).

Biofilms are composed primarily of microbial cells and EPS, these last accounting for 50% to 90% of the total organic carbon in biofilms and being considered the primary matrix material of biofilms. These substances are considered key compounds that determine physicochemical properties of biofilms, and are formed by polysaccharides, proteins, nucleic acids, and lipids. EPS supplies a matrix that allows the cells to stand firm with regard to planktonic cells and form the morphology and internal structure of biofilms, being responsible therefore for the functional and structural integrity of biofilms (173). They may vary in chemical and physical properties, but are primarily composed of polysaccharides, some of which are neutral or negatively charged, as is the case of Gram-negative bacteria. Studies indicated that different organisms produce different amounts of EPS, which increases with age of the biofilm (174). Moreover, these substances may associate with metal ions, divalent cations, and other macromolecules (such as proteins, desoxyribonucleic acid (DNA), lipids, and even humic substances) (173), and the nutrient level of the growth medium affects their production. Excess of available carbon and limitation of nitrogen, potassium, or phosphate promotes the synthesis of EPS (175), while slow growth of bacteria will also enhance their production (175). Because these substances are highly hydrated, they can prevent desiccation in biofilms. Moreover, EPS may also render biofilms antimicrobial resistance properties by impeding the mass transport of disinfectants through the biofilm, probably by binding directly to these agents (176).

1.3.2.1 *Listeria* and *Salmonella* biofilms on food contact surfaces

Foodborne pathogens like *E. coli* O157:H7, *L. monocytogenes*, *Yersinia enterocolitica*, and *C. jejuni* form biofilms on food surfaces and food contact equipment, leading to serious health problems and economic losses due to recall of food (101). Biofilms have been associated with a number of foods and food processing surfaces, with foodborne pathogens gaining entry into the food from processing surface biofilms (177). Subsequently, microorganisms colonize and grow on the surface of food, turning biofilms into a potent threat to the safety of food by being a source of contamination. Food items are contaminated with undesirable spoilage and pathogenic bacteria from sloughed portions of biofilms, which lead to serious hygienic problems and economic losses due to food spoilage and the presence of foodborne pathogens (178, 179).

The capacity of *L. monocytogenes* to adhere to the animate or inanimate surfaces, and subsequently form biofilms in the food-processing environment, has been well documented.

However, it has been noted that there are differences in both the extent and rate of attachment and biofilm formation depending on the surface selected, pre-treatment of the target surfaces, environmental and growth conditions, pH, temperature, etc. Moreover, Kalmokoff *et al* reported that a majority of *L. monocytogenes* strains might not form biofilms in monoculture (126), and no relation was found between processing environment persistence, strain source (food or clinical), and strain subtype (serotype or lineage) to attachment and biofilm formation. Other reports examining longer-term biofilm formation have noted that *L. monocytogenes* is a poor organism for cell attachment and biofilm formation, and this has led to the suggestions that these strains may use a primary colonizing bacterium of a different species to form a biofilm consortium on a surface (180, 181). Both Djordjevic *et al* (182) and Borucki *et al* (40) reported that biofilm formation could correlate with phylogenetic division but not serotype, while Djordjevic *et al* (182) reported that lineage I strains were significantly better at biofilm formation than strains belonging to lineage II, suggesting a possible relationship between biofilm formation and the phylogenetic division most closely associated with foodborne outbreaks. However, Borucki *et al* (40) found a increased biofilm formation in lineage II strains (serotypes 1/2a and 1/2c), which are not normally related to foodborne outbreaks. These conflicting reports might be due to differences in methodology, sample size, and specific strains used in the studies. On the other hand, the relation between formation of biofilm and the virulence of *L. monocytogenes* remains unclear (16).

S. Enteritidis has emerged as one of the most significant foodborne pathogens during the past three decades (80, 183). It is important that the majority of the strains of this organism can grow on surfaces and interfaces to form biofilms composed of self-secreted exopolysaccharide or exopolymeric material (184), including on the food processing and food contact surfaces. *S. Enteritidis* has been shown to form biofilms on materials of different nature and under different growth conditions (178, 184, 185, 186). Moreover, it was found that in rich medium (broth) and at room temperature (28°C), this bacterium produces a pellicle whose matrix is mainly composed of curli or thin aggregative fimbriae and cellulose (184, 187). Disruption of any of the two operons responsible for cellulose biosynthesis, *bcsABZC* and *bscEFG*, impaired pellicle formation and significantly increased the susceptibility of *S. Enteritidis* to disinfectants (184). It was believed, until recently, that unlike other Gram-negative bacteria, where various surfaces or intercellular adhesion factors were shown to participate in biofilm formation, only curli and

cellulose production has been described to be involved in *S. enterica* biofilm formation process (94, 188). Nevertheless, recent reports have shown that a large cell wall-associated secreted protein, BapA, having sequence homology with Bap (biofilm-associated protein) of *S. aureus*, is also required for biofilm formation and host colonization (94, 189).

Biofilm-forming *S. Enteritidis* isolates are considered to be more virulent, given that the ability to form biofilms correlates with enhanced oral invasiveness, although not with epithelial cell disruption and egg contamination (190, 191). However, Parker *et al* (192) reported that biofilm-producing *S. Enteritidis* might act as a 'helper' phenotype that aids access of less orally-invasive strains to the post-mucosal environment of the bird, with subsequent enhanced recovery of contaminated eggs.

Increased inherent resistance of biofilm bacteria to sanitizers or antimicrobial agents is the major factor affecting plant sanitation and product safety. Frank and Koffi (95) reported the increased resistance of *L. monocytogenes* in biofilms and Holah *et al* (193) reported that *P. aeruginosa*, *S. aureus*, and *P. mirabilis* biofilms were 10 to 100 times more resistant to food surface disinfectants than their planktonic counterparts. Thus, foodborne pathogens growing as biofilms are more important than those growing as planktonic cells in foods.

1.4 Control of foodborne pathogens

It should be assumed that any surface or material that comes in contact with food is a potential source of microbial contamination. Some microorganisms, such as *Listeria* and *Salmonella*, pose a particular challenge in this regard as they are common environmental pathogens that can become established in a food processing environment and repeatedly contaminate work surfaces. In the case of RTE foods, the challenges are greatest because production frequently involves extensive processing and packaging after cooking. In addition, there may be an opportunity for foodborne pathogens proliferation in the product during storage and distribution and consumers are typically not expected to perform any antimicrobial step before consumption.

There is good evidence indicating that the biofilm mode of life leads to increased resistance to antimicrobial products (194, 195, 196). Biofilms are more resistant to antimicrobials compared to planktonic cells and this makes their elimination from food

processing facilities a big challenge (195, 196). Moreover, the emergence of resistant bacteria to conventional antimicrobials clearly shows that new biofilm control strategies are required (196, 197).

In the following sections some approaches to prevent bacterial colonization of food contact surfaces are presented, focusing on those that were studied in this thesis.

1.4.1 Surface coatings

An effective and desirable approach to decrease the adhesion process is to modify the food processing surface character by making it less attractive for microorganisms by the use of surface coating techniques (198), which prevents biofilm formation and consequently improves the surface hygiene process. One of the strategies that has the potential of inhibiting the early stages of biofilm formation involves the utilization of a low surface energy polymeric coating, which functions by presenting a non-stick surface to bacterial and other colonizing microorganisms (199). It has been suggested that the constituent polymer must possess a flexible linear backbone onto which side chains with low intermolecular interactions are attached via suitable linking groups (200).

Diamond-like carbon (DLC) coatings have also been attracting interest due to their excellent properties, including low friction and chemical inertness, and are a good base coating to be alloyed with different elements. The amorphous nature of DLC opens the possibility of introducing certain amounts of additional elements, such as Si, F, N, O, W, V, Co, Mo, Ti, and Ag, and their combinations into the film and still maintain the amorphous phase of the coating (201). Liu *et al* (198) prepared Si- and N-doped DLC coatings with various silicon and nitrogen contents on 316 stainless steel substrates. These authors evaluated the adhesion of *P. aeruginosa* (ATCC 33347) on the modified substrates. They observed that the addition of N or Si to the DLC coating had a significant influence on bacterial adhesion. In general, the altered DLC coating with N or Si performed better than the pure DLC coating in inhibiting bacterial adhesion.

1.4.1.1 Titanium dioxide

Titanium dioxide (TiO₂) is a photocatalyst and widely utilized as a self-cleaning and self-disinfecting material for surface coatings in many applications (202, 203). The photocatalytic

reaction of TiO_2 has been used to inactivate a wide spectrum of microorganisms (202, 204, 205, 206, 207). The first work on the microbiocidal effect of TiO_2 photocatalyst was carried out with *E. coli* in water (208). These authors reported that *E. coli* was killed by contact with a TiO_2 photocatalyst upon illumination with light. Hydroxyl radicals ($\bullet\text{OH}$) and reactive oxygen species generated on the illuminated TiO_2 surface (Figure 1.7) play a role in inactivating microorganisms by oxidizing the polyunsaturated phospholipid component of the cell membrane of microbes (202, 209, 210, 211, 212, 213). OH radicals are approximately one thousand or possibly ten thousand times more effective for *E. coli* inactivation than common disinfectants such as chlorine, ozone and chlorine dioxide (213).

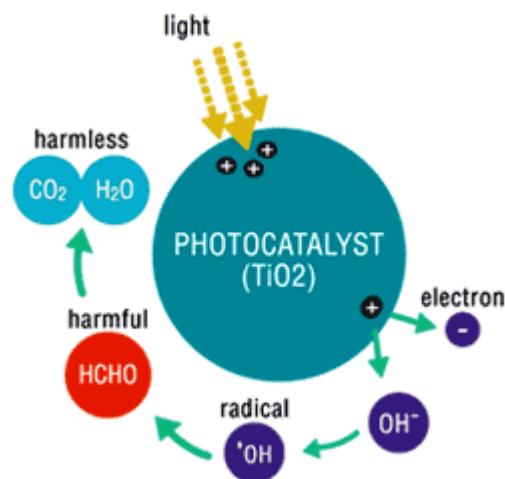


Figure 1.7 Titanium dioxide photocatalysis reaction. Adapted from: www.phototroph.com.hk/techno.html.

TiO_2 is non-toxic and has been approved by the American Food and Drug Administration (FDA) for use in human food, drugs, cosmetics and food contact materials. Currently there is considerable interest in the self-disinfecting property of TiO_2 for meeting hygienic design requirements in food processing and packaging surfaces. Bactericidal and fungicidal effects of TiO_2 on *E. coli*, *Salmonella choleraesuis*, *Vibrio parahaemolyticus*, *L. monocytogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Diaporthe actinidiae* and *Penicillium expansum* have been reported (204, 205, 207, 208, 210, 213, 214, 215, 216). Application of TiO_2 photocatalytic disinfection for drinking water production was investigated by Wist *et al* (217).

The development of TiO₂-coated or -incorporated food packaging and food preparing equipment has also received attention.

1.4.2 Antimicrobial incorporated materials

Antimicrobial packaging is a form of active packaging and one promising approach to prevent both contamination by pathogens and growth of spoilage microorganisms on the surface of food. Active packaging interacts with the product or the headspace between the package and the food system, to obtain a desired outcome (218, 219). Likewise, antimicrobial food packaging acts to reduce, inhibit or retard the growth of microorganisms that may be present in the packed food or packaging material itself. Antimicrobial packaging materials have to extend the lag phase and reduce the growth rate of microorganisms to prolong the shelf life and maintain food quality and safety (220). The number of published articles and patents suggest that research on the incorporation of antimicrobials into packaging for food applications has more than doubled in recent years. Generally recognized as safe (GRAS), non-GRAS and 'natural' antimicrobials have been incorporated into paper, thermoplastics and thermosets, and have been tested against a variety of microorganisms including *L. monocytogenes*, pathogenic *E. coli*, and spoilage organisms including molds (221, 222, 223).

Antimicrobial agents may be incorporated into the packaging materials initially and migrate into the food through diffusion and partitioning (220). Some typical compounds that have been proposed and tested for antimicrobial activity in food packaging include organic acids such as sorbate, propionate and benzoate or their respective acid anhydrides bacteriocins (e.g., nisin and pediocin) or enzymes such as lysozyme. Of all the antimicrobials, silver substituted zeolites are the most widely used as polymer additives for food applications, especially in Japan. Sodium ions present in zeolites are substituted by silver ions, which are antimicrobial against a wide range of bacteria and molds. These substituted zeolites are incorporated into polymers like polyethylene, polypropylene, nylon and butadiene styrene at levels of 13% (219). Silver ions are taken up by microbial cells disrupting the cells' enzymatic activity. Commercial examples of silver substituted zeolites include Zeomic, Apacider, Aglon, Bactekiller and Novaron.

Combinations of more than one antimicrobial incorporated into packaging have also been investigated. For example, it is hypothesized that compounds active against Gram-positive

bacteria (i.e. lysozyme) combined with chelating agents (i.e. ethylene diamine tetracetic acid (EDTA)) can target Gram-negative bacteria. Addition of EDTA to edible films containing nisin or lysozyme, however, had little inhibitory effect on *E. coli* (224) and *S. Typhimurium* (225). All antimicrobial agents have different activities which affect microorganisms differently. There is no 'Magic Bullet' antimicrobial agent effectively working against all spoilage and pathogenic microorganisms. This is due to the characteristic antimicrobial mechanisms and due to the various physiologies of the microorganisms (220).

1.4.2.1 Microban®

Microban is both a company and brand name. Microban® anti-bacterial protection technology was developed in 1969 and used in industrial and medical products from 1988. From 1994 its applications were extended to a broader range of consumer products. Microban International developed the proprietary technology to incorporate Microban into solid plastics and synthetic fibers and fabrics. In the late 1990's the Microban company teamed up with Sainsbury's, to develop a range of products with Microban® anti-bacterial protection. This was in response to the consumer's perceived need for reassurance and peace of mind about food safety (226). Since then, the availability in the UK of products claiming antibacterial protection has increased rapidly (227). In the USA a similar trend has been driven by increased public awareness and fear of microbial infections (228).

The active ingredient in Microban, triclosan, is permanently added to the structure of products during manufacturing (229). A wide range of domestic products incorporating these agents is now available, including dishcloths, food boxes, toothbrushes, washing-up liquid and hand-washing gels. Manufacturers claim these products give "permanent protection against bacteria" (230). However, there is little independent scientific evidence of either efficacy or possible adverse effects. Previous investigations of triclosan-incorporated plastics and polymers involved experimental systems based on pure cultures and were not conclusive as to the antimicrobial utility of such polymers. Triclosan released from polystyrene initially reduced growth of *Bacillus thuringiensis* and *E. coli*, but was less effective at growth inhibition over extended time (231). Triclosan-incorporated plastic storage boxes were demonstrated to be effective against *E. coli* when grown in rich liquid medium in contact with the plastic at 30 and 22°C but no

difference was observed when grown at 4°C (227). Using plate growth assays, it was demonstrated that triclosan-containing polymer coating a food packaging material was effective against *Enterococcus faecalis* (232), whereas a triclosan-incorporated plastic wrap did not effectively reduce bacterial numbers on refrigerated and vacuum packed meat surfaces (233). The presence of triclosan in a soft denture liner did not reduce the adherence of viable *Candida albicans* after 24h of exposure (234). Others have demonstrated that triclosan in solid substrates was deactivated by soil bacteria and this deactivation provided a niche for sensitive bacteria to grow (235).

Microban® anti-bacterial protection can work in a number of ways. One way is to permanently introduce Microban® into the structure of the product, as bin liners, food cutting boards, food storage containers, plastic utensils, polyester type dish cloths, tea towels and other textiles used for cleaning. The anti-bacterial molecules cannot penetrate thick-walled skin cells of mammals and so are safe for human use. However, they do penetrate thin-walled cells like those of bacteria, yeasts and fungi and interrupt their ability to function, grow and reproduce. Microban® anti-bacterial protection can be incorporated into virtually any polymer resin, plasticiser or colouring/dye process and works in cast, blow moulded, injection moulded, extruded, blown or powder coated processes. Its use does not disrupt the manufacturing process and has no effect on the tensile strength, colour or texture of the end product. Microban® anti-bacterial protection exists in an equilibrium distribution throughout the product. It migrates from the inside of the product to the surface, as required, to create an anti-bacterial surface which helps to minimize the growth of bacteria. It can only be removed by abrasion, as during washing up, or in use. Products are engineered to contain exactly the right amount of Microban® to provide protection for the lifetime of the product (226).

Before new products can be approved for manufacture, various safety and legal checks have to be made. Microban® is fully approved by the EU (under EU Directive 90/128/EC) for use in food contact applications, and has been proven not to taint food in contact with plastic surfaces containing Microban®. It is registered with the Environmental Protection Agency and approved by FDA for use in medical and food-related products (229).

1.4.3. Disinfectants in food industry

Disinfection is the use of antimicrobial products to kill microorganisms. The aim of disinfection is to reduce the surface population of viable cells left after cleaning and prevent microbial growth on surfaces before production restart. Disinfectants are more effective in the absence of organic material (fat, carbohydrates, and protein based materials). Interfering organic substances, pH, temperature, water hardness, chemical inhibitors, concentration and contact time generally control the disinfectants efficacy (236, 237).

Table 1.2 gives a summary on biocide targets and effects of some common disinfectants, sporicides and sanitizers.

Table 1.2 Antimicrobial targets, mechanism of interactions and antimicrobial effects of selected biocides

Adapted from: Block (238), Denyer and Stewart (239).

