Microbial biofilms in food environments: study approaches and intervention strategies

A Ph.D. dissertation presented by

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to the

University of Udine

for the degree of Ph.D. in the subject of

Food Science (Cycle XXVI)

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UNIVERSITY OF UDINE

Italy

March 2014

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SUMMARY

The main objective of this study was to examine in-depth the problems related to the formation of microbial biofilms in food plants. Although it has long been known that the ability to form biofilms is a common trend in natural environments including food processing environments, most of the studies in the literature concern the medical context, where microbial biofilms are often the cause of serious hospital infections. In the food field, however, the interest in biofilms has only recently arisen, even though the formation of microbial biofilms appears to be the major cause of cross-contamination in food products. Currently, data on microbial communities in the different food processing areas are only limited, and the influence of environmental parameters on the characteristics of biofilms have been studied for only a few microbial species. This knowledge is, however, necessary for the development of intervention strategies for the prevention and removal of biofilms, which allows at the same time to obtain a high degree of hygienic-sanitary safety of the surfaces, as well as to reduce the impact conventional strategies have on the environment and on the safety of operators.

The study discussed in this thesis was carried out on specific groups of organisms known to be pathogenic or food spoilers, including *Listeria monocytogenes*, *Staphylococcus aureus* and *Pseudomonas* spp. The need for the availability of appropriate study models that allow to obtain, in as little time as possible, a high number of biofilm samples with homogeneous characteristics was addressed in Chapter 2, where the use of a microtiter plate system and a reactor able to grow microbial biofilms was evaluated on materials widely used in the food industry. In addition, to facilitate the microbiological laboratory activities, two techniques of plate counts were compared in order to highlight problems and benefits in the context of the study of biofilms for both. The microtiter plate assay and the CDC biofilm reactor assay showed to be sufficiently reliable and repeatable tools to produce a number of samples large enough to provide sufficient information on the ability of food microorganisms to produce biofilms under different operating conditions. Moreover, the drop plate method has proved particularly suitable, sufficiently accurate and reliable, as well as advantageous from the economic point of view, for the quantification of viable cells present in the biofilm.

The influence of environmental parameters on biofilm formation was studied in Chapter 3 and Chapter 4. In particular, in Chapter 3 the effect of the synergy of multiple parameters on the formation of biofilms in a static model system was studied, both in terms of quantification of total the biomass (dead and live cells and EPS matrix) and of the only evaluation of the cell count. The use of a Central Composite Design allowed to mimic the real environmental conditions in the food industry and to obtain the greatest amount of information limiting the number of experiments to be carried out. Therefore, useful data were obtained to increase the information in the literature about the synergistic effects of the environmental parameters on biofilm formation regarding the food sector. In Chapter 4 the effect of temperature on the adhesion and on the biofilm structure, as well as on resistance to disinfectants commonly used in the food industry sanitation plans was studied. The study showed that temperature significantly affects the kinetics

of adhesion, but also the cell density and the amount of EPS produced, and consequently the resistance to biocides. The use of CLSM technique for microscopic observation allowed the study of biofilms in undisturbed conditions, and thus it is well suited to a possible use during the biofilm growth. The evaluation of different strategies for the removal of biofilms was the subject of Chapter 5 and Chapter 6, in which conventional and non-conventional approaches were considered. A comparison between chemical, physical and biological treatments shows that a hurdle-approach, in which different strategies are used in sequence, could help in limiting the health and hygiene problems related to microbial biofilms in the production of foodstuffs. Finally, in Chapter 7 the gained knowledge was used to study the problem of biofilms in specific food contexts.