Mechanisms of Interaction	Antimicrobial Agent	Antimicrobial Targets	Antimicrobial Effect
Halogenation	Hypochlorites chlorine-releasing agents	Amino groups in proteins	Metabolic inhibition
Free-radical oxidation	Peroxygens	Enzyme and protein thiol groups	Metabolic inhibition
Electrostatic (ionic) interaction w/ phospholipids	QAC's, chlorhexidine, polyhexamethylene, biguanides	Cell membrane integrity, membrane-bound enzyme environment and function	Leakage, respiratory inhibition, protoplast lysis, intracellular coagulation, ATPase inhibition
Penetration/partition into phospholipid bilayer, displacement of phospholipid molecules	Phenols, weak acids parabens	Transmembrane pH gradient, membrane integrity	Leakage, disruption of transport, respiratory and energy coupling processes Possibly cells lysis
Solution of phospholipids	Aliphatic alcohols	Membrane integrity	Leakage
Membrane protein solubilization	Anionic surfactants Antifungal imidazoles	Cell membrane integrity, membrane bound enzyme, environment and function	Leakage, uncoupling of energy processes, lysis, inhibit ergosterol synthesis, induce gross membrane damage
Oxidation of thiol groups	Isothiazolinones, organomercurials, hypochlorites, organochlorine derivatives, heavy metal salts oxides, bronopol	Thiol containing cytoplasmatic membrane bound enzymes	Metabolic inhibition
General alkylation reactions	Glutaraldehyde, formaldehyde, oxides, chloroacetamide	Biomolecules (DNA, proteins, RNA) containing amino, imino, amide, carboxyl and thiol groups	Metabolic and replicative inhibition Cell wall damage may occur by interaction with NH ₂ groups
Metal ion chelation	EDTA, oxines	Divalent cation-mediated outer membrane integrity, Gram-negative cell wall principle target, metal iron requiring enzyme processes	Leakage, increased susceptibility to applied stress Induce release of LPS Metabolic inhibition

Three types of chemical sanitizers that are most commonly used in current food industry are reviewed in this section. These chemicals are chlorine compounds, quaternary ammonium compounds (QACs), and peroxygen compounds. Additionally, information about triclosan is also discussed since it is widely used worldwide and was included in studies presented in this thesis.

CHLORINE COMPOUNDS – SODIUM HYPOCHLORITE

Chlorine and products that produce chlorine comprise the largest and most common group of food plant disinfecting agents due to its low cost, ease of application, and ability to inactivate a wide variety of microorganisms. Commonly used chlorine compounds include: liquid chlorine, hypochlorite, inorganic chloramines and organic chloramines (240). Chlorine exists in more than one chemical state when dissolved in water, and hypochlorous acid is the most effective chemical form of chlorine (241). Although it works well at cold temperatures and tolerates hard water, the effectiveness of chlorine is reduced if the pH of solutions is elevated as well as if organic soiling materials are present. Moreover, at low pH levels, bactericidal efficiency of these disinfectants is very unstable (242). The disadvantages of chlorine compounds are that they are corrosive to many metal surfaces (especially at higher temperatures), and they are potentially irritant to skin (especially at low pH). Additionally, they may form potentially carcinogenic trihalomethanes under appropriate conditions (243).

Chlorine compounds are broad-spectrum germicides which act on microbial membranes; inhibit sulfhydryl enzymes and enzymes involved in glucose metabolism. They have a destructive effect on DNA by oxidation of purine and pyrimidine bases (243). In spite of being widely studied, the actual mechanism of action of chlorine compounds is not fully known. Vegetative cells are mostly more susceptible to chlorine inactivation than spores. Chlorine compounds have been found to be less effective on Gram-positive bacteria than Gram-negative bacteria. At 50 ppm, chlorine could inactivate *C. jejuni* in biofilms, resulting in 3 log reduction within 45 s (244).

Sodium hypochlorite is the best example of a chlorine compound used as a disinfectant and its bactericidal effect is based on the penetration of the chemical and its oxidative action on essential enzymes in the cell (245). It is known to be very active in killing most bacteria, fungi and viruses, and it is also known as a strong oxidizing agent (246). Nevertheless, the effectiveness of sodium hypochlorite against a number of pathogens, including *L. monocytogenes*, *C. jejuni*, and *Yersinia enterocolitica* was evaluated and found to vary among different organisms (247).

QUATERNARY AMMONIUM COMPOUNDS – BENZALKONIUM CHLORIDE

Quaternary ammonium compounds (QACs) are a class of compounds, which have the general structure as shown in Figure 1.8. The properties of these compounds depend upon the covalently bound alkyl groups (R-groups), which can be highly diverse (240).

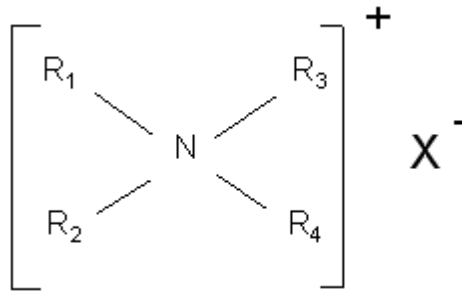


Figure 1.8 The general structure of quaternary ammonium compounds. Adapted from: Schmidt (240).

QACs are widely used in disinfection operations in food processing industries because they have several advantages over other commonly used disinfectants (248). They are cationic surfactant sanitizers and also have some cleaning activity (240), being effective against molds, yeast (249), Gram-positive and Gram-negative bacteria except *Pseudomonas* spp., a dominant bacteria in the seafood processing environment (194). QACs are non-corrosive, non-irritating, and their activity is unaffected by organic load. Under recommended usage and precautions, they pose little toxicity or safety risks (240). QACs require a relatively long contact time to achieve significant kill and are therefore often applied as foam (250). However, their broad application in food industries can cause the possibility of microbial growth and adaptation (194, 251). To reduce the resistance of bacteria to QACs, the study by Sundheim *et al* (251) recommended that the use of higher temperature should be considered as an alternative or a supplement to using higher concentrations of QAC based disinfectants.

The formation of an antimicrobial film on exposed surfaces is an advantage in the application of QACs. However, this may be a disadvantage in operations such as cultured dairy products, cheese, beer, etc. where microbial starter cultures are used (252). A common feature of QACs is their ability to cause membrane damage and cell leakage, primarily due to their

adsorption to the bacterial membrane in large amounts (253). Monoalkyl QACs bind via ionic and hydrophobic interactions to microbial membrane surfaces, with the cationic head group facing outwards and the hydrophobic tails inserted into the lipid bilayer, causing rearrangement of the membrane and subsequent leakage of intracellular constituents (254). Ioannou *et al* (254) also reported that generally QACs are initiators of autolysis at low biocide concentrations (9 to 18 $\mu\text{g ml}^{-1}$), which, together with bactericidal activity, contribute to cell death.

Benzalkonium chloride (BAC) is a synthetic derivative of ammonium chloride (NH_4Cl); it is a second generation, substituted QAC with high biocidal activity. These synthetic compounds are derived from NH_4Cl with the hydrogen atoms being replaced by organic groups such as methyl, ethyl, and/or benzyl groups. The chemical name of BAC is alkyl dimethyl benzyl ammonium chloride (254, 255). The appearance of methicillin-resistant *S. aureus* (MRSA), a major nosocomial agent which tends to be cross-resistant to BAC, a disinfectant widely used in hospitals, has been reported (256). The increase in resistance of MRSA to β -lactam antibiotics, including cefmetazole, cloxacillin, flomoxef, moxalactam, and oxacillin, has been suggested to be due to gene mutations (affecting the efficiency of uptake, activating an efflux pump, or encoding elements regulating the expression of methicillin resistance) conferring resistance to BAC and benzethonium chloride, another cationic detergent (257). The *E. coli* MdfA (multidrug transporter) protein was identified and shown to confer greater tolerance to both antibiotics and BAC (258, 259).

PEROXIDES – HYDROGEN PEROXIDE

Peroxides, also named peroxygen compounds, contain at least one pair of covalently bonded oxygen atoms (-O-O-). One of the oxygen atoms is loosely bound in the molecule and is readily detached as freely active oxygen. Generally, peroxides can be divided into two groups: the inorganic group, containing hydrogen peroxide and related compounds; and the organic group, containing peroxyacetic acid and related compounds. Both organic peroxides and inorganic peroxides are strong oxidizing agents and exhibit varying degrees of antimicrobial activities.

Hydrogen peroxide (HP), though widely used in the medical field, it has become commonly used as a sanitizer in food industry. It is stable and has low toxicity at recommended concentrations, and safely decomposes to oxygen and water. FDA approval has been granted for

the use of HP in sterilizing equipment and packages for the aseptic manufacture of food and drink products (260). The primary mode of action for HP is to create an oxidizing environment and to generate singlet or superoxide oxygen (261). As a high-energy form of oxygen, superoxide oxygen ($O_2 \bullet$) is very reactive and toxic to living organisms. It causes oxidative destruction of lipids and other biochemical components. HP is a fairly broad spectrum compound, with slightly higher activity against Gram-negative than against Gram-positive organisms. HP was reported to be more effective against anaerobes because they are incapable of generating catalase, which destroys the peroxide (261). There are several factors affecting the efficacy of HP. Physical or chemical factors, such as concentration, pH, temperature, and organic contamination are influential in determining efficacy of the antimicrobial activity of HP. Temperature has a pronounced effect on the germicidal activity of HP. The higher the temperature, the stronger killing effectiveness of HP is (261).

PHENOLS AND BIS-PHENOLS – TRICLOSAN

Phenolic-type antimicrobial agents have long been used for their antiseptic, disinfectant, or preservative properties, depending on the compound. It has been known for many years (262) that, although they have often been referred to as “general protoplasmic poisons,” they have membrane-active properties that also contribute to their overall activity (263). With phenols at low concentrations, inactivation of essential enzymes is observed. However, at high concentrations, these compounds penetrate and disrupt the cell wall and precipitate cell wall proteins (238). Low concentrations of phenols have been shown to lyse growing cells of *E. coli*, streptococci and staphylococci (264).

Phenol induces progressive leakage of intracellular constituents, including the release of K⁺, the first index of membrane damage (265), and of radioactivity from ¹⁴C-labelled *E. coli* (266, 267). Pulvertaft and Lumb (268) demonstrated that low concentrations of phenols (0.032%, 320 mg/ml) and other (nonphenolic) agents lysed rapidly growing cultures of *E. coli*, staphylococci, and streptococci and concluded that autolytic enzymes were not involved. Srivastava and Thompson (269, 270) proposed that phenol acts only at the point of separation of pairs of daughter cells, with young bacterial cells being more sensitive than older cells to phenol. The bis-phenols are hydroxy-halogenated derivatives of two phenolic groups connected by various bridges (271, 272). In general, they exhibit broad-spectrum efficacy but have little activity against

P. aeruginosa and molds, and are sporostatic toward bacterial spores. Triclosan and hexachlorophane are the most widely used biocides in this group, especially in antiseptic soaps and hand rinses. Both compounds have been shown to have cumulative and persistent effects on the skin (273).

Triclosan is a bisphenol antimicrobial agent that has a broad range of activity (274). It is bacteriostatic at concentrations ranging between 0.025 and 100 µg/ml, and bactericidal at higher levels (275, 276). It is used as a preservative, antiseptic and disinfectant in a diverse range of products. The inhibitory activity of triclosan results from blocking lipid synthesis through specific inhibition of the NADPH-dependent enoyl-acyl carrier protein reductase FabI (277, 278). At higher concentrations, triclosan is likely to damage the bacterial membrane (279). Gram-negative bacteria use multiple mechanisms to develop resistance to triclosan, including mutations in the enoyl reductase, alteration of the cell envelope, active efflux and expression of triclosan-degradative enzymes (280, 281). The main physiological change resulting from adaptation to triclosan, as described so far in *E. coli* and *Salmonella*, is the overexpression of efflux pumps, particularly the AcrAB efflux pump (282, 283). As active AcrAB was also associated with increased resistance to many other structurally unrelated antimicrobials (284, 285), there might be a link between triclosan usage and antibiotic resistance (286, 287).

1.4.3.1 Bacterial biofilms and disinfectants interaction

It is important to note that most of the disinfection processes that are implemented are based upon the results of planktonic tests (288). However, such tests do not mimic the behaviour of biofilm cells and can be highly ineffective when applied to control biofilms. Biofilms have been reported as possessing susceptibilities towards antimicrobials that are 100–1000 times less than equivalent populations of free-floating counterparts (289). If a microbial population faces high concentrations of an antimicrobial product, susceptible cells will be inactivated. However, some cells may possess a degree of natural resistance and physiological plasticity or they may acquire it later through mutation or genetic exchange. These processes allow the microorganism to survive and grow (290, 291). The increased biofilm resistance to conventional treatments enhances the need to develop new control strategies (195, 292).

There is mounting evidence that microorganisms in biofilms actively respond to antimicrobial challenges (293). There are also reports that bacteria in biofilms can respond to antibiotic treatment by increasing the synthesis of EPS that contribute to the matrix of the biofilm (294, 295). While biofilms are exposed to antimicrobial agents, reaction-diffusion limited penetration might result in only low levels of the antimicrobial agent reaching the deeper regions of biofilms (Figure 1.9) (293). Thus, the sheltered cells are then able to enter an adapted-resistant state if the local time scale for adaptation is faster than that of disinfection, and this mechanism is not available to a planktonic population (293). The authors illustrated a mathematical model that investigated the potential for an adaptive stress response to contribute to the protection of cells in a biofilm. If an antimicrobial-induced stress response is more effectively deployed in a biofilm, there must be either unique regulation that occurs in the biofilm mode of growth or the conditions in a biofilm must favour induction of the stress response over killing of the cell. The results indicated that for a sufficiently thick biofilm, cells in the biofilm implement adaptive responses more effectively than do planktonic cells (293). Based on the results of the study, the authors concluded that effective disinfection of the biofilms requires an applied biocide concentration that increases quadratically or exponentially with biofilm thickness (293).

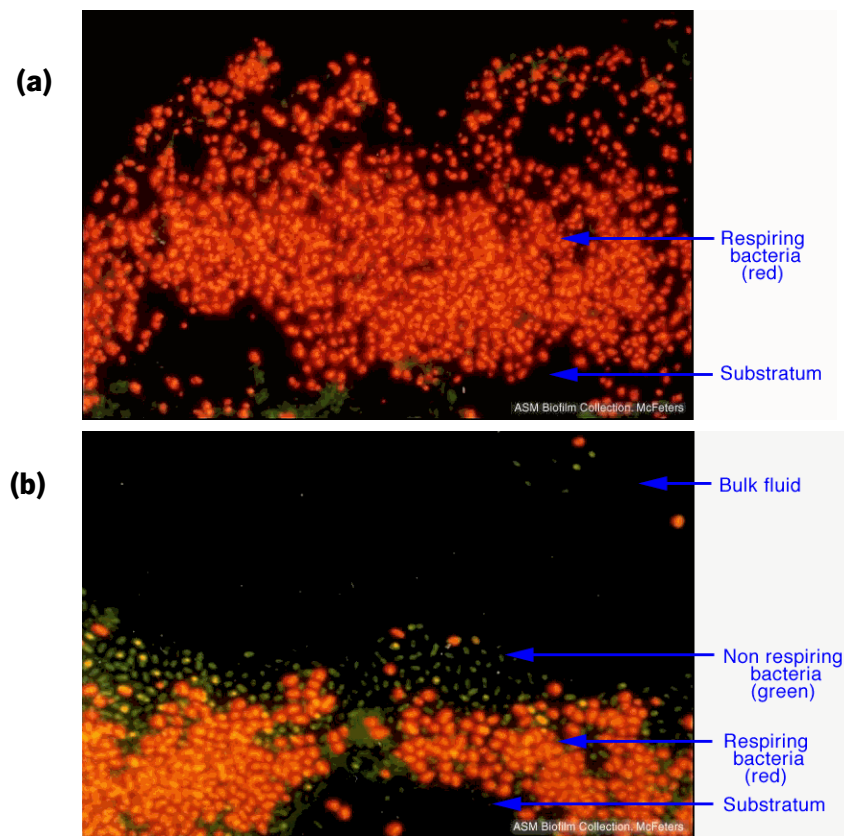


Figure 1.9 Micrographs of biofilm cross-sections composed of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* with progressive exposure to chloramines showing **(a)** untreated control biofilm, which is predominantly composed of respiring bacteria, and **(b)** biofilm which is predominantly composed of respiring bacteria, after 30 min. exposure to disinfectant.

Adapted from: <http://wvlc.uwaterloo.ca/biology447/Biofilms/biofilmsoverview.htm>.

Mah and O'Toole (296) reported that owing to the heterogeneous nature of the biofilms, it is likely that multiple resistance mechanisms are at work within a single community, such as slow growth and/or induction of an *rpoS*-mediated stress response, along with the physical and/or chemical structure of EPS or other aspects of biofilm architecture could confer biofilm resistance to biocides. Some of the phenomena that are postulated to contribute to the biofilm defense include expression of biofilm-specific biocide-resistant phenotypes and the recognition of antimicrobial challenge and active deployment of protective stress responses by a subpopulation of the biofilm cells (293, 297).

1.5 Stress-response and virulence of bacterial foodborne pathogens

The term stress has been used to describe the effect of sublethal treatments and is universally used in reference to the agents or treatments causing injury. Although there is a tendency to perceive food matrices as metabolically supportive environments, food is frequently bacteriostatic or bactericidal due to intrinsic factors such as water activity (a_w), pH, oxidation-reduction potential, competitive exclusion by protective cultures, and other environmental and processing stresses (298). Other types of stress encountered in food environments may include exposure to acids, bases, bioactive antimicrobial peptides, oxidants, osmotic pressure differences, starvation, heating, freezing, thawing, and the presence of other innate and supplemented antimicrobial compounds (299). Some emerging technologies (e.g. high hydrostatic pressure) cause sublethal injury, although some have argued that other technologies (e.g., pulsed electric field) do not induce injury (300, 301). Bacterial stresses, which generally fit into three categories – physical, chemical, or nutritional – can occur throughout the farm-to-fork continuum and lead to different types of bacterial cell damage.

The presence of injured microorganisms in food poses significant public health concerns. Injured cells may initially go undetected during routine quality control checks and at critical control points during manufacturing. However, subsequent cellular repair in the food may allow for growth and the ensuing results, including spoilage and the production of toxins and other virulence factors (302). As an example, three virulence factors of *E. coli* O157:H7, verotoxins 1 and 2, and the attaching and effacing gene were retained after starvation and heat stress (303). According to Singh and McFeters (304), virulence of *Yersinia enterocolitica* in orally inoculated mice also was unaffected by chlorine stress. A bacterium's pathogenicity or virulence may be considered the end result of its ability to repair injury (305). Mekalanos (306) defines virulence determinants as those factors contributing to infection and disease, but not to general "housekeeping" functions. A clear line of distinction is not always seen between the two, but virulence genes, to some extent, are part of an adaptive response to stresses encountered in a host (307). Many of the stresses that are intrinsically part of a host's defense system are similar to those encountered in the natural environment. Pathogenic microorganisms may see exposure to stress in both natural environments and food processing facilities as a signal for the expression of virulence factors (308). A strain of *S. Enteritidis* possessing enhanced acid and heat tolerance

was shown to be more virulent for mice and more invasive for chickens than was a non-resistant reference strain (73).