LIST OF BACTERIAL STRAINS USED IN THIS STUDY

Strain	Microbial species	Collection[a]	Source	Accession Number	ST[b]	Lineage	serotype	SEs-coding gene[c]
Lm_1	*Listeria monocytogenes*	DIAL	Ground beef	FJ774254.1	580	II	1/2c	n.a.
Lm_4	*Listeria monocytogenes*	DIAL	Sushi	CP002004.1	155	II	1/2a	n.a.
Lm_5	*Listeria monocytogenes*	DIAL	Cheese	CP001604.1	121	II	1/2a	n.a.
Lm_7	*Listeria monocytogenes*	DIAL	Poultry food	CP001604.1	4	I	4b	n.a.
Lm_23	*Listeria monocytogenes*	DIAL	Pork meat	CP002816.1	new			n.a.
Lm_29	*Listeria monocytogenes*	DIAL	Chopping board	CP002816.1	320	II	2a	n.a.
Lm_174	*Listeria monocytogenes*	CESA	Slaughtering equipment	CP001604.1	363	I	1/2b	n.a.
Lm_254	*Listeria monocytogenes*	CESA	Conveyor belt	CP001175.1	204	II	2a	n.a.
Lm_278	*Listeria monocytogenes*	CESA	Working surface	CP001604.1	121	II	1/2a	n.a.
Lm_284	*Listeria monocytogenes*	CESA	Fish plant surface	CP001604.1	204	II	2a	n.a.
Lm_287	*Listeria monocytogenes*	CESA	Fish plant surface	CP001604.1	4	I	4b	n.a.
Lm_288	*Listeria monocytogenes*	CESA	Ham processing equipment	JF967620.1	9	I	4b	n.a.
Lm_SB	*Listeria monocytogenes*	DIAL	Cow milk	FJ774256.1	6	I	4b	n.a.
Lm_1E	*Listeria monocytogenes*	DIAL	Dairy product	CP002816.1	37	II	1/2a	n.a.
Lm_5E	*Listeria monocytogenes*	DIAL	Dairy product	CP002816.1	4	I	4b	n.a.
Lm_6E	*Listeria monocytogenes*	DIAL	Dairy product	JF967201.1	26	II	1/2a	n.a.
Lm_Scott A	*Listeria monocytogenes*	n.a.	Listeriosis outbreak[1]	CM001159.1	2	I	4b	n.a.
St_037	*Staphylococcus aureus*[d]	DIAL	Food handler	DQ647044.1	n.a.*	n.a.	n.a.	seb
St_059	*Staphylococcus aureus*	DIAL	Food handler	DQ647042.1	n.a.	n.a.	n.a.	-
St_117	*Staphylococcus aureus*	DIAL	Cow milk	JX560992.1	n.a.	n.a.	n.a.	sea, sed
St_132	*Staphylococcus aureus*	DIAL	Food handler	EF463060.1	n.a.	n.a.	n.a.	see
St_137	*Staphylococcus aureus*	DIAL	Food handler	JN390831.1	n.a.	n.a.	n.a.	-
St_174	*Staphylococcus aureus*	DIAL	Food handler	JN315154.1	n.a.	n.a.	n.a.	-
St_231	*Staphylococcus aureus*	DIAL	Ready-to-eat food	JN315149.1	n.a.	n.a.	n.a.	seb, sec
St_DSMZ20231	*Staphylococcus aureus*	DSMZ	Human pleural fluid	EU259819.1	n.a.	n.a.	n.a.	-
Ps_019	*Pseudomonas fluorescens*	DIAL	Raw milk	GU198116.1	n.a.	n.a.	n.a.	n.a.
Ps_053	*Pseudomonas fragi*	DIAL	Raw milk	GU549487.1	n.a.	n.a.	n.a.	n.a.
Ps_071	*Pseudomonas putida*	DIAL	Mozzarella cheese	GU060497.1	n.a.	n.a.	n.a.	n.a.

[a]DIAL, Department of Food Science, Udine, Italy; CESA, Center of Excellence of Aging, University of Chieti-Pescara, Italy; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany)

[b]Sequence Type as assessed by Multilocus Sequence Typing (MLST) by Department of Comparative Biomedicine and Food Science, University of Padova, Italy

[c]presence of Staphylococcal Enterotoxin (SE) coding genes as assessed by PCR ([2]Johnson et al., 1991)

[d]the diversity within the *Staphylococcus aureus* strains was assessed by RAPD-PDR using M13 primer ([3]Pinto et al., 2005)

[*]n.a., not applicable

The strains were identified by sequencing a part of 16S rRNA gene according to Carraro et al. ([4]2011). The stock cultures were maintained in Tryptone Soya Broth (TSB, Oxoid, Italy) added with 30% glycerol at -80 °C. For each test, the inocula were performed culturing each strain overnight in TSB at 30 °C for *Pseudomonas* sp. and at 37 °C for *L. monocytogenes* and *S. aureus*. At the end of incubation, the viable counts were evaluated by plate count assay in Tryptone Soya Agar (TSA, Oxoid, Italy) plates.

[1] Fleming, D. W., Cochi, S. L., MacDonald, K. L., Brondum, J., Hayes, P. S., Plikaytis, Holmes, M.B., Audurier, A., Broome, C.V., Reingold, A. L. 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. New England J Medicine 312, 404-407

[2] Johnson W., Tyler, M.S., Ewan, S.D., Ashton, E.P., Polland, F.E., Rozee, K.R. 1991. Detection of genes for enterotoxins, exfoliative toxins and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by polymerase chain reaction. J Clin Microbiol 29, 426-430

[3] Pinto, B., Chenoll, E., Aznar, R. 2005. Identification and typing of food-borne *Staphylococcus aureus* by PCR-based techniques. Syst Appl Microbiol 28, 340-352

[4] Carraro, L., Maifreni, M., Bartolomeoli, I., Martino, M. E., Novelli, E., Frigo, F., Marino, M., Cardazzo, B. (2011). Comparison of culture-dependent and-independent methods for bacterial community monitoring during Montasio cheese manufacturing. Res Microbiol 162, 231-239

LIST OF ABBREVIATIONS

BHI	Brain Heart Infusion (Oxoid, Milan, Italy)
CBR	CDC Biofilm Reactor
CFU	Colony Forming Unit
CIP	Cleaning In Place
CLSM	Confocal Laser Scanning Microscopy
eDNA	Extracellular DNA
EPS	Extracellular Polymeric Substances
GSFA	Gelatin Sugar Free Agar (Oxoid, Milan, Italy)
LB	Luria Bertani broth (10 g/L Tryptone; 5 g/L Yeast Extract; 5 g/L NaCl)
MRD	Maximum Recovery Diluent (Oxoid, Milan, Italy)
MRS-A	de Man Rogosa Sharpe Agar (Oxoid, Milan, Italy)
OD	Optical Density
PCA	Principal Component Analysis
PSA	Pseudomonas Agar Base (Oxoid, Milan, Italy)
PTFE	Polytetrafluoroethylene
TSA	Tryptone Soya Agar (Oxoid, Milan, Italy)
TSB	Tryptone Soya Broth (Oxoid, Milan, Italy)

Chapter 1. GENERAL INTRODUCTION

Microorganisms are traditionally studied, characterized and identified as planktonic, freely suspended cells and described on the basis of their growth characteristics in nutritionally rich culture media. Nowadays, however, all detailed studies of microbial communities in different environments have led to the conclusion that planktonic microbial growth rarely exists in nature. As a matter of fact, it is now accepted that it is a natural tendency of microorganisms to attach on wet surfaces, to multiply and to embed themselves in a matrix composed of extracellular polymeric substances (EPS) that they produce, forming the so called *biofilm*. Biofilms are defined as a matrix-enclosed bacterial populations which are attached to each other and/or to surfaces or interfaces (Costerton et al., 1995).

The inclination of bacteria to colonize surfaces is a double-edged sword that can prove either beneficial or potentially destructive. While nitrogen fixation and bioremediation of wastewater are beneficial functions of biofilms, the contamination of medical devices and of food equipment as well as the obstruction of fluid flow through conduits, over surfaces, through filter, and corrosion are major economic and public health risks of the medical and food field, as well as maritime and petroleum industries (Costerton et al., 1987; Carpentier and Cerf, 1993).