Expression of many virulence factors depends on environmental cues (306, 309). Several environmental conditions have been identified that induce expression of Spv (*Salmonella* plasmid virulence) proteins, including glucose starvation, low pH, elevated temperature, and iron limitation (310, 311). The *spv* genes in several serovars of *Salmonella* (e.g., Typhimurium, Dublin, and Enteritidis) are thought to facilitate rapid multiplication in host cells, systemic spread, and infection of extra-intestinal tissues (310). An invasion gene in *S. Typhimurium*, *invA*, is reportedly induced by high osmolarity (298, 306) and expression of listeriolysin, a major virulence factor in *L. monocytogenes*, by heat shock, oxidative stress, and transition to the stationary phase (306, 312, 313). Production of thermostable direct hemolysin, a major virulence factor of *V. parahaemolyticus*, is enhanced by heat shock at 42°C (314).

Temperature-regulated virulence factors have been identified in enteroinvasive *E. coli* (315), *S. flexneri* (306, 316), *L. monocytogenes* (317, 318), *Y. enterocolitica* (319), and heat shock has been linked to virulence in *L. monocytogenes* (298, 306), *S. Typhimurium* (298, 311), and *Shigella* spp. (320). As pathogens traverse from the natural environment, through contaminated food, water, or insect vectors into mammalian hosts, a sudden increase in body temperature triggers strong heat shock–like responses that intensify when host defense mechanisms (including fever) are encountered (321).

Acid tolerance is thought to enhance virulence in one or both of the following ways: (i) resistance to strong acid conditions facilitates survival in the stomach, thereby decreasing the requisite infective dose (322, 323), and (ii) resistance to moderately acidic conditions improves pathogen survival in acidic foods dependent on low pH for microbial inactivation (324). Acid tolerance of *E. coli* 0157:1-17 likely contributes to its low infective dose. Acid-sensitive strains of *S. Typhimurium* exhibit reduced virulence (311), whereas acid-tolerant mutants of *L. monocytogenes* exhibit increased virulence in the mouse model (31). Disruption of the RpoS system in *Salmonella*, which is involved in acid and general stress tolerance, may offer insight into the relationship between stress and virulence. *rpoS* null mutants are attenuated for mice after both oral and intraperitoneal infection (325). For many pathogens, acid tolerance seems to enhance survival in the host macrophage (298, 307).

The preceding examples indicate that alterations in cellular physiology, including stress protein synthesis in response to environmental stresses, may strongly impact virulence. An extension of this is the purported role of alternative sigma factors (e.g., σ^B) in the regulation of virulence factors (326, 327). A bacterium's ability to successfully handle environmental stress partially defines its virulence, since the response to such stress often includes the expression and control of various virulence factors (298). These consequences led Archer (298) to question whether a "reduction in preservation might not in fact lead to a reduction in the immediate virulence of certain pathogens, and, additionally, to a lowering of the rate of emergence of new or better host-adapted pathogens." Nevertheless, there is no available information about stress-response and virulence gene expression by disinfection surviving biofilm cells, and only recently it was reported the effect of disinfection on virulence gene expression by *L. monocytogenes* planktonic cells (328).

1.6 Scope and aims of this thesis

The main goal of this work was to provide a better understanding of the phenomena that involves foodborne contaminations caused by *L. monocytogenes* and *S. enterica* Enteritidis and also to give an insight into their response regarding yet unexplored growth conditions and exposure to antimicrobial agents. Moreover, to study the effect of disinfection on biofilm cells' genetic response was another important goal of this work. To accomplish these objectives *L. monocytogenes* biofilm formation ability was studied under different growth modes at different temperatures, and its survival on antimicrobial-coated food contact surfaces was tested. *S. enterica* Enteritidis was evaluated for its biofilm formation ability and viability on regular and antimicrobial incorporated materials. The final stage of this work focused on biofilm cells susceptibility, from both bacterial species, to chemical disinfection and aimed at giving the first insights of their response in terms of stress and virulence gene expression.

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Chapter 2

Effect of batch and fed-batch growth
modes on biofilm formation by
Listeria monocytogenes at different
temperatures

2.1 Introduction

Several studies have already been published regarding adhesion and biofilm formation by different *L. monocytogenes* strains (1, 2, 3). However, it is important to note that final conclusions about biofilm formation capability, exopolysaccharide production and biofilms viability, among others, may differ not only due to differences between specific strains tested (2, 4) but also because of the different methods and conditions applied in each work (1, 2, 5, 6). Until now, some of the most studied parameters involved in biofilm formation by *L. monocytogenes* have been: medium composition (6, 7), material surfaces (8, 9), incubation temperature (9, 10) and incubation time (1). However, even though researchers seem to arbitrarily choose batch or fed-batch conditions to assess biofilm formation by *L. monocytogenes* (1, 3, 10), to our knowledge nothing is known on the effect of these two growth modes on this biological process.

In this work, biofilm formation by five *L. monocytogenes* strains was assessed under batch and fed-batch conditions at three different temperatures (4°C, 25°C and 37°C) in order to evaluate how these distinct growth modes might affect biofilm development on an abiotic surface, in terms of biomass and cells' viability.

2.2 Materials and methods

Bacterial Strains and Culture Conditions

All assays were performed with five *L. monocytogenes* strains: 747, 925, 930 and 994 are food isolates belonging to distinct serotypes - 747, 925 and 930 present serotype 1/2b, while strain 994 presents serotype 4ab - whereas 1562 is a clinical isolate presenting serotype 4b. All strains were kindly provided by Dr. Paula Teixeira (Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal). For each assay, strains were subcultured on trypticase soy agar (TSA; Merck) for 24 - 48 h at 37°C and then grown in 30 ml of tryptic soy broth (TSB, Merck) for 18 ± 2 hours at room temperature with agitation at 120 rpm. Cells were harvested by centrifugation (5 min, 9000 rpm, 22°C), washed twice with sterile phosphate buffer saline (PBS 0.1 M, pH 7) and cell suspensions were standardized to an optical density (OD_{640nm}) \approx 0.3 corresponding to a concentration of approximately 1×10^9 CFU/ml.

Biofilm Formation in Fed-batch Mode

Biofilm formation assays were performed in sterile 96-well flat-bottomed uncoated polystyrene tissue culture plates (Orange Scientific, Belgium). Each well was filled with 240 μl of TSB supplemented with 0.25% (w/v) of glucose (Merck) and 10 μl of cell suspension. Negative controls consisted of wells filled only with culture medium without any bacterial cells. The plates were incubated at 4°C, 25°C and 37°C, for 5 days, with constant agitation at 120 rpm. The culture medium was refreshed twice a day by carefully pipetting 240 μl from each well (with care not to touch the bottom and the sides of the well) and gently adding the same volume of fresh medium. Four independent assays were performed for each strain at each condition with eight wells per strain per assay.

Biofilm Formation in Batch Mode

Biofilms were formed on microtiter plates as described above, except that there was no replacement of medium during all the incubation period.

Determination of Biofilm Biomass

Biofilm biomass was assessed as previously described (11) with some modifications. Briefly, at each sampling point medium was removed by pipetting, and each well washed with PBS also by pipetting. Biofilms were then fixed with 200 μl of methanol (Merck) per well for 15 minutes. Following this, the liquid phase was removed and the plates were left to dry at room temperature until they were completely dehydrated. Biofilm in each well was then stained with 200 μl of an aqueous 1% (v/v) CV solution (Merck) for 5 minutes at room temperature, and the excess dye rinsed off by washing with PBS. Once again, the plates were left at room temperature until a complete drying was achieved. The dye bound to biofilms in each well was resolubilized with 200 μl of 33% (v/v) acetic acid (Merck) and the optical density (OD) of each well measured at 570 nm in a microplate reader (BIO-TEK® Synergy HT, IZASA Portugal).

Determination of Cellular Metabolic Activity

Cellular metabolic activity was assessed by the reduction of tetrazolium salt (XTT) as described previously (12) with some modifications. Briefly, biofilms were gently washed with PBS and then 250 μl of an aqueous solution containing 50 $\mu\text{g/ml}$ XTT (Sigma) and 10 $\mu\text{g/ml}$

phenazine methosulphate (PMS; Sigma) was added to each well. Microtiter plates were incubated for 3 hours at 37°C in the dark and the OD measured at 490 nm. Ratio ($OD_{490\text{nm}}/OD_{570\text{nm}}$) was calculated in order to evaluate cell activity per biofilm biomass.

Epifluorescence microscopy

In order to obtain microscopic observations of cell's viability, biofilms were formed on polystyrene coupons under the same batch and fed-batch conditions described above. After five days of incubation, coupons were carefully washed with PBS, mounted on a glass slide and stained with LIVE/DEAD (L/D) BacLight Kit (Molecular Probes). The two reagents (syto9 and propidium iodide) were prepared according to the manufacturer's instructions and mixed in equal proportions. The mixture (50 μl per coupon) was then applied to each coupon and incubated for 15 minutes in the dark. Biofilms were visualized under an epifluorescence microscope (Olympus BX 51) equipped with a filter block that simultaneously detects the two components of the mixture.

Statistical Analysis

The statistical analysis was performed using the statistical program SPSS (Statistical Package for the Social Sciences). The results were compared using the non-parametric Mann-Whitney U-test at a 95% confidence level.

2.3 Results

Biofilms Biomass

The analysis of the effect of distinct growth modes on *L. monocytogenes* biofilm formation on polystyrene showed different performances for batch and fed-batch conditions, since at refrigeration temperature (Figure 2.1a) batch conditions lead to greater biomass amounts than fed-batch conditions, while at higher temperatures (Figure 2.1b and Figure 2.1c) the fed-batch mode was the more effective in enhancing biofilm formation ($p < 0.05$). Although not easily seen in the figures it is worth noting that, for most strains, biofilms grown under batch conditions had a general decrease of $OD_{570\text{nm}}$ values at 25°C and 37°C between the 3rd and 4th

day, the same period when biofilms formed under batch mode at refrigeration temperature achieved a significant biomass increase for most strains.

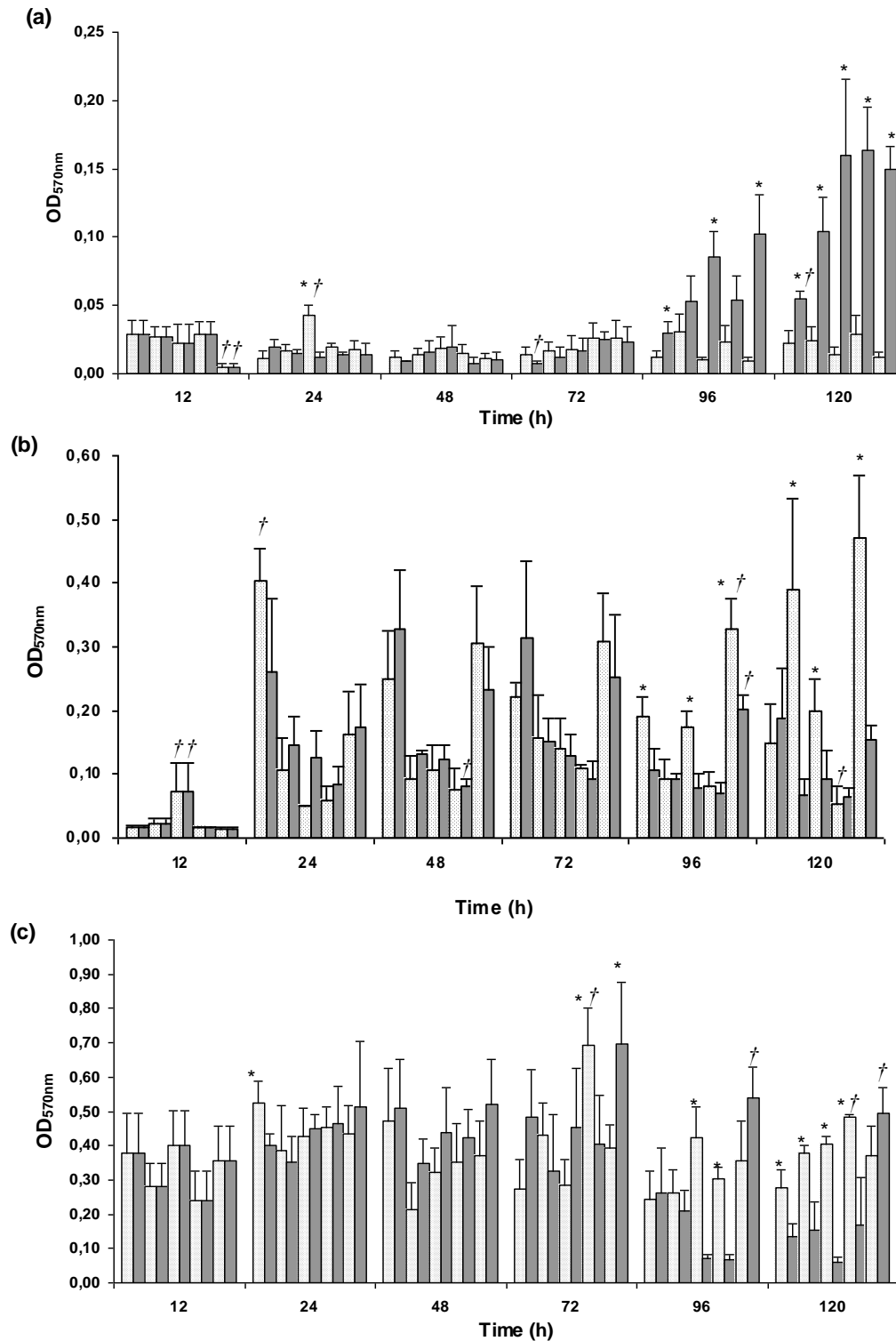


Figure 2.1 Biofilm formation measured by crystal violet destaining on (■) batch mode and (▨) fed-batch mode at (a) 4 °C, (b) 25 °C and (c) 37 °C. Bars represent average CV-OD₅₇₀ values and standard errors. Each pair of bars represents one strain, from left to right: 747, 925, 930, 994 and 1562. Symbols indicate statistically different values ($p < 0.05$) within each strain considering different growth modes (*) and between strains considering the same growth mode (†).

Cellular Metabolic Activity

Concerning the effect of distinct growth modes on biofilms' metabolic activity, and despite few exceptions, after 12 hours incubation, biofilms formed under fed-batch conditions were significantly more active than biofilms formed under batch conditions, independently of temperature or incubation time (Figure 2.2). This was corroborated by the microcopy images obtained after L/D staining, where biofilms formed under fed-batch mode (Figure 2.3a and Figure 2.3c) exhibited more green cells - which indicates that most cells have an unaltered cell membrane integrity - while biofilms formed under batch conditions (Figure 2.3b and Figure 2.3d) presented more red cells - which indicates that most cells have a damaged membrane. Centering the attention on the graphs scales, it is also worth noting that, in contrast to what was observed in biomass assays, metabolic activity results were significantly lower ($p < 0.05$) at 25°C and 37°C compared to the values found at refrigeration temperature (Figure 2.2).

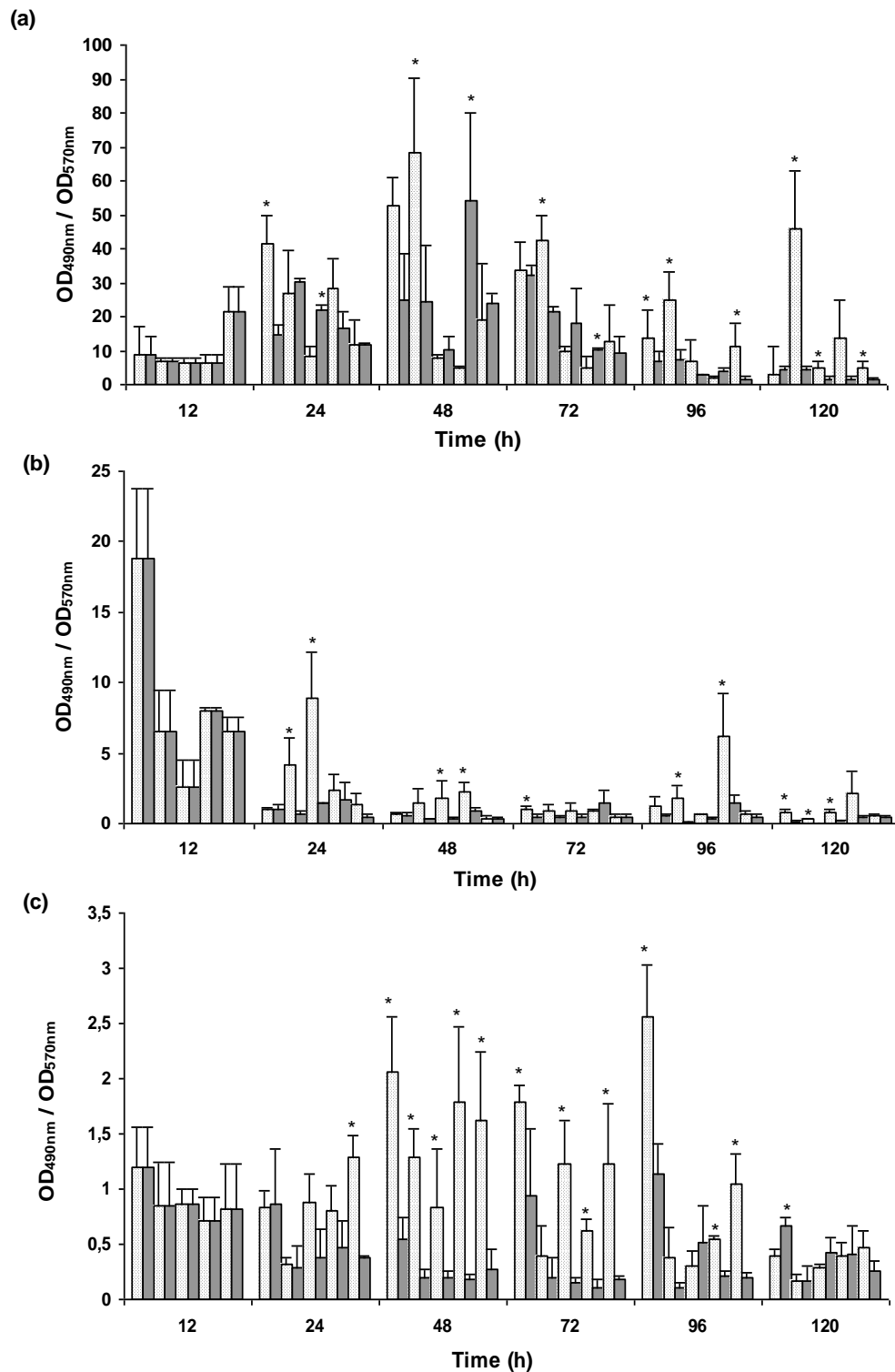


Figure 2.2 Biofilms cellular activity estimated by (OD_{490nm} / OD_{570nm}) ratio on (■) batch mode and (□) fed-batch mode at (a) 4 °C, (b) 25 °C and (c) 37 °C. Bars represent average (OD_{490nm} / OD_{570nm}) values and standard errors. Each pair of bars represents one strain, from left to right: 747, 925, 930, 994 and 1562. Symbol * indicates significantly different values ($p < 0.05$) within each strain considering different growth modes.

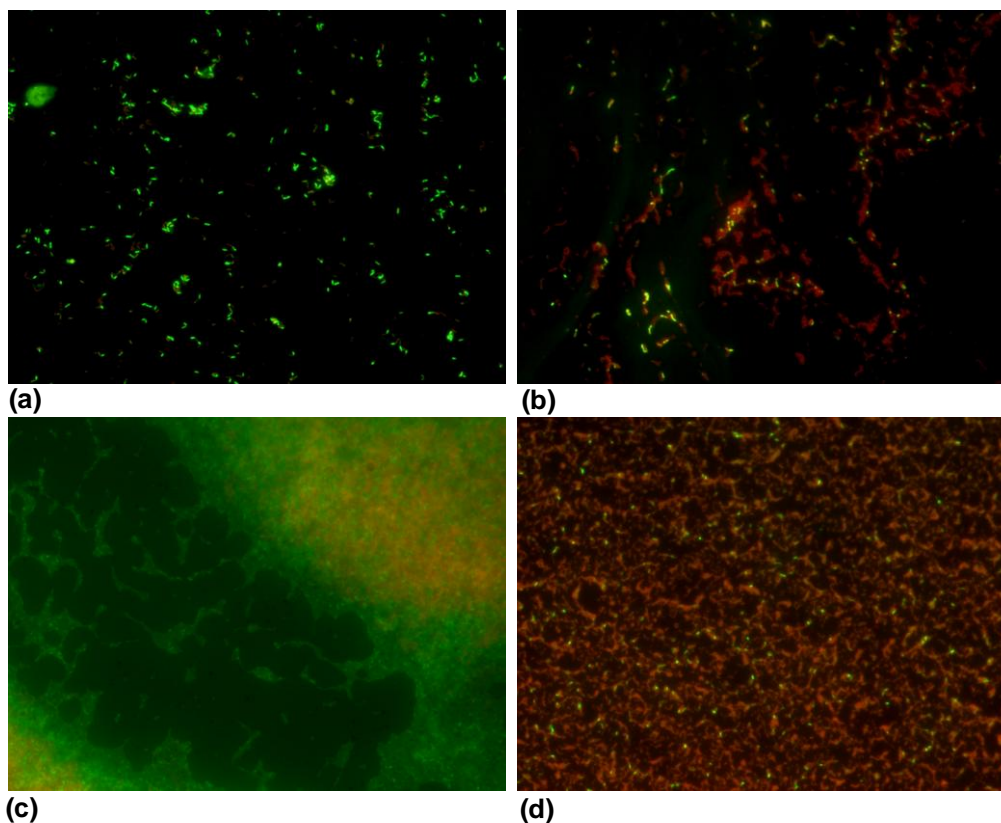


Figure 2.3 – Visualization of metabolically active cells by epifluorescence microscopy on five days old *L. monocytogenes* biofilms formed on polystyrene coupons under fed-batch **(a)** and batch mode **(b)** at 4 °C, and under fed-batch **(c)** and batch mode **(d)** at 37°C. Pictures were taken under a 40x objective after L/D staining.

2.4 Discussion

A general overview of the data obtained with both growth modes revealed that incubation temperature played a crucial role in *L. monocytogenes* biofilm development on polystyrene. It is worth noting that the highest biomass amount developed at 37°C is in agreement with other researches that showed that *L. monocytogenes* produces more biofilm as temperature increases (8, 10, 13, 14). On the other hand, apart from optimal growth temperature *L. monocytogenes* is also able to grow over a wide range of temperatures including refrigeration (2 – 4°C), as was confirmed in this work by the significantly high OD_{490nm}/OD_{570nm} values observed in biofilms formed at 4°C (Figure 2.2a) and the microscopy images (Figure 2.3). This means that, although at this temperature a low amount of biomass is formed, cells within the biofilms are metabolically more

active than those of biofilms formed at 37°C. The fact that biofilms formed at 4°C presented low biomass values can be due to a bacterial slow growth and a low accumulation of exopolymers. Indeed, quantification of total exopolysaccharides by Dubois method (15), after matrix extraction by sonication, showed that biofilms formed at refrigeration temperature did not have a detectable amount of polysaccharides and only biofilms formed at 37°C under fed-batch conditions were shown to have some polysaccharides in their matrix (data not shown). Moreover, the epifluorescence images (Figure 2.3b) are in agreement with Bonaventura *et al.* (16) studies in which it was reported that biofilms formed on polystyrene at 4°C (in batch condition) consisted of sparse clusters of cells with minimum amounts of exopolymers. The results obtained are also in accordance with Chavant *et al.* (10), in which they assessed *L. monocytogenes* adhesion and biofilm formation on polytetrafluoroethylene (a hydrophobic surface as is polystyrene) under fed-batch conditions at three temperatures (8°C, 20°C and 37°C) and had found that at the lowest temperature the colonization of the surface was very slow and no bacterial mat could be formed. In that same work, the researchers concluded that the nature of the surface (hydrophobicity) and the temperature were the main factors which significantly affected adhesion and biofilm formation.

Considering the biomass results for each growth mode, the differences found reflect how biofilms react to environments with different amounts of available nutrients. In fact, biofilms grown at higher temperatures seem to have higher growth rates (attested by their high biomass levels) and, thus, must demand a larger amount of nutrients available. So, although cells under batch mode at 25°C and 37°C had managed to grow in the first few days, the growing biomass amount together with the lack of nutrients might have caused biofilms' deterioration and/or detachment. This deterioration could also be responsible for the low (OD_{490nm}/OD_{570nm}) values (Figure 2.2b and Figure 2.2c). Previous studies have showed that restrictions in essential nutrients occurring in solid structures may result in a considerable decrease in bacterial metabolic activity (17, 18), which is in agreement with the microscopy images obtained in this work, where the large amount of red cells on biofilm formed at 37°C under batch conditions is a clear sign of cells' membrane damage (Figure 2.3d). On the other hand, and as stated above, cells at refrigeration temperatures display a slow growth, produce lower amounts of exopolymers and need longer adaptation periods to start growing. So, unlike what may happen in fed-batch mode, in which loosely adhered cells may be washed out every time the medium is refreshed

(19), in batch conditions cells remain in the system and, despite the slow growth, a higher amount of biomass might be accumulated.

2.5 General conclusions

The assessment of *L. monocytogenes* biofilm formation under different growth modes and different temperatures revealed that at refrigeration temperature (4°C) a higher amount of biofilm was produced when batch conditions were applied, while at higher temperatures the fed-batch feeding condition was the most effective on biofilm formation. Moreover, independently of the temperature used, biofilms formed under fed-batch conditions were metabolically more active than those formed in batch mode. In general, this work shows that different growth modes and temperatures significantly influence *L. monocytogenes* biofilm formation on abiotic surfaces as well as the metabolic activity of cells within biofilms.

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Chapter 3

Bacterial adhesion and biofilm
formation on materials with
antimicrobial properties

Section 3.1

Food contact surfaces coated with nitrogen-doped titanium dioxide: effect on *Listeria monocytogenes* survival under different light sources

3.1.1 Introduction

Disinfection plays a crucial role in food processing environments since it reduces the number of pathogenic microorganisms and, thus, prevents infectious diseases. Conventional chemical disinfection methods are effective in killing harmful microorganisms but are also related with an unintentional health hazard because of the dangerous disinfection by-products (DBPs) that are formed (1), and this is one of the reasons why the development of efficient but harmless sterilization procedures has become a critical subject.

Due to their extremely strong oxidation capability, photocatalytic titanium dioxide (TiO₂) substrates exhibit a self-cleaning function by being able to decompose various types of organic matter (2, 3, 4) and also act as disinfectants by injuring both the cell envelope and intracellular components of the microorganisms in contact with those substances. In fact, cell wall damage followed by cytoplasmic membrane injury leading to a direct intracellular attack has been proposed as the sequence of events when microorganisms undergo TiO₂ photocatalytic challenge (5, 6). This is mostly achieved through the displacement of Ca²⁺, Na⁺ and K⁺ ions, which are vital for bacterial metabolism. Since the microbiocidal effect of TiO₂ photocatalytic reactions was reported for the first time in 1985 (7), several studies have been published regarding TiO₂ photocatalytic elimination of a wide spectrum of organisms, including bacteria - *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella* spp., etc. -, fungi - *Candida albicans*, *Aspergillus niger*, etc. -, algae and cancer cells (5, 6, 8, 9).

Since TiO₂ photocatalyst is only efficient upon irradiation by ultraviolet (UV) light at levels that would provoke severe injury to human cells, the emergence of nitrogen-doped TiO₂ (N-TiO₂) brought a significant improvement in photocatalytic activity under visible-light (10, 11), with an active wavelength range (below 520 nm), covering a wider irradiation energy range for white fluorescent and incandescent light than that of TiO₂ (12). This innovation has raised the potential to develop TiO₂-coated surfaces for use in our living environments, which are of particular interest in places where disinfection plays a crucial role in the prevention of infectious diseases, such as hospitals, microbiological laboratories, pharmaceutical industry and food-processing environments. Although fluorescent and incandescent lights are the most commonly used for indoor lighting, and several researchers have used them to study photocatalytic reactions (12, 13, 14) to the authors' knowledge there is no report concerning the application and performance comparison of both these visible light sources under the same experimental conditions. In this context, the present work aimed at comparing the bactericidal effect of N-TiO₂ coated materials

under these two visible light sources and to evaluate the application of this surface treatment on food-contact materials as a way of improving foodborne pathogens control. *L. monocytogenes* was the bacterium chosen to represent such microorganisms, as it is responsible for severe food contamination worldwide leading to serious and potentially fatal diseases both in humans and animals. Due to its high efficiency in promoting TiO₂ photocatalysis, and to have comparison between different kinds of light, assays with UV-light irradiation were also performed. Moreover, given that some TiO₂ coatings are known to become super-hydrophilic under UV light irradiation (15, 16, 17, 18), surfaces' hydrophobicity was determined through contact angle measurement after exposure to UV-light to verify if this phenomenon occurred on the tested surfaces and, consequently, may have affected surfaces disinfection.

3.1.2 Materials and methods

Coupons with Photocatalyst

Stainless steel and glass coupons used in these experiments were coated with N-TiO₂ by pulsed direct current reactive magnetron sputtering, from a high purity Ti target in an Ar/N₂:O₂ atmosphere and subsequently subjected to a post heat treatment at 500°C in a vacuum furnace. The level of nitrogen doping in the TiO₂ lattice was adjusted by controlling the amount of nitrogen gas in the reactive flow upon sputtering; details of these experiments can be obtained elsewhere (19). Square glass slides of 2.0 x 2.0 cm and stainless steel discs with a 2 cm diameter were used after being cleaned by immersion in a 0.2% solution of a commercial detergent (Sonazol Pril, Alverca, Portugal) followed by immersion in ethanol. Each coupon was then rinsed with ultrapure water and dried at 60°C. Control coupons had exactly the same characteristics except the coating with N-TiO₂.

Bacterial Culture

For each assay, *L. monocytogenes* clinical isolate 1562 was subcultured on trypticase soy agar (TSA; Merck) for 24 - 48 h at 37°C and then grown in 30 ml of tryptic soy broth (TSB, Merck) for 18 ± 2 hours at room temperature with agitation at 120 rpm. Cells were harvested by centrifugation (5 min, 9000 rpm, 22°C), washed twice with 0.9% saline and cell suspensions

were standardized to an optical density (OD_{640nm}) \approx 0.3 corresponding to a concentration of approximately 1×10^9 CFU/ml.

Photocatalytic Reactions and Enumeration of Viable Bacteria

For each photocatalytic reaction, 50 μ l of bacterial suspension were placed on a coupon's surface and then covered with a coverslip to improve contact between bacteria and the surface and to prevent the suspension from drying (20). After optimization of experimental conditions taking into consideration irradiation time and bacterial suspension drying, a 30 min exposure period was selected to perform the assays, which were all done at room temperature ($20 \pm 2^\circ\text{C}$). Three different lights were used - two fluorescent lamps of 4 W each (irradiance of 0.13 mW/cm^2), one incandescent lamp of 60 W (irradiance of 8.93 mW/cm^2) and two UV lamps (irradiance of 0.83 mW/cm^2); the irradiances were measured with a portable photo radiometer (Photo/Radiometer HD 2102.1, Delta Ohm). The same procedure was conducted for both control and coated coupons. These assays also included coated and non-coated coupons kept in the dark, to be compared with those submitted to irradiation.

After the photocatalytic reactions, surviving bacteria were recovered from each coupon by washing with 1 ml of 0.9% saline. The resultant suspension was serially diluted and the bacterial concentration determined by the standard plating method on TSA plates. Colony forming units (CFUs) were counted after 24 hours incubation at 37°C . At least three independent assays were performed for each material with three coupons per assay.

Hydrophobicity

The hydrophobicity was determined through contact angle measurement (OCA 20, Dataphysics) with Millipore water, using the advanced type technique on air. According to this method, a surface is considered hydrophobic if the water contact angle exceeds 65° and hydrophilic if it does not (21). Measurements were done on glass and stainless steel coupons (coated and non-coated) after 30, 60, 120 and 300 min of UV light exposure, as well as on coupons kept in the dark (controls).

Statistical analysis

Data analysis was performed using the statistical program SPSS (Statistical Package for the Social Sciences). Contact angle results were compared through one-way ANOVA, whereas bacterial survival was compared using the non-parametric Mann-Whitney U-test. All tests were performed with a confidence level of 95%.

3.1.3 Results

Bacterial Loss of Viability under Visible and UV Light Irradiation

Results presented in Figure 3.1.1 express the bacterial survival in percentage, where 100% corresponds to viable cells collected from the coupons that were kept in dark (data not shown), which number was not significantly different from the initial inoculum ($\approx 1 \times 10^9$ CFU/ml). All experimental conditions had reduced the bacterial survival on control and coated coupons, and in both cases it was UV-light that lead to the most effective disinfection. Regarding uncoated surfaces, UV was the only light that gave significantly different results ($p < 0.05$) between both materials, with 3.38% survival on glass and 41.18% survival on stainless steel. Moreover, the most efficient photocatalytic reaction was also accomplished by UV-light irradiation, which achieved the highest levels of disinfection ($p < 0.05$) with *L. monocytogenes* survival percentages of 0.15% and 2.37% on N-TiO₂ coated glass and stainless steel, respectively. Nevertheless, except for glass coupons when exposed to fluorescent light, visible light had also significantly affected cell survival on N-TiO₂ coated coupons of both materials when compared to controls.

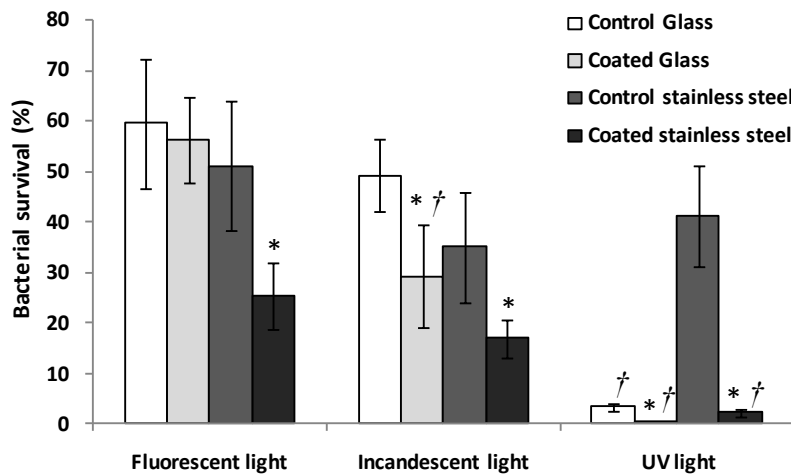


Figure 3.1.1 *L. monocytogenes* survival on uncoated and N-TiO₂ coated glass and stainless steel surfaces after 30 min exposure to fluorescent, incandescent and UV light. Symbols indicate statistically different values ($p < 0.05$) between control and coated surfaces of the same material considering the same light irradiation (*) and between the same surface considering different light irradiation (†).

Although not as effective as UV-light irradiation, fluorescent light had promoted disinfection on coated stainless steel surface, while incandescent light was able to reduce the bacterial load on both coated surfaces ($p < 0.05$). The performance of different kinds and sources of light is in accordance with the respective lamp(s) spectra (Figure 3.1.2), which shows that at 380 nm (wavelength below which the photocatalyst's absorbance rapidly increases) fluorescent light has a marginal relative intensity, whereas incandescent light presents a moderate relative intensity. In the same way, UV-light efficiency is corroborated by a higher relative intensity value at 380 nm. Consequently, the different performances of both coated materials are also in agreement with the corresponding diffuse reflectance spectra (Figure 3.1.3), given that for wavelengths higher than 380 nm, in particular between 400 and 450 nm, N-TiO₂ films on stainless steel tend to reflect less diffuse light and to absorb more than in comparison to the same films on glass.