Biofilms can comprise single or multiple microbial species, and can be formed on a wide variety of surfaces, both biotic and abiotic, including living tissues, medical devices, industrial environment or natural aquatic systems (Donlan, 2002). Although mixed-species biofilms predominate in most environments, single-species biofilms exist in a variety of infections and on the surface of medical implants (Adal and Farr, 1996). Bacteria in biofilm (sessile form) profoundly differ from their free-floating (planktonic) counterparts. It has been shown that when microorganisms attach to a surface and adopt a sessile growth state, they show a modified gene expression which makes them phenotypically different from their planktonic counterparts. As a matter of fact, both up- and down-regulation of a number of genes of cells occurs during the attachment step of the biofilm formation upon initial interaction with the substratum. In this regard, Davies and Geesey (1995) demonstrated in *Pseudomonas aeruginosa*, the up-regulation of the *algC* gene for expression of essential enzyme for biosynthesis of alginate and a key point in the regulation of the alginate pathway. Prigent-Combaret et al. (1999) opined that the expression of genes in biofilms is evidently modulated by the dynamic physicochemical factors external to the cell and may involve complex regulatory pathways. Due to these different gene expressions, bacteria grown in a biofilm can be up to 1000 times more resistant to antibiotics, biocides and immune chemicals compared to the same bacteria grown in liquid culture (Gristina et al., 1987; Prosser et al., 1987). Therefore, it can be said that microorganisms prefer to live as sessile organisms as they will be protected from antimicrobial agents by the EPS matrix of their own synthesis whereby they are encased (Donlan, 2000).

1.1 HISTORICAL BASIS OF BIOFILM STUDY

Antonie van Leeuwenhoek first found microorganisms attached on tooth surfaces and forming sessile communities using his primitive light microscope, which could be considered as the first observation

of a microbial biofilm (Leeuwenhoek, 1684). The subsequent studies, which started from the 1920s, were related to marine bacteria on the surface of ship hulls. From these studies, it was found that for marine microbes, growth and activity were enhanced by the presence of a surface onto which they could adhere (the so called "bottle effect") (Heukelekian and Heller, 1940). However, only the electron microscope allowed a detailed examination of biofilms. By using a scanning and transmission electron microscopy and a specific polysaccharide-stain called ruthenium red, Jones et al. (1969) showed that the matrix material surrounding and enclosing cells in these biofilms was polysaccharidic. Costerton coined the term biofilm in 1978 explaining the mechanisms whereby microorganisms adhere to biotic and abiotic materials and the benefits accrued by this ecologic niche.

Over the last decades the study of biofilms has been based on the use of different techniques, such as scanning electron microscopy (SEM) or standard microbiologic culture techniques. Although biofilm formation has been a recognized and scientifically documented aspect of microbial physiology for more than 50 years, only in the recent years the utilization of the confocal laser scanning microscopy (CLSM) to characterize biofilm ultrastructure and the investigation of the genes involved in cell adhesion and biofilm formation help to better understand the microbial biofilm structure and development.

1.2 BIOFILM STRUCTURE

The biofilm structure has been the subject of several studies; information collected by these works allowed to propose three conceptual models of biofilm structure (Figure 1.1).