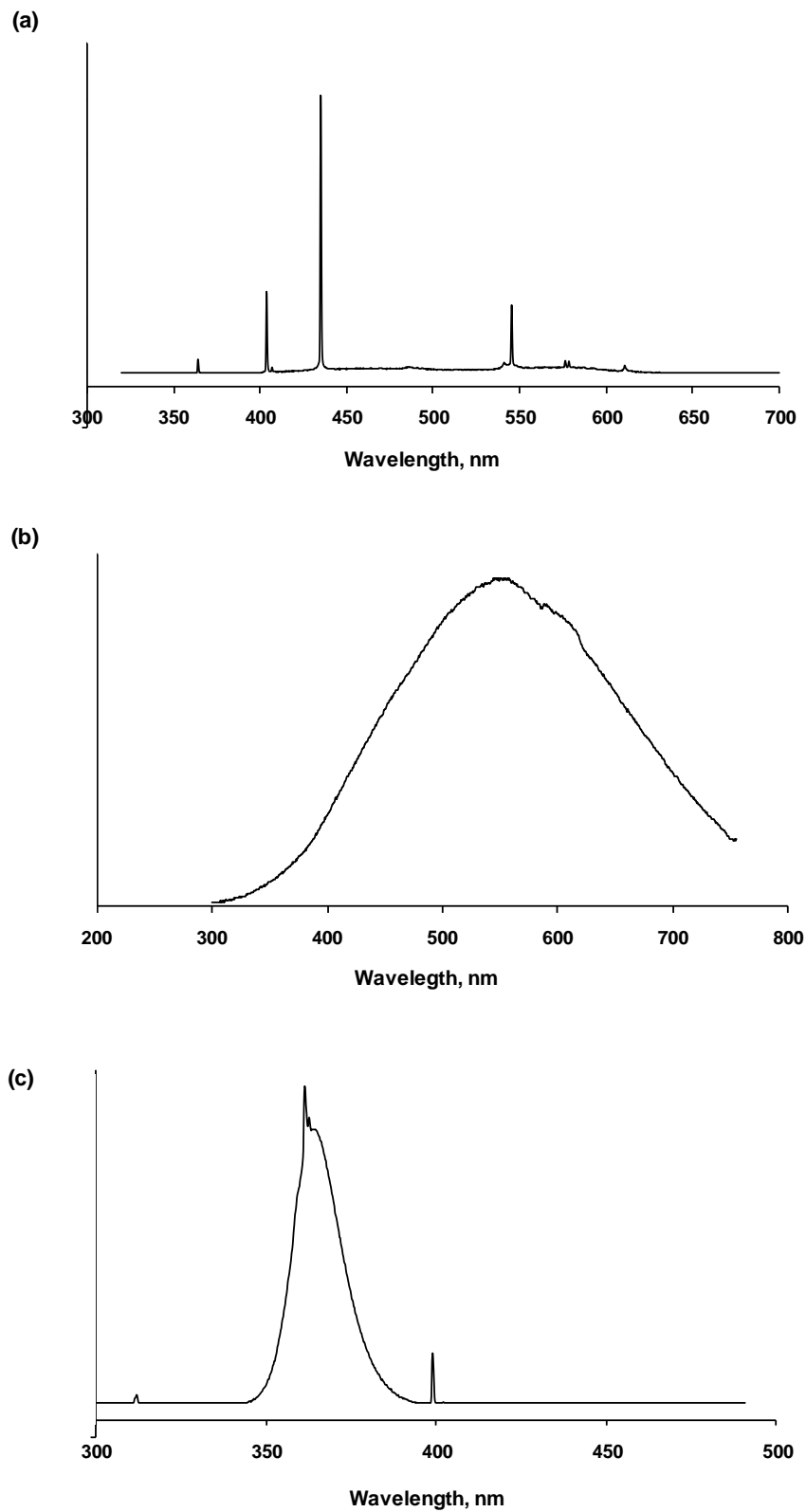


Figure 3.1.2 Light spectra of (a) fluorescent, (b) incandescent and (c) UV lamps.

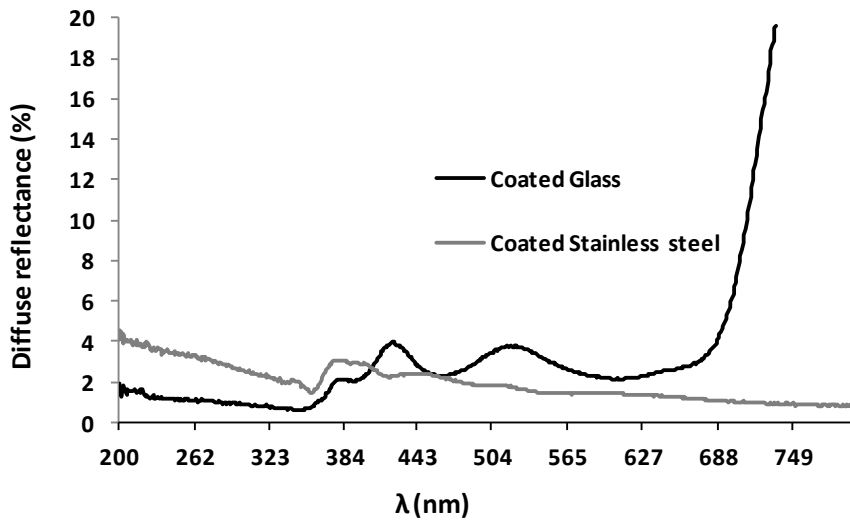


Figure 3.1.3 Diffuse reflectance of N-TiO₂ coated glass and stainless steel.

Effect of UV-light Exposure on Hydrophobicity

Contact angle measurements, which results are presented in Figure 3.1.4, revealed that both materials coated with N-TiO₂ have a hydrophobic surface and no significant change occurred after UV-light irradiation for 30 minutes (exposure time used for photocatalytic reactions). In fact, it took two and five hours exposure, for glass and stainless steel respectively, to find a statistically significant reduction ($p < 0.05$) of hydrophobicity values between controls and coated coupons'. Moreover, hydrophilicity (contact angle smaller than 65°) was only found in N-TiO₂ coated glass after one, two and five hours UV-light irradiation, while coated stainless steel coupons kept a hydrophobic surface even after those exposure times. Since contact angles of control surfaces were identical for all the conditions tested (at dark and after the different exposure times), only the mean value of those measurements was used and represented in the respective chart (Figure 3.1.4).

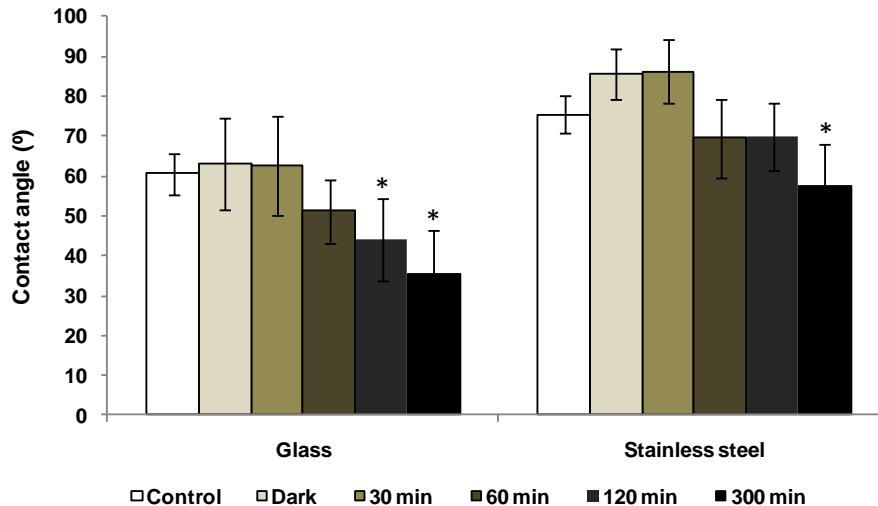


Figure 3.1.4 Water contact angles of uncoated and N-TiO₂ coated glass and stainless steel surfaces at dark and after different exposure times to UV-light. Symbol * indicates statistically different values ($p < 0.05$) between control and coated surfaces of the same material.

3.1.4 Discussion

In the pursuit of a harmless and chemical-free disinfection of food processing environments, photocatalytic disinfection of glass and stainless steel (two materials commonly used in kitchens and food processing environments) coated with N-TiO₂ was evaluated under the two light sources most frequently used indoors – fluorescent and incandescent -, as well as under UV-light irradiation. After 30 min of light exposure, bacterial viability was assessed and the survival percentage compared between the different experimental conditions. The results showed that *L. monocytogenes* survival was reduced on all coupons used, controls included (Figure 3.1.1). Such a result on uncoated surfaces may be at least partially due to surface heating during the assays, because of the heat emitted by lamps, given that excessive heating changes the morphological and physiological state of bacteria and, ultimately, can lead to their death (22). Nevertheless, the significantly lower ($p < 0.05$) survival on uncoated glass exposed to UV-light, comparing to all other controls, must be related not only with heating but with the combination of heat and the antimicrobial capability of UV radiation absorbed by glass. In fact, this material absorbs UV-light with greater efficiency than other materials, since electrons in the glass absorb the energy of photons in UV range, in comparison with the weaker energy of photons in the visible light spectrum.

The analysis of the results regarding photocatalytic reactions under visible light revealed a higher effectiveness of the incandescent light, since it had efficiently promoted *L. monocytogenes* elimination on both coated surfaces, while fluorescent light did not accomplish a significant decrease of cell survival on coated glass. Given that the active radiation spectrum of these N-TiO₂ films shows that its major photocatalytic activity occurs on wavelengths below 450 nm (Figure 3.1.3), the better disinfection performance of incandescent light must be related with its higher relative intensity values compared to fluorescent light spectrum (Figure 3.1.2). Nevertheless, fluorescent light was found to emit trace amounts of UV-A, UV-B and UV-C sufficient for bacterial inactivation (23) as well of visible light from the intense discrete peaks at 404 and 435 nm which, all-together, may be the reason why good disinfection results on coated stainless steel surfaces were obtained with this light source. This gives hope for the use of such a photocatalyst in most indoor environments, namely hospitals. Considering its features and the results obtained for fluorescent light, it is possible to infer that the statistical disparity on *L. monocytogenes* survival between N-TiO₂ coated glass and stainless steel may be a consequence of different interactions between surfaces tested and fluorescent light, where both light and surfaces' characteristics are involved. In fact, analyzing the way each material interacts with visible light, diffuse reflectance values showed that the absorption limit of both coated materials corresponds approximately to an absorption edge located at ≈ 380 nm (3.26 eV), below which the absorbance rapidly increases. However, for wavelengths above 380 nm, in particular between 400 and 500 nm (visible light range), N-TiO₂ films deposited on stainless steel tend to reflect less diffuse light and absorb more than those deposited on glass, which explains the better performance of the metal substrate material. It is also worth noting that, although Morikawa *et al* (12) had reported these films to absorb radiation below 520 nm, N-TiO₂ films used in the present work absorb radiation below 450 nm, albeit to a less extent than that registered below the absorption edge. On the other hand, while still concerning visible light reflectance of both materials, it is important to note that, although both surfaces exhibit a combination of diffuse and specular reflectance, N-TiO₂ films deposited on stainless steel substrates have a higher proportion of specular reflectance and less of diffuse reflectance, in comparison to glass substrates, which inevitably results in a larger dispersion of light on the bacteria and more effective elimination. Specular reflectance implies light rays to be reflected and remain concentrated in a bundle upon leaving the surface, while diffuse reflectance implies the light rays to be reflected and diffused in many different directions. Such different behavior between the two substrate materials may

influence the elimination of bacterial cells in contact with N-TiO₂ films and, thus, contribute to the different results between the two surfaces. On the other hand, contact angle measurements on control coupons and on coated surfaces kept in the dark showed that glass was significantly less hydrophobic ($p < 0.05$) than stainless steel. Taking into account studies where less hydrophobicity has been related with less microbial interaction with surfaces (24, 25, 26, 27) it is possible to deduce that cell-surface interaction was stronger on stainless steel than on glass, which may have enhanced the photocatalytic disinfection performance on the former material.

Although results obtained with fluorescent and incandescent light proved that visible light was able to promote *L. monocytogenes* elimination on both materials used, photocatalytic disinfection was significantly higher when UV-light was employed. This was already expected due to the disinfection properties of this light, and is in accordance with Irie *et al* (28) that reported N-TiO₂ photocatalytic activity generated by visible light to be inferior to that induced by UV light. Moreover, and in contrast to what happens under visible light, N-TiO₂ and TiO₂ exhibit a similar activity under UV-light (12). This means that N-TiO₂ photocatalysis under UV-light irradiation has a highly effective bactericidal capability similar to that reported by many authors concerning TiO₂ photocatalytic reactions (5, 6, 7, 8, 9, 29, 30, 31). Since there are several reports on TiO₂ coated surfaces becoming super-hydrophilic under UV irradiation (15, 16, 17, 18), contact angles were measured in all control and coated surfaces under UV-light irradiation in order to comprehend if that phenomenon was occurring on the materials used in this work and how it could be influencing disinfection. Results have shown that UV disinfection performance was not influenced by changes in surfaces' hydrophilicity (Figure 3.1.4), since neither materials suffered significant differences on contact angles, regardless of being coated or uncoated and the measurements being done in the dark or after UV irradiation for 30 minutes (exposure time used in the assays). The apparent lack of better hydrophilicity of N-TiO₂ coated coupons used in this work is not in agreement with previous reports that found that this coating tends to increase surface hydrophilicity, especially after light exposure (32, 33). This disparity might be due to the fact that surface properties are different from those reported in the literature, in particular the surface area of the crystalline grains, which in the present case is very low ($< 50 \text{ m}^2/\text{g}$).

3.1.5 General conclusions

UV irradiation was the most effective in reducing *L. monocytogenes* viability on N-TiO₂ coated glass and stainless steel coupons, but both visible light sources also promoted a reduction of the bacterial load, with incandescent light achieving better results than fluorescent light. Hence, although UV-light was the most effective on promoting photocatalytic reactions on N-TiO₂ coated coupons, good levels of disinfection were also accomplished under visible light, meaning that this surface coating represents a safe complementary sanitation tool against foodborne pathogens, on both domestic and industrial food-processing facilities.

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Section 3.2

Salmonella enterica Enteritidis biofilm formation and viability on regular and triclosan incorporated bench cover materials

3.2.1 Introduction

Bacterial adhesion and subsequent biofilm formation on food contact surfaces is the major cause of economic costs in food industry and is also responsible for transmission of diseases, both from industrial and domestic environments. Nowadays the importance of good cleaning, hygiene and use of separate surfaces and equipment for raw and cooked foods is well known to reduce the risk of cross-contamination, which is an important factor in transmission of microbiological food-borne illness (1). However, bacterial food poisoning continues to be an important health problem worldwide with numerous foodborne disease outbreaks and deaths being registered every year. The last report published by the European Food Safety Authority declared a total of 5,332 foodborne outbreaks in the European Union, causing 45,622 human cases, 6,230 hospitalisations and 32 deaths (2). The same document states that most of the reported outbreaks were caused by *Salmonella* (35.4%), which confirms that this bacterium is still one of the most important foodborne pathogens. As *E. coli* (3), *Campylobacter* (3), *Pseudomonas* (4) and *Listeria* (5, 6), *Salmonella* has been reported to adhere and form biofilms that, when growing on food-contact surfaces, represent a major source of food contamination. Various food-contact surfaces, such as glass, rubber, metal and plastic have been considered in studies about *Salmonella* adhesion and biofilm formation (7, 8, 9, 10, 11, 12, 13) but little information is available concerning contamination of kitchen bench stones, even though these are materials commonly present in food processing environments, especially in domestic kitchens of European Mediterranean countries.

Like many other surfaces, kitchen bench stones are now available as regular and antimicrobial incorporated materials, with granite and marble being the most frequently used regular stones, while Silestone® is now the world leader in quartz surfaces with an antimicrobial integrated. Silestones have the feel and the weight of a natural stone but are synthetic materials composed of 94% quartz, available in the market worldwide and whose composition includes triclosan as antibacterial agent (14). Among compounds that restrain bacterial development and that are frequently applied to control bacterial contamination in the home and during food processing, triclosan is one of the most commonly used. It is a polychloro-phenoxy-phenol compound with broad-spectrum antimicrobial activity (15) first used in the early 1970s (16, 17). Triclosan acts as a broad-spectrum antimicrobial agent by targeting lipid biosynthesis and inhibiting cell growth (18, 19, 20) with the minimal inhibitory concentrations for a variety of tested organisms ranging from less than one part per million to parts per thousand for

Pseudomonas (21). Nowadays it is widely found in many domestic products such as shower gels, deodorants, toothpastes, hand soaps and creams (21), as well as in impregnated surfaces of refrigerators, chopping boards and plastic lunchboxes. Triclosan has also been used in industrial environments, such as food processing facilities, where exposed equipment, floors and walls have been treated with this compound to decrease microbial contamination (22).

Since, in the authors' knowledge, there is a lack of information concerning biofilm formation on kitchen bench stones, this work aimed at assessing such biological process by *Salmonella* Enteritidis on granite, marble and triclosan incorporated silestones. To have a comparison between different food-contact surfaces, stainless steel was also included in this study, as it has been the most used material for working surfaces and kitchen sinks because of its ease of fabrication, mechanical strength, corrosion resistance and durability (23). Given that attachment to the surface is the first stage in the formation of a biofilm, *Salmonella* Enteritidis adhesion was evaluated in order to obtain some information about the initial interaction between bacteria and the different surfaces. Cellular viability within biofilms was also assessed to determine whether triclosan had any effect on biofilm-cells during biofilm development.

3.2.2 Materials and methods

Bacteria and Culture Conditions

In order to cover the behavior of different strains from different sources, five *Salmonella* Enteritidis strains were used in this work: 1 food isolate (355), 3 clinical isolates (357, 358, CC) and 1 reference strain (NCTC 13349). All isolates were kindly provided by Dr. Paula Teixeira (Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal). For each assay, strains were sub-cultured on Luria Bertani Broth Miller agar (LBA; Sigma-Aldrich, Inc., St. Louis, Mo.) for 24–48 h at 37°C and then grown in 30 ml of Luria Bertani Broth Miller (LB; Sigma-Aldrich) for 18 ± 2 h at room temperature with agitation at 120 rpm. Cells were harvested by centrifugation (5 min, 9000 rpm), washed twice with saline 0.9 % and cell suspensions were standardized to a concentration of approximately 1×10^9 CFU/ml ($OD_{640nm} \approx 0.5$).

Materials

Surfaces tested were granite “Pedras Salgadas” (Vila Pouca de Aguiar, Portugal), marble (Sivec), stainless steel (SS) (304, finishing 2B) and two kinds of silestone – white (wST) and beige (bST) (Cosentino). Squares of 2.0 by 2.0 cm² of each material were used after being cleaned by immersion in a 0.2% solution of a commercial detergent (Sonazol Pril) followed by immersion in ethanol. Each square was then rinsed with ultrapure water and dried at 60°C.

Adhesion Assays and Cells Enumeration

Each square of the tested materials was placed in six-well tissue culture plates (Orange Scientific) containing 7.5 ml of LB supplemented with 0.25% (w/v) glucose (Merck) and 50 µl of cell suspension. Negative controls consisted of wells filled only with culture medium without any bacterial cells. After 2 h at room temperature (22°C) with constant shaking at 120 rpm, squares were rinsed three times by soaking for 10 s in 0.9% saline in order to remove unattached cells. These washing steps were carefully performed to remove only the bacteria that were suspended in the liquid interface formed along the surface and to minimize cell detachment from the surface (24). Four independent assays were performed for each strain on each material with three squares per strain per assay.

Adhered cells were scraped from each surface with a sterile cell scraper (Orange Scientific) and collected in 1 ml of sterile Maximum Recovery Diluent (MRD; 1.0 g peptone + 8.5 g sodium chloride per liter of water, pH 7.0 ± 0.2). The efficiency of this washing procedure was confirmed by visual inspection through epifluorescence microscopy (data not shown). Each obtained suspension was serially diluted in MRD and spread on LBA plates. Colony-forming units (CFUs) were counted after 24 h incubation at 37°C.

Biofilm Assays and Quantification

Biofilm formation methodology was the same used for adhesion assays except for the incubation time, which was extended to 48 h. After the washing procedures described above, total amounts of biofilm grown on each surface was evaluated through crystal violet (CV) staining as follows. Squares were transferred to new six-well plates and fixed by submersion in methanol (Merck) for 15 min. After withdrawing the methanol, squares were allowed to dry at room temperature before being submerged in an aqueous 1% (v/v) CV solution (Merck) for 5 min at

room temperature. Squares were then gently washed with 0.9% saline and transferred to a new six-well tissue culture plate. A 33% (v/v) acetic acid solution (Merck) was then added to each well to release and dissolve the stain. 250 µl of the eluted dye from each square was transferred to a 96-well microtitre plate and its absorbance read in triplicate in an ELISA reader (BIO-TEK® Synergy HT, Izasa) at 570 nm.

Bacterial Viability Assays

Since CV staining is a basic dye that binds to negatively charged surface molecules and polysaccharides in the extracellular matrix (25) and stains both living and dead cells (26), a different methodology was used to assess cellular viability. Biofilms formed on the surfaces were washed as described above and the number of viable cells assessed following the same procedure described for adhered cells enumeration, except that biofilm suspensions were longer and more vigorously vortexed in order to promote cells disaggregation. Once again, the efficiency of this washing procedure was confirmed by visual inspection through epifluorescence microscopy (data not shown).

Statistical Analysis

Data analysis was performed using the statistical program SPSS (Statistical Package for the Social Sciences). The results were compared using the non-parametric Mann–Whitney U-test at a 95% confidence level.

3.2.3 Results

Bacterial Adhesion

Results presented in Figure 3.2.1 show that all surfaces were largely colonized by all *Salmonella* Enteritidis strains, with most strains achieving 10^5 CFU/cm² after two hours of incubation. Strains 358 and NCTC 13349 adhered significantly more to marble than to any other surface ($p < 0.05$), while the food isolate 355 and the clinical isolate 357 exhibited a greater propensity to adhere to marble and bST than to the other materials ($p < 0.05$). These data are in accordance with the mean adhesion of *Salmonella* Enteritidis strains to the same material, which

shows that marble was more readily colonized than other surfaces, while granite, both silestones and SS had similar extents of adhesion, with less adhered cells than on marble.

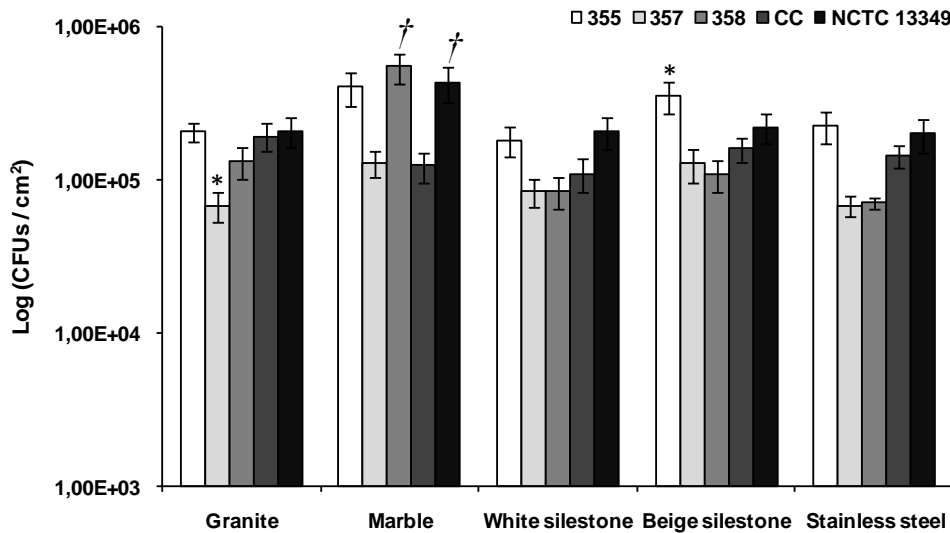


Figure 3.2.1 Number of *Salmonella enterica* Enteritidis adhered cells per square centimeter of the different materials after 2 hours incubation. Symbols indicate statistically different values ($p < 0.05$) concerning the adhesion of different strains to the same material (*) and concerning the adhesion of the same strain to different materials (†).

Concerning adhesion of individual strains to the same material, a significantly different number of adhered cells from all other strains was found only on granite and bST, where clinical isolate 357 had the lowest number of adhered cells and food isolate 355 achieved the highest adhesion value ($p < 0.05$), respectively. Nevertheless, *Salmonella* Enteritidis strains 355 and NCTC 13349 were always found to be among the most adherent strains on all materials, while clinical isolates tended to have lower a number of adhered cells. The only exception to this fact was observed on marble, where strain 358 reached an adhesion extent similar to that achieved by the food isolate and collection strain.

Biofilm Formation

Salmonella Enteritidis biofilm formation assessed through CV staining (Table 3.2.1) showed that strains 355, 357 and NCTC 13349 formed more biofilm on marble than on any other surface ($p < 0.05$). Moreover, the other two strains also had high biofilm amounts on this same material, with clinical isolate 358 forming significantly more biofilm on marble than on wST or SS. Mean results concerning biofilm formation by all *Salmonella* Enteritidis strains on the same material confirm marble as the material on which higher amounts of biofilm were formed ($p < 0.05$), while both silestones and SS showed similar optical density (OD) values. Biofilms on granite were smaller than those formed on marble but significantly higher biofilm amounts were produced by strain 355 on granite than on the other three surfaces. bST was the only material where biofilm formation achieved statistically lower values, with clinical isolate 357 presenting its lowest biofilm amount on bST.

The comparison of biofilm formation by *Salmonella* Enteritidis on each material pointed out NCTC 13349 as the strain that formed the lowest amount of biofilm on granite ($p < 0.05$). Moreover, together with food isolate 355, this strain was also one of the weakest biofilm formers on wST and SS, and only had accomplished high biofilm amounts on marble. The three clinical isolates presented similar OD values for all surfaces except for bST, where strain 357 formed significantly less biofilm than the other two ($p < 0.05$).

Bacterial Viability within Biofilms

Table 3.2.1 also shows the quantification of viable cells within *Salmonella* Enteritidis biofilms and shows that bacterial viability was significantly higher on granite and marble than on both silestones. In fact, isolates 355 and CC had fewer viable cells on wST than on any other surface, while strains 357 and NCTC 13349 had similar amounts of viable cells on both silestones, but which were lower than those found on all other materials ($p < 0.05$). An intermediate level of *Salmonella* Enteritidis viability was found on SS, with strains 357 and NCTC 13349 achieving numbers of viable cells significantly lower than those registered on both regular stones and significantly higher than those registered on both silestones. Comparing both silestones performance in terms of antimicrobial effect, bST was slightly less successful since isolates 355 and CC had higher numbers of viable cells on this surface than on wST ($p < 0.05$).

Concerning cellular viability within biofilms formed by different *Salmonella* Enteritidis strains on the same material it is possible to see that food isolate 355 was related to low

numbers of viable cells on all surfaces, with significantly lower results on granite and wST than any other strains. Conversely, clinical isolate 358 had always high viability values, achieving higher numbers of viable cells on wST and SS than any other strains ($p < 0.05$). Except for granite, clinical isolate 357 was one of the *Salmonella* Enteritidis strains with the lowest viability on all surfaces, while clinical isolate CC was one of the strains with a higher number of viable cells on all materials except for wST and SS. Viability within biofilms formed by NCTC 13349 was higher on both regular stones than on both silestones, while SS presented intermediate numbers of viable cells of this strain ($p < 0.05$).

Table 3.2.1 Total biomass and viability of *Salmonella* Enteritidis biofilms

Strains	Granite		Marble		White Silestone		Beige Silestone		Stainless steel	
	Biomass ^a	Viability ^b	Biomass	Viability	Biomass	Viability	Biomass	Viability	Biomass	Viability
355	0.06±0.02 [†]	6.78±0.12*	0.11±0.03 [†]	6.99±0.22	0.02±0.00	5.67±0.41 ^{*,†}	0.04±0.01	6.22±0.10 [†]	0.03±0.01	6.89±0.05
357	0.08±0.02	6.98±0.10	0.16±0.04 [†]	6.90±0.15	0.07±0.02	6.18±0.11	0.03±0.01 [†]	6.09±0.08	0.06±0.02	6.78±0.19 [†]
358	0.08±0.02	7.06±0.08	0.12±0.03	7.15±0.09	0.06±0.01	6.90±0.10*	0.08±0.02	6.86±0.10	0.07±0.02	7.08±0.10*
CC	0.08±0.01	7.21±0.05	0.11±0.02	7.12±0.05	0.08±0.01	6.57±0.15 ^{*,†}	0.08±0.02	6.88±0.19	0.07±0.01	6.77±0.21
NCTC13349	0.04±0.01*	7.12±0.05	0.15±0.03 [†]	7.14±0.05	0.02±0.00	6.23±0.12	0.02±0.00	6.19±0.01	0.03±0.01	6.85±0.15 [†]

^a OD570nm mean values ± SD.

^b Log (CFU/cm²) mean values ± SD.

Symbols indicate statistically different values ($p < 0.05$) concerning biofilm formation of different strains to the same material (*) and concerning biofilm formation by the same strain to different materials (†).

3.2.4 Discussion

Since limited information is available concerning bacterial adhesion on both regular and antimicrobial incorporated stones (27, 28, 29, 30, 31), and no reports have been made concerning biofilm formation on any of these surfaces, the present work reports the study of the attachment and biofilm formation ability of five *Salmonella* Enteritidis strains on granite, marble and on two silestones impregnated with triclosan. SS was also included for comparative purposes, since it is widely used not only in domestic kitchens but also in the food processing industry, where working surfaces and machinery (7, 32, 33) as well as tanks and pipelines (34) are made of this material.

As previously reported (29, 35) this work showed that, although all strains were able to colonize all surfaces, *Salmonella* adhesion was strongly strain dependent and the number of adhered cells varied according to the different materials tested (Figure 3.2.1). Marble was the stone more prone to bacterial colonization and, thus, the less advisable material in terms of food safety, while no advantage was found for silestones comparing to granite and SS since all of them had similar amounts of adhered cells. These results are not in agreement with other studies that found higher adhesion extent on SS than on stones and no differences concerning the number of adhered cells on granite, marble and both silestones (29, 30). However, both studies referenced had used DAPI staining and epifluorescence microscopy while in the present work CFUs enumeration was performed, which also explains the generally lower amount of adhered *Salmonella* Enteritidis cells observed comparing to other reports. Since only adhered bacteria that remain viable are the actual cause of post-process contamination, CFUs enumeration seems to be more accurate for these types of studies than epifluorescence methods. On the other hand, the higher levels of adhesion on marble are in agreement with a study that suggested a correlation between the substrate electron acceptor parameter of this material and the number of adhered cells, since marble was the surface with the highest adhesion level and the highest electron acceptor values (28). Porosity is another property to take into account, since it is the most important factor of absorption and fluid transport in stone material (36) and it influences many physical properties of rocks (37). Given that marble has higher porosity than granite, this may have enhanced *Salmonella* Enteritidis adherence to the former material.

Since it was not possible to test silestones without incorporated triclosan, we cannot be sure that different performances between silestones and the other surfaces are reflecting triclosan action. Nevertheless, the results obtained are supported by previous findings that allow

us to make comments about the possible role of this antimicrobial agent upon *Salmonella* Enteritidis cells. Accordingly, the absence of significant differences between adhesion results of silestones and most of the other materials is supported by the fact that cells used for the adhesion assays were in stationary-phase, which are known to have a higher resistance to triclosan than cells in log-phase (38). Moreover, it has been reported that polymers impregnated with high concentrations of triclosan had accomplished just some initial slowing down of bacterial growth rates through the compound released to the liquid medium, while triclosan that remained immobilized in the material did not contribute to the antibacterial character of the polymer (39). It is then possible to infer that the release rate of triclosan from silestones to the surrounding media was too low to achieve a significant effect on *Salmonella* Enteritidis cells after only two hours of contact.

All strains were able to form biofilm on all surfaces tested, but total biomass amount was strain dependent and different for each strain on the different materials (Table 3.2.1). Marble was the surface on which most *Salmonella* Enteritidis strains were able to form more biofilm ($p < 0.05$). Granite had some higher OD values than those registered for SS and silestones, which were the materials with lower biofilm amounts. Differences between adhesion and total biofilm biomass results were not surprising, since it is already established that initial adhesion extent does not always correlate with biomass amount after biofilm development (11, 40, 41).

Results concerning *Salmonella* Enteritidis viability within biofilms have shown granite and marble to bear the highest numbers of viable cells and, in contrast with OD values, no significant differences were observed between these surfaces (Table 3.2.1). In turn, most biofilms formed on SS had higher cellular viability than biofilms formed on silestones, even though similar amounts of total biomass were found between these materials. Such observations confirm the importance of using different methods for biofilm analysis, as most authors have done (42, 43, 44, 45, 46, 47, 48), not only to get more information about the biofilms formed on each material but also to prevent erroneous interpretation and conclusions of results. It is also possible to deduce that different OD values reflect different biofilms constitution, which is in agreement with the fact that, although extracellular matrices are always present in biofilms, there is a huge diversity in their composition and in the timing of their synthesis. Furthermore, this diversity was found not only between biofilms formed by different species but also among biofilms formed by different strains of a single species (49).

Salmonella Enteritidis biofilms formed on silestones had the lowest numbers of viable cells ($p < 0.05$) indicating that, even though biofilm formation was able to take place on silestones, triclosan seems to play a role in inhibiting or retarding this biological process. It is also important to note that CFU enumeration does not detect viable but non-culturable (VNC) cells and that triclosan, as an antimicrobial agent, might induce that kind of cellular state. So, it must be taken into account that the actual total numbers of viable cells may be larger than those reported here. Although it was not possible to know the concentration of triclosan available at the silestones surface or within the biofilm, previous works had shown that at low concentrations triclosan has a bacteriostatic effect, while at higher concentrations it becomes bactericidal regardless of the bacterial phase of population growth (50, 51, 52). Moreover, the lethal activity of triclosan was found to be concentration and contact time dependent (50), which allows us to infer that during the 2 hours adhesion the active concentration of triclosan was too low to achieve a considerable effect upon *Salmonella* Enteritidis cells, while during the 48 hours period of biofilm formation concentrations became high enough to affect both biofilm growth and cellular viability.

3.2.5 General conclusions

Enumeration of adhered cells on granite, marble, stainless steel and silestones revealed that all materials were prone to bacterial colonization and no considerable effect of triclosan was observed. Conversely, results concerning biofilm formation highlighted a possible bacteriostatic activity of triclosan, since smaller amounts of *Salmonella* Enteritidis biofilms were formed on silestones and with significantly lower numbers of viable cells than those found on the other materials. Summarizing, all surfaces tested failed in promoting food safety and imply a cautious utilization with appropriate sanitation when used in food-processing environments. Nevertheless, triclosan gives silestones some advantage in controlling microbial contamination due to its bacteriostatic effect.

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Chapter 4

Listeria monocytogenes and
Salmonella enterica Enteritidis
biofilms susceptibility to different
disinfectants and stress-response and
virulence gene expression of surviving
cells

4.1 Introduction

Inadequate cleaning and disinfection of food processing environments is the cause of major economic losses and represents a serious danger to public health. In fact, several studies have shown that the presence of microorganisms on food contact surfaces is one of the most common causes of food spoilage and transmission of foodborne diseases (1, 2, 3, 4, 5, 6), and their ability to adhere and form biofilms makes disinfection even more difficult and challenging (7, 8, 9, 10, 11, 12). *L. monocytogenes* and *S. enterica* are two of the most common foodborne pathogens responsible for numerous disease outbreaks worldwide every year (13, 14, 15, 16, 17, 18, 19, 20, 21) and numerous authors have reported that both these bacteria have the ability to adhere and form biofilms on many different surfaces (22, 23, 24, 25, 26, 27). Moreover, the increased difficulty in eliminating adhered and biofilm forms of these microorganisms compared to planktonic cells has also been shown in several reports (24, 28, 29, 30, 31).

The bactericidal character of most commercial products used for surfaces cleaning and disinfection is mainly based on phenolic compounds, organic acids, alcohols, chlorine, quaternary ammonium compounds and iodophors, the efficacy of which has been reported to be higher against bacterial suspensions than against adhered cells and biofilms (32, 33, 34, 35, 36). This fact has raised the need to reformulate the standard procedures used to test disinfectants' efficacy in order to include adhered cells and biofilms as targets together with planktonic cells (28, 37, 38). Among the various different methods that have been used to study biofilm communities (39, 40, 41), the Calgary Biofilm Device (CBD) is a high-throughput microtitre plate-based technology for screening antimicrobial susceptibility of microbial biofilms (42). This is a very versatile and high-throughput technique that allows the determination of minimum biofilm eradication concentration (MBEC) of a wide range of products and compounds such as antibiotics, biocides, metals, and disinfectants (43, 44, 45, 46), and the reasons why it was the selected method to perform this work.