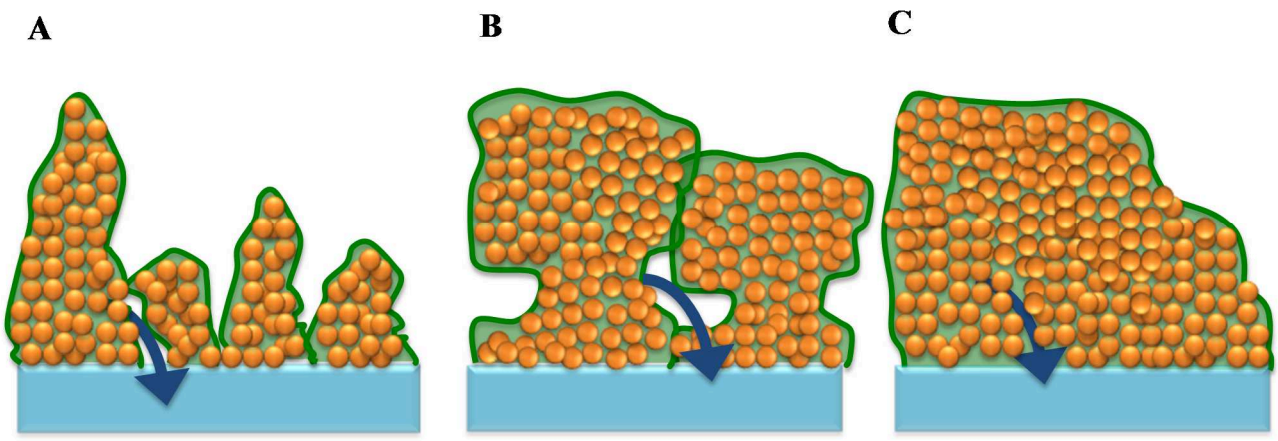

Figure 1.1 Schematic diagram of three distinct biofilm models, (A) comprising separated microbial stack, (B) penetrated water channel and (C) dense confluent structures. Microbial cells are in orange; the EPS matrix is in green; the solid surface is in light blue. The arrows indicate water channels.

The first is the heterogeneous mosaic model described by Walker et al. (1995), where the individual microbial stacks, well separated from one another, are surrounded by water. The second form is the water-channel model constructed by Costerton et al. (1994) in which microcolonies form mushroom-like structures may coalesce and are penetrated by branching water channels. The last one is a dense biofilm model

apparent in some medically important biofilms, where channels and fluid-filled voids were detectable (Lawrence et al., 1998). In all these systems, the water channels permit the flow of nutrients, enzymes, metabolites, waste products and other solutes, throughout the biofilm community. In the water channel, transport is facilitated with passive diffusion or with the help of water. Facilitated transport also aids in the transport of molecules to the inside of the biofilm. It is believed that the water channels participate in the transport of oxygen to the inner areas (Costerton et al., 1995). Although these models represent three distinct forms, in reality biofilms are a combination of all three, depending on many extrinsic factors. In particular, the structure is largely dependent on substratum concentration. Moreover, the presence of polysaccharide-synthesizing and -degrading enzymes in the biofilm means that the matrix composition will be constantly changing (Sutherland, 2001a).

1.3 THE BIOFILM MATRIX AND ITS FUNCTIONS

The biofilm matrix is the extracellular material, mostly produced by the organisms themselves, in which the biofilm cells are embedded (Figure 1.2) .

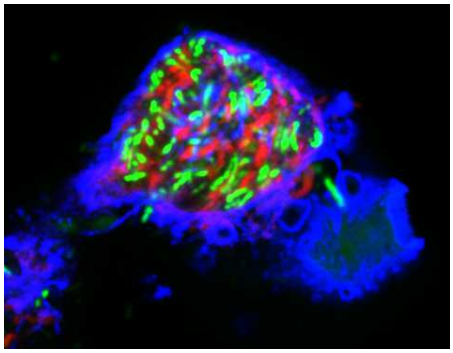

Figure 1.2 CLSM image of *Pseudomonas fluorescens* 5-days-old biofilm formed on stainless steel AISI 304. Biofilm matrix is in blue, viable cells are in green and dead cells are in red