Another important issue related to surfaces disinfection is the acquisition of bacterial resistance to disinfectant agents and, furthermore, the possible relation between chemical biocides and the emergence of resistance to antibiotics. In fact, it has been thought that some biocides and antibiotics may have similar behaviours and characteristics in the way they act and in the way bacteria develop resistance to them (47, 48, 49). *L. monocytogenes* and *S. enterica* susceptibility and resistance to different kinds of antimicrobials has been widely studied, both in

planktonic cells (31, 50, 51, 52, 53, 54, 55, 56, 57) and biofilms (24, 31, 58, 59, 60, 61, 62, 63, 64, 65). However, the effect of disinfection challenge on the expression of stress-response and virulence genes in these bacteria has not been so extensively studied, since only a few reports are available on this theme and all of them concern only planktonic cells (66, 67, 68, 69, 70). Moreover, to the authors' knowledge there is no report on genetic expression analysis of *L. monocytogenes* or *S. enterica* biofilm cells after disinfection challenge. Since improved knowledge about the relation between exposure to decontaminants and genetic responses would provide additional information for cautious sanitizers usage in food processing environments, the aims of the present work were to evaluate *L. monocytogenes* and *S. enterica* biofilms susceptibility to four commonly used disinfectants, and to investigate how their action may alter surviving cells' stress-response and virulence genes expression.

4.2 Materials and methods

Bacterial Strains and Culture Conditions

In order to assess the behaviour of different strains from different sources, this work included three *L. monocytogenes* (food isolate 994, clinical isolate 1562 and reference strain CECT 4031^r) and three *S. enterica* Enteritidis strains (food isolate 355, clinical isolate CC and reference strain NCTC 13349). All isolates were kindly provided by Dr. Paula Teixeira (Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal). From a cryogenic stock at -70°C, strains were streaked out twice on trypticase soy agar (TSA, EMD Chemicals), and colonies were suspended in sterile saline (0.9 %) to match the optical density of a 0.5 McFarland standard. Suspensions were then diluted 1:30 in Mueller-Hinton II Broth cation adjusted (MH, Becton, Dickinson and Company) to a final concentration of $\approx 1.0 \times 10^7$ CFU/ml, which subsequently served as inocula for the assays. The starting cell number was always confirmed by plating 3 or 4 replicates of serial ten-fold dilutions of a sample of the inoculum.

Calgary Biofilm Device

The CBD was created in 1996 by microbiologists working at the University of Calgary and consists of a batch culture technique to grow 96 equivalent biofilms at a time (42, 71). It is commercially available as the MBEC™ physiology and genetics assay (Innovotech Inc., Edmonton, Alberta, Canada) and consists of 96 independent pegs mounted on the inside surface

of the lid of a 96-well microtiter plate. Each peg fits the corresponding well when the CBD is placed over a microtiter plate, without contacting the well surface, allowing microorganisms to grow as 96 identical biofilms. By placing the biofilms on the pegs into the wells of a microtiter plate, it is possible to assess an array of antimicrobial compounds with varying concentrations.

Biofilm Formation

Single strain biofilms were grown in CBD, the pegs of which were submerged in 200 μ l of inoculum placed in each well of the 96-well tissue culture plate. The device was placed on a gyratory shaker in a humidified incubator, where biofilms were left to grow at 37°C, for 24 h at 125 rpm. After this incubation period, culture medium was discarded and biofilms on the pegs were washed for 1 min using 200 μ l saline (0.9%) in each well of a microtiter plate. For biofilm growth control, 8 individual pegs were broken off the MBEC peg lid using sterile forceps, placed into 200 μ L of recovery medium (MH + Tween 1%) and sonicated for 8 min on high with an Aquasonic (model 250HT; VWR Scientific) (42) for biofilm disruption. Serial dilutions of the bacterial suspensions were made in 0.9% saline, plated on TSA and incubated for 24 - 48 h at 37°C for subsequent CFUs count. Final data, given as log CFU/peg, resulted from at least three independent experiments with 8 replicates each. It is important to note that all experimental conditions regarding biofilm formation were optimized to achieve a final biomass of 6 log CFU/peg for all biofilms, in order to have countable amounts of cells even after a 3 log reduction caused by the disinfection assays.

Biofilms Susceptibility Tests

Disinfectants and Neutralizer Preparation

Four disinfectants were chosen for this study: (1) sodium hypochlorite (SH) solution, 4.99% wt/v available chlorine, Sigma-Aldrich; (2) Polycide™ a commercial product in which the active agent is benzalkonium chloride (BAC) at 6.5% w/v, Pharmax Limited; (3) hydrogen peroxide (HP) 30% wt/v solution in water, Sigma-Aldrich; and (4) triclosan, Sigma-Aldrich. Working solutions were prepared fresh at maximum concentrations of 800 μ g/ml for SH and BAC, 90 mg/ml for HP, and 4000 μ g/ml for triclosan. To inactivate disinfectants after biofilms challenge, a universal neutralizer was used composed of L-histidine (Sigma Aldrich), L-cysteine (Sigma Aldrich) and reduced glutathione (Sigma Aldrich) dissolved in double distilled water. For

each disinfection challenge, a fresh solution of recovery medium + neutralizer was prepared by adding 1 volume of universal neutralizer per 40 volumes of recovery medium.

Disinfection Challenge

For disinfection assays, after identical biofilms were formed as described above, biofilms were washed for 1 min with 0.9% saline to remove free cells. The disinfection challenge was then performed by submerging the biofilms in the wells of 96-well tissue culture plates containing disinfectants solutions serially diluted (twofold) in phosphate buffer solution (PBS) for 15 min, at room temperature and without agitation. The pegs were then washed for 1 min with 0.9% saline to remove residual disinfectant solution and incubated for 1 min with the recovery medium + neutralizer (prepared as mentioned above) to inactivate the disinfectants. In the same plate, biofilms were sonicated for 8 min to promote disruption and recovery of surviving cells. Bacterial suspensions dilutions and CFUs/peg counts were performed as described above.

Since for a disinfectant agent to be considered effective against adhered and biofilm cells it has to reach a 3 log units reduction (72), only the cells from biofilms that suffered such viability reduction were collected for later genetic expression analysis, as well as the corresponding biofilm cells that were not exposed to disinfection challenge (control). Moreover, for each bacterial species only the most resistant strain to each disinfectant was selected for gene expression analysis. When different strains had the same MBEC value, the strain with the highest log CFU/peg value at the concentration immediately below MBEC was selected (data not shown). Collected cells were stored at -80°C in microtubes containing 500 µl of RNAlater® solution (Ambion, Canada).

Genetic Expression Analysis

Primer Design

Primers used for *L. monocytogenes* and *S. enterica* stress-response and virulence genes analysis by quantitative real time-PCR (qPCR) were designed using the software Primer 3 (73) and are listed in Table 4.1. In order to verify the specificity of each primer pair for its corresponding target gene, PCR products were first amplified from genomic DNA (data not shown).

Table 4.1 Primers used for the assessment of gene expression by qPCR.

Bacteria	Gene	Sequence (5'- 3')	Product size (bp)
<i>L. monocytogenes</i>	<i>cplC</i>	F: CTTGGACCTACTGGTGTTG R: TTGCCGAACTTTTCTGTC	197
	<i>prfA</i>	F: GGTAGCCTGTTTCGCTAATGA R: TAACCAATGGGATCCACAAG	193
	<i>16S rRNA</i>	F: GGAGCATGTGGTTTAATTCG R: CCAACTAAATGCTGGCAACT	199
<i>S. enterica</i> Enteritidis	<i>ropS</i>	F: GAATCTGACGAACACGCTCA R: CCACGCAAGATGACGATATG	171
	<i>avrA</i>	F: GAGCTGCTTTGGTCCTCAAC R: AATGGAAGGCGTTGAATCTG	173
	<i>16S rRNA</i>	F: CAGAAGAAGCACCGGCTAAC R: GACTCAAGCCTGCCAGTTTC	167

RNA Extraction

Total ribonucleic acid (RNA) of each sample was extracted using the PureLink™ RNA Mini Kit (Invitrogen) according to manufacturer’s recommended protocol. Potential DNA contamination was removed during RNA purification procedure by On-column PureLink™ DNase treatment (Invitrogen). RNA concentration (ng/μl) and purity (OD_{260nm}/OD_{280nm}) were assessed by spectrophotometric measurement using a NanoDrop device (NanoDrop 1000 Spectrophotometer, V3.6.0, Thermo Fisher Scientific, Inc.).

cDNA Synthesis

To ensure equivalent starting amounts of RNA from control and respective treated samples to be converted to complementary DNA (cDNA), the proper dilutions in RNase-free water were performed. cDNA of each sample was synthesized using the iScript™cDNA Synthesis Kit (BioRad). Each reaction contained 2.5 μl of iScript Reaction Mix + iScript Reverse Transcriptase and 7.5 μl of RNA template, respecting the proportions recommended by the kit manufacturer in a final reaction volume of 10 μl. Complete reaction mix was incubated in a thermocycler (MyCycler™ Thermal Cycler, BioRad) with the following reaction protocol: 5 min at 25°C, 30 min at 42°C and 5 min at 85°C.

Quantitative Real-Time Polymerase Chain Reaction

qPCR reactions were performed on a CFX96™ Real-Time PCR Detection System Bio-Rad system (Bio-Rad Laboratories, Inc.). Each 20 µl of reaction mixture contained 2 µl of cDNA (diluted 1:20 from the cDNA synthesis reaction), 1 µl of each primer, 10 µl of 2x SSoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc.), and 6 µl of nuclease-free water. Thermal cycling conditions were as follows: 3 min initial denaturation at 95°C, followed by 40 cycles of 10 s denaturation at 95°C, 10 s annealing at 50°C (for *L. monocytogenes* samples this step was performed at 53°C, concerning primers efficiency previously determined – data not shown) and a 15 s extension at 72°C. A melt curve was performed at the end of each run, with readings from 65°C to 95°C every 1°C for 5 s, in order to confirm that only the desired product was amplified.

Gene Analysis and Expression

Samples for qPCR reactions were run in triplicate. Data were analysed using the Bio-Rad CFX Manager™ version 1.6 (Bio-Rad Laboratories, Inc.) and the relative quantification method ($2^{-\Delta\Delta_{CT}}$; (74), which describes the change in expression of the target genes relative to the 16S ribosomal RNA (rRNA) reference genes from untreated control samples (75, 76). Data were analysed by averaging the CT values (cycle at which each sample amplification curve crosses a specific threshold) for triplicate samples. The ΔCT values of the target genes were determined by normalizing to the endogenous control genes 16S rRNA. These samples were subsequently subtracted from the 16S rRNA genes from the untreated control samples. The $\Delta\Delta CT$ was used to calculate relative expression using the formula $2^{-\Delta\Delta_{CT}}$ (74, 77, 78). No-reverse transcriptase (no-RT) controls - RNA samples not submitted to the reverse transcriptase reaction – were used in order to check for possible DNA contamination. All no-RT controls showed $\Delta\Delta CT$ values above 10 cycles, confirming the quality and purity of cDNA.

Statistical Analysis

qPCR data were analysed by means of the Student's t-test, at a 95% confidence level, using the statistical program SPSS (Statistical Package for the Social Sciences).

4.3 Results

Minimum Biofilm Eradication Concentration

Results of biofilms susceptibility to each disinfectant presented in Table 4.2 revealed SH to have the lowest MBEC values for all biofilms tested, ranging from 3.125 to 12.5 µg/ml. On the other hand, the lower susceptibility was found in disinfection with triclosan, since it did not eradicate any of *S. enterica* biofilms even at the maximum concentration used (4000 µg/ml). An intermediate susceptibility to BAC was found comparatively with the other compounds, with notably higher MBEC values than SH but considerably lower than those registered for HP and triclosan.

Table 4.2 MBEC values of each disinfectant agent

Strains	SH (µg / ml)	BAC (µg / ml)	HP (mg / ml)	Triclosan (µg / ml)
<i>Listeria monocytogenes</i>				
994	3.1	100.0	22.5	500.0
1562	6.3	50.0	45.0	500.0
CECT4031^r	3.1	6.3	11.3	250.0
<i>Salmonella enterica</i> Enteritidis				
355	6.3	100.0	5.6	> 4000
CC	12.5	400.0	90.0	> 4000
NCTC13349	6.3	100.0	90.0	> 4000

Intraspecies variability was found to influence the response to each chemical agent, with some strains being predominantly more resistant to disinfection while others were more susceptible. In this way, *L. monocytogenes* clinical isolate 1562 and *S. enterica* clinical isolate CC were the most resistant strains to SH and HP, and SH and BAC, respectively. On the other hand, *L. monocytogenes* collection strain CECT 4031^r was the most susceptible to BAC, HP and triclosan actions, while among *S. enterica* strains only food isolate 355 revealed a lower MBEC

value concerning disinfection with HP (Table 4.2). Interspecies variability was also observed since, taking into account the average results of related strains in the same disinfection challenge, MBEC values against *L. monocytogenes* biofilms were inferior to those registered for *S. enterica*.

Stress-response and Virulence Gene Expression

Results concerning gene expression by the most resistant *L. monocytogenes* and *S. enterica* strains to each disinfectant agent are presented in Figure 4.1a-b and Figure 4.1c-e, respectively. It was chosen to present these results graphically and per strain in order to enable an easier and faster visualization of how each disinfectant has affected genetic expression. The first finding was that none of *L. monocytogenes* strains expressed the virulence gene *prfA* under any condition, neither before nor after disinfection (Figure 4.1a-b), although its presence in genomic DNA was previously confirmed by PCR, as stated above. The same was also observed concerning expression of *rpoS* stress-response gene by *S. enterica* 355, before and after challenge with triclosan (Figure 4.1c). In this way, only alterations of stress expression were registered for *L. monocytogenes* strains showing that, except for triclosan, all disinfectants lead to a significant increase of *cpfC* gene expression by food isolate 994 and clinical isolate 1562. Stress expression by *S. enterica* strains was only notably altered in NCTC 13349 surviving cells after disinfection with HP (Figure 4.1e), while CC biofilms treated with SH and BAC did not suffer significant alterations of *rpoS* gene expression (Figure 4.1d). Except for SH, all disinfectants tested lead to a significant increase of virulence expression by *S. enterica* biofilm surviving cells, with triclosan promoting the highest increment on *avrA* expression, followed by HP and, finally, BAC.

The overall results showed HP to be the disinfecting agent with more effect on stress-response and virulence gene expression, followed by BAC, while SH had only affected stress expression by *L. monocytogenes* surviving cells. Triclosan was the only disinfectant that did not interfere with *cpfC* gene expression but, on the other hand, it was responsible for the highest *avrA* up-regulation in *S. enterica* surviving cells.

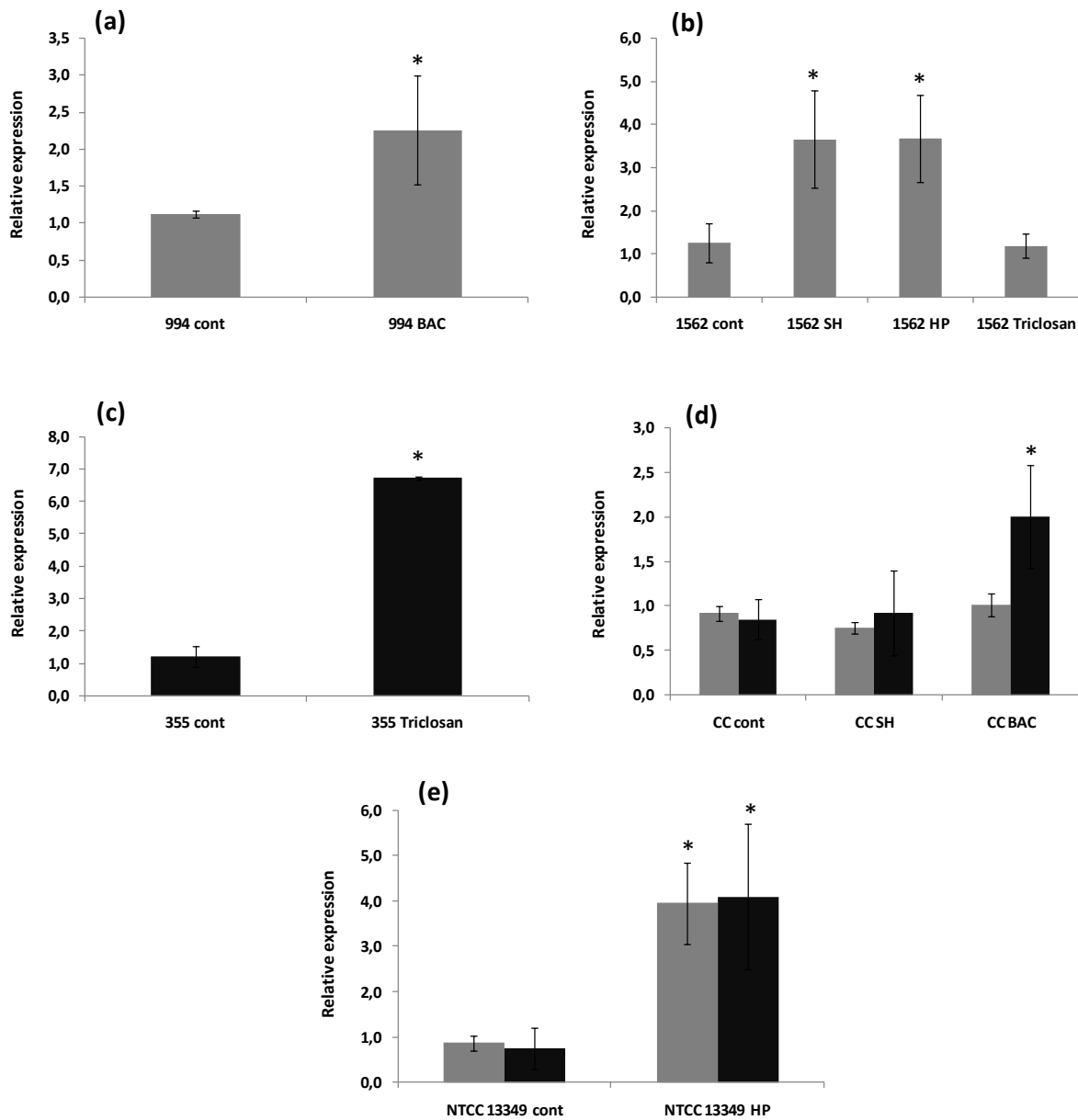


Figure 4.1 Genetic expression analysis of *L. monocytogenes* and *S. enterica* biofilm cells. The relative expression of stress-response (■) and virulence (■) genes was assessed by qPCR using biofilm cells of the most resistant strains to each disinfectant, namely (a) *L. monocytogenes* strains 994 and (b) 1562, and *S. enterica* strains (c) 355, (d) CC and (e) NCTC 13349. Abbreviations BAC, SH and HP stand for benzalkonium chloride, sodium hypochlorite and hydrogen peroxide, respectively. Symbol * indicates significantly different values ($p < 0.05$) when comparing the relative expression of control (cont) and surviving biofilm cells.

4.4 Discussion

Biofilms have been pointed out as a possible source of persistent contamination in food processing environments, being very difficult to control and leading to premature product deterioration or postprocess contamination with pathogens (79). Among foodborne pathogens, *L. monocytogenes* and *S. enterica* are two of the most common and dangerous to public health. Although their biofilm resistances to sanitizers have been largely reported (e.g., 31, 80), only a few studies have been done regarding the effect of disinfection on genetic expression by such bacteria, and all of them concern only planktonic cells (66, 67, 68, 69, 70). In order to improve knowledge about biofilms' susceptibility to disinfectants and gain some insights about the effect of disinfection on stress-response and virulence gene expression by biofilm surviving cells, this work evaluated *L. monocytogenes* and *S. enterica* biofilms susceptibility to four commonly used disinfectant agents, and analysed stress and virulence expression by the surviving cells.

Biofilms from both bacterial species were more susceptible to SH than to any other disinfectant tested. Moreover, all SH MBEC values were way below the in use recommended concentration (200 µg/ml), ranging between 3.13 and 12.5 µg/ml. This biocidal agent is a chlorine compound used as a disinfectant, the bactericidal effect of which is based on the penetration of the chemical and its oxidative action on essential enzymes in the cell (81). The antimicrobial efficiency of SH has been reported against most bacteria, but it is also known to be very active in killing fungi and viruses, and is a strong oxidizing agent (36). As far as biofilm disinfection is concerned, its efficacy might be related to the fact that, as a chlorine compound, it has the ability to depolymerise biofilms' matrix EPS (82), thereby interfering with the integrity and stability of those microbial communities, making them more susceptible to the chemical disinfection.

On the other hand, *S. enterica* biofilms were resistant to triclosan, since this was the only disinfectant tested that did not achieve biofilm eradication. This compound is a bisphenol antimicrobial agent that has a broad range of activity (83), being used as a preservative, antiseptic and disinfectant in a diverse range of products (84). This biocide is also one of the most commonly used compounds that are frequently applied to control bacterial contamination in domestic settings and during food processing (85). In this study, a concentration range of 4000 - 1.95 µg/ml was used based on the fact that triclosan was been reported to be bacteriostatic at concentrations ranging between 0.025 and 100 µg/ml, and bactericidal at higher levels (86, 87, 88). Although MBEC values concerning *L. monocytogenes* biofilms varied

between 250 and 500 µg/ml, no *S. enterica* biofilms eradication was achieved by triclosan even at the maximum concentration used. This performance disparity concerning the two bacterial species used might be due to the fact that Gram-negative bacteria use multiple mechanisms to develop resistance to this antimicrobial agent, including mutations in the enoyl reductase, alteration of the cell envelope and expression of triclosan-degradative enzymes (89, 90). Moreover, it has been described that the main physiological change resulting from adaptation to triclosan in *Salmonella* is the over-expression of efflux pumps (91, 92). So, it is likely that at least some of these defensive mechanisms were taking place in *S. enterica* biofilm cells during disinfection and, thus, had prevented biofilm eradication. Although higher triclosan concentration could be tested in order to determine its MBEC values against *S. enterica* biofilms, it was reported that even a concentration of 20,000 µg/ml might not be effective in killing *Salmonella*, particularly not within biofilms (93), which emphasizes the importance to reconsider the antimicrobial efficacy of this compound against bacterial biofilms when incorporated into products such as kitchen utensils, dishwashing liquids and food storage containers.

Although not so susceptible as to SH, *L. monocytogenes* and *S. enterica* biofilms were also susceptible to BAC; most MBEC values were within the in-use recommended concentration for quaternary ammonium compounds (QACs) - 200 µg/ml. BAC is a nitrogen-based surface-active QAC with a broad-spectrum antimicrobial activity, commonly used as a cationic surfactant and disinfectant for processing lines and surfaces in the food industry. Due to their positive charge, QACs form electrostatic bonds with negatively charged sites on bacterial cell walls, destabilizing the cell wall and cytoplasmic membrane, which leads to cell lysis, leakage and death (94, 95). These compounds are known to be bacteriostatic at low concentrations and bactericidal at high concentrations (96), and have been reported to be ineffective against most Gram-negative microorganisms (37, 97, 98), with *Salmonella* being one of the few exceptions. Accordingly, overall results obtained in this work showed a higher susceptibility of *L. monocytogenes* to BAC compared to *S. enterica* biofilms, although all *S. enterica* biofilms were also eradicated by this chemical agent, with only one case (CC strain) requiring a higher BAC concentration than that generally recommended.

Susceptibility tests performed with HP showed that some of its MBEC values were much higher than the 3% concentration that is generally present in disinfectants for surface wiping (99). This chemical agent is known to be a very powerful oxidizing agent, being effective against a wide spectrum of microorganisms including bacteria, yeasts, molds, viruses and spore-forming

organisms (100). It acts as a disinfectant by producing reactive oxygen species (hydroxyl radicals, superoxide anions), which attack essential cell components such as DNA, lipids and proteins (99). Although the effectiveness of peroxides against biofilms has been recognized, previous reports have also shown that HP elicited a significant microbial reduction only at concentration ranges way above the target concentrations in the commercial mixtures (101, 102, 103, 104).

Having determined the MBEC of each disinfectant tested, and identified the respective *L. monocytogenes* and *S. enterica* most resistant strains, the expression of stress-response and virulence genes was analysed as a way to gain some new insights about the effect of disinfection on gene regulation in biofilm cells. In order to do so, a stress-response gene and a virulence gene of each bacterial species were chosen, and their expression compared between control and biofilm disinfection surviving cells. The first finding was that both control and surviving biofilm cells from the *L. monocytogenes* strains analysed did not express the selected virulence gene – *prfA* – under the conditions studied; it was also possible that the expression was below the limit of detection of the assay. This specific gene is the transcriptional activator of the main virulence genes of *L. monocytogenes* (105, 106, 107, 108), with the known PrfA-regulated products including surface proteins involved in host cell invasion and cell-to-cell spread, secreted membrane-damaging factors mediating escape from the phagocytic vacuole and a transporter by which *Listeria* steal sugar phosphates that mediates rapid growth in the host cytosol (105, 109, 110, 111). While it is clear that PrfA is a key regulatory element required for the control of virulence gene expression in *L. monocytogenes*, it is not clear what controls its activity or how *prfA* expression is regulated. Nevertheless, it has been reported that the regulation of PrfA and virulence gene expression is influenced by several environmental factors. One example is the temperature-dependent control of translation of the *prfA* messenger, which is processed only at 37°C and not at 30°C (112, 113). In the present work, although biofilm were grown at 37°C, disinfection challenges and collection of cells were performed at room temperature, which could be a reason why *prfA* expression was not detected. Moreover, intraspecies genetic expression variability is also another factor that may have caused this result, since it has been shown that genes with important functions can vary in their expression levels between strains grown under identical conditions (114). This intraspecies variability is also the reason why there is always the possibility that a reagent may be effective with some strains of an organism and not with others. In general, and although no further considerations can be made regarding virulence of *L. monocytogenes* biofilm cells assessed, it can be said that disinfection with all disinfectants tested

in this work did not significantly affect the expression of one of the main transcription factors that controls key virulence determinants of this pathogen.

On the other hand, disinfectants' actions lead to significant differences concerning the expression of stress-response genes by both bacterial species. As far as *L. monocytogenes* *cpIC* gene is concerned, up-regulations of almost three-fold concerning SH and HP action, and two-fold concerning BAC action were observed. In contrast, triclosan was the only disinfectant that did not interfere with *cpIC* expression. This gene encodes a protein (CpIC ATPase) that is produced under stress conditions and that promotes early bacterial escape from the phagosome of macrophages, enhancing intracellular surviving (115). So, SH, HP and BAC actions upon *L. monocytogenes* biofilm cells may have triggered the same kind of stress conditions as those experienced by bacterial cells when inside a phagosome. In fact, one of the antimicrobial functions of phagocytic cells has been classified as an oxygen-dependent mechanism, which results in the generation of reactive oxygen molecules such as superoxide anion, hydroxyl radicals, hypochlorite ion, hydrogen peroxide, and singlet oxygen within a phagosome. Accordingly, and as stated above, the mechanisms of action of SH and HP are mainly based on oxidative action, producing reactive oxygen species that attack essential cell components. In contrast, BAC acts mostly at the bacterial cells' wall and cytoplasmic membrane, destabilizing them and leading to death through cell lysis. A similar threat is presented to *L. monocytogenes* inside a phagosome where, among the antimicrobial proteins that take part in the attack against the intruder, lysozyme acts directly on the bacterial cell wall proteoglycans present especially in the exposed cell wall of Gram-positive bacteria (116). Moreover, it is known that *L. monocytogenes* escape from the phagosome occurs within 30 minutes following phagocytosis (117), which means that this bacterium is able to rapidly respond to the stress condition implied by the anti-microbial attack by the macrophage and, thus, must be able to do the same within 15 min of disinfection challenge.

Regarding the genetic analysis of *S. enterica* biofilms, the expression of the stress-response gene *rpoS* was only significantly increased after disinfection with HP. This gene is the general stress response regulator sigma factor, being required for survival of bacteria under starvation and stress conditions (118, 119, 120), and is also related with the regulation of adhesins (121) and other genes (120, 122). Moreover, *rpoS* has been reported to play an important role in biofilm formation (123), which infers that its up-regulation after treatment with HP may be a response to the damage caused by the free radicals produced by this chemical agent in the biofilm matrix (38). Among *S. enterica* biofilms that were genetically analysed, those

formed by strain 355 were the only ones that did not express the *rpoS* gene. As stated above concerning *prfA* gene expression, interspecies gene expression variability is a likely reason of this occurrence.

Finally, the analysis of *avrA* gene expression by *S. enterica* biofilms showed that disinfection with triclosan, HP and BAC lead to significant up-regulations of about 6-, 5- and 2-fold, respectively, compared to controls. However, SH was the only disinfectant that did not promote notable modifications on the expression of this gene. *avrA* is a virulence-associated gene located within *Salmonella* pathogenicity island 1 - which is necessary for the invasion of epithelial cells and induction of macrophage apoptosis (124, 125, 126) -, and is involved in the induction of programmed cell death and the inflammatory response of hosts against infection (127). The substantial up-regulation of this gene observed after treatment with triclosan is in agreement with a previous study that reported *S. typhimurium* biofilms response to this antimicrobial to include changes of gene expression (93). In this way, our results not only corroborate these previous findings but also highlight that such bacterial response is not exclusively triggered by triclosan, since the same kind of genetic alteration was observed regarding *S. enterica* biofilms disinfection with HP and BAC.

4.5 General conclusions

SH had the lowest MBEC values, while triclosan had the worst performance since no *S. enterica* biofilm eradication was achieved even at the maximum concentration used. Both intraspecies and interspecies variability were found to influence disinfection efficacy, and most MBEC values related to *L. monocytogenes* were lower than those found for *S. enterica*. In general, *L. monocytogenes* stress-response gene and *S. enterica* virulence gene were significantly up-regulated in surviving cells when compared to bacteria not subjected to disinfection challenge. Although ineffective on eradicating *S. enterica* biofilms at the concentrations tested, triclosan lead to the highest increase in their virulence expression, while HP had also significantly increased virulence and/or stress-response gene expression, depending on the bacterial species. On the whole, this work showed SH to be the most effective disinfectant against biofilms of both species used, and *L. monocytogenes* biofilms to be more susceptible to disinfection than *S. enterica* biofilms. Moreover, it was found that, even at concentrations considered effective for biofilm elimination (3 log reduction), disinfection surviving cells seem to develop a stress response

and/or become more virulent, which may compromise food safety and represent a potentially increased risk for public health.

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Chapter 5

Main conclusions & Suggestions for future work

In this last chapter the most important conclusions drawn from the present thesis are addressed. Also, considering the conclusions of the work developed, some suggestions for future research in this field are given.

5.1 Main conclusions

The aim of the present thesis was to improve the knowledge about the phenomena involved in foodborne contaminations caused by *Listeria monocytogenes* and *Salmonella enterica* Enteritidis, particularly regarding biofilm formation ability and the effect of different antimicrobial challenges. In order to achieve these goals, several aspects were studied throughout, namely: the influence of different growth modes at different temperatures on the biofilm formation by *L. monocytogenes* (**a**); bacterial adhesion and biofilm formation on materials with antimicrobial properties, namely glass and stainless steel coated with nitrogen-doped titanium dioxide (**b**) and triclosan incorporated bench cover stones (**c**). Lastly, a study was carried out regarding chemical disinfection in order to evaluate the susceptibility of biofilms formed by both bacteria to different antimicrobial agents, and analyse the genetic expression of the surviving cells (**d**). The main conclusions that can be extracted from the work presented are the following:

- a)** In long term assays (longer than 2 days) fed-batch conditions were the most prone to promote biofilm formation by *L. monocytogenes* on polystyrene when high incubation temperatures are used, while in a refrigerated environment it was batch mode that enhanced a higher biomass formation. Moreover, the growth mode applied also affected the metabolic activity of cells within biofilms, since fed-batch mode lead to biofilms metabolically more active at all temperatures. So, when assessing biofilm formation by *L. monocytogenes* strains on such abiotic surfaces, it should be recognized that different growth modes do lead to divergent results determining the extent to which a strain will produce biofilm and influencing the metabolic activity of biofilms' constituent cells.

- b)** Photocatalytic reactions induced by visible light on glass and stainless steel surfaces coated with N-TiO₂ were effective in killing *L. monocytogenes*. Moreover, the comparison between the two most commonly used indoor light sources showed a better capability of incandescent light on promoting photocatalytic disinfection than fluorescent light. In this way, this study has contributed to the interesting and important field of investigation that approaches different photocatalytic surface coatings, lights' performance and microorganisms' susceptibility as an attempt to improve visible light photocatalytic disinfection. In fact, this sanitation tool not only is appropriate for indoor environments

but is also safer and more cost effective than disinfection using UV and chemical agents, which imply hazardous irradiation and byproducts production, respectively. So, although not yet as effective as that induced by UV-light irradiation, N-TiO₂ coated surfaces' disinfection through visible light still remains a valid tool in food protection and cross-contamination control that can be applied on both domestic and industrial food-processing environments.

- c)** All surfaces tested - regular and triclosan incorporated - were prone to bacterial colonization and biofilm development by *S. enterica* Enteritidis, although different materials had different biofilm biomass amounts and viable cell counts. Viability results revealed granite and marble to have the highest numbers of viable cells, whereas silestones had less viable cells than both regular stones and stainless steel. Nevertheless, as far as food safety is concerned, silestones do not represent a significant improvement on food contact surfaces, since they are not able to prevent bacterial colonization, requiring a cautious and rigorous cleaning just like any other regular material. Thus, the pursuit of more secure materials to improve food-safety continues to be an actual need and a demanding challenge.
- d)** *L. monocytogenes* and *S. enterica* biofilms were more susceptible to sodium hypochlorite than to any other disinfectant tested, while all *S. enterica* biofilms were resistant to triclosan within the concentration range used. Save this case, all disinfection challenges were influenced by intra- and inter-species variability, as denoted by the different MBEC values observed after challenge with each disinfectant. Moreover, the overall results showed that the most resistant strains to each disinfection challenge had undergone genetic adjustments in terms of stress-response and/or virulence, depending on the bacterial species and strain. Consequently, the main finding of this work is the interesting and worrying fact that, even at concentrations that lead to significant reduction in biofilm biomass, disinfectants may induce virulence of the surviving cells and, thus, increase their infectious potential in case of contact with a host. Nevertheless, further studies including a wider range of target genes and disinfectants need to be studied in order to confirm these conclusions and to clarify which specific factors inherent to disinfection can be triggering the genetic changes of biofilm surviving cells.

5.2 Suggestions for future work

The work described in this thesis provided an insight into several aspects of *Listeria monocytogenes* and *Salmonella enterica* Enteritidis interaction with different conditions and materials, leading to interesting new questions for further research. Some of the suggestions that should be considered for future investigation are given below:

- Since most biofilms are found as mixed microbial cultures, and given the knowledge herein acquired about *L. monocytogenes* and *S. enterica* biofilms, it would be very interesting to study the general response to different antimicrobial challenges by mixed biofilms composed of different combinations of pathogens and other organisms (e.g., food spoilage organisms) commonly found in food processing environments, as well as the effects of such challenges on each of the bacterial species involved.
- Since surfaces are commonly exposed to some kind of abrasion during food processing and/or sanitation procedures, another suggestion would be the study of the process of *L. monocytogenes* and *S. enterica* Enteritidis bacterial adhesion and biofilm formation on worn surfaces, to mimic the conditions under which bacterial colonization normally takes place.
- Other materials used as food contact surfaces should be assayed, such as packaging materials and edible films, both with and without antimicrobial properties. Given their self-cleaning character, super-hydrophobic materials are another interesting surface to be addressed.
- Since it has been suggested that microorganisms resistant to biocides might also acquire resistance to antibiotics, the development of alternative disinfection methods involving the use of bacteriophages, enzymes and/or antimicrobial peptides, constitutes an attractive research challenge.