Generally, it can be said that resident cells of biofilm, which may include many different species, only account for about 5% of the total biomass. The remaining part of the biofilm is composed of matrix, which has water as the major component, accounting for up to 97%. Apart from water, the other components of the matrix include, in varying amounts, EPS (1-2%), globular glycoproteins and proteins, which include lytic products and secreted enzymes (1-2%), extracellular DNA (eDNA) from lysed cells (1-2%), lipids, phospholipids and sequestered ions from the surrounding environment (Godwin and Foster, 1989; Flemming and Wingender, 2001). Recently it was found that eDNA is a significant component of EPS, as it plays a very important role in the biofilm development (Spoering and Gilmore, 2006). It is believed that eDNA is involved in maintaining the three-dimensional structures of biofilms and enhancing the exchange of genetic materials (Molin and Tolker-Nielsen, 2003). Although it is commonly accepted that eDNA is released manly from bacterial cell lysis (Webb et al., 2003), several studies have revealed that some active secretion mechanisms may exist (Draghi and Turner, 2006). However, Whitchurch et al. (2002) showed the possibility that eDNA is secreted actively via transport vescicles for the purpose of creating the biofilm

matrix. The EPS is regarded as the major structural component of the matrix, providing a framework for the biofilm complex. EPS may account for 50% to 90% of the total organic carbon of biofilms and may vary in chemical and physical properties, but it is primarily composed of homo- and heteropolysaccharides, in particular, of glucose, fructose, mannose, galactose, pyruvate and mannuronic acid- or glucoronic acid-based complexes (Johansen et al., 1997). Essentially, the EPS provide the skeleton into which microbial cells and their bioactive products are placed in. As a matter of fact, the EPS determine the immediate conditions of life of biofilm cells living in this microenvironment by affecting porosity, density, water content, charge, sorption properties, hydrophobicity and mechanical stability (Flemming and Wingender, 2002). EPS produced by the microorganisms vary depending on whether the microorganisms are Gram-negative or Gram-positive cells. Moreover, different organisms produce different amounts of EPS and the amount of EPS increases with age of the biofilm (Leriche et al., 2000).

As reported by Sutherland (2001a), the biofilm matrix composition is influenced by a combination of intrinsic factors, such as the genotype of the attached cells, and extrinsic factors, which include the surrounding physico-chemical environment. Moreover, since the biofilm matrix is constantly changing as it is influenced by changes in the surrounding macro-environment, it may be considered as dynamic. So, the specific composition for any biofilm varies depending upon the organisms present, their physiological status, the nature of the growth environment, the bulk fluid-flow dynamics, the substratum and the prevailing physical conditions. The biofilm matrix allows the resident microorganisms to form stable aggregates of different cell types, leading to the development of a functional, synergistic microconsortium. The spatial arrangement of microorganisms gives rise to nutrient and gaseous gradients, as well as those of electron acceptors, products and pH. Thus, aerobic and anaerobic habitats can arise in close proximity, and as a consequence, the development of large variability of species can occur.

The biofilm matrix performs several functions for the benefit of the cells within the biofilm itself. In general, it can be said that the biofilm matrix plays an important role in the structural stability due to the occurrence of non-covalent interactions (electrostatic interactions and hydrogen bonds). These interactions occur between the matrix components, in the attachment of cells to a surface thanks to the presence of the EPS that are involved in the initial adhesion events, and in the protection, as the matrix acts as a protective umbrella that physically prevents the access of antimicrobials to the cell surface (Allison, 2003). Another function of the biofilm matrix is the protection of the biofilm cells against dehydration under water-limited conditions, and other environmental conditions such as temperature fluctuations and osmotic shock thanks to the high water content of the matrix. The outer layer of EPS can dry out under water-deficient conditions and form a hard, protective layer, preventing dehydration of the inner cells (Sutherland, 2001b).

1.4 PROCESS OF BIOFILM FORMATION

The biofilm formation is a stepwise and dynamic process involving the initial attachment of the bacteria to a solid surface, the formation of micro-colonies on the surface, the differentiation of the micro-colonies into mature biofilms encased in exopolysaccharides and the consequent detachment (Figure 1.3).

The process of bacterial adhesion is controlled by a number of variables. These include the species of bacteria, environmental factors, essential gene products and the surface characteristics (Carpentier and Cerf, 1993).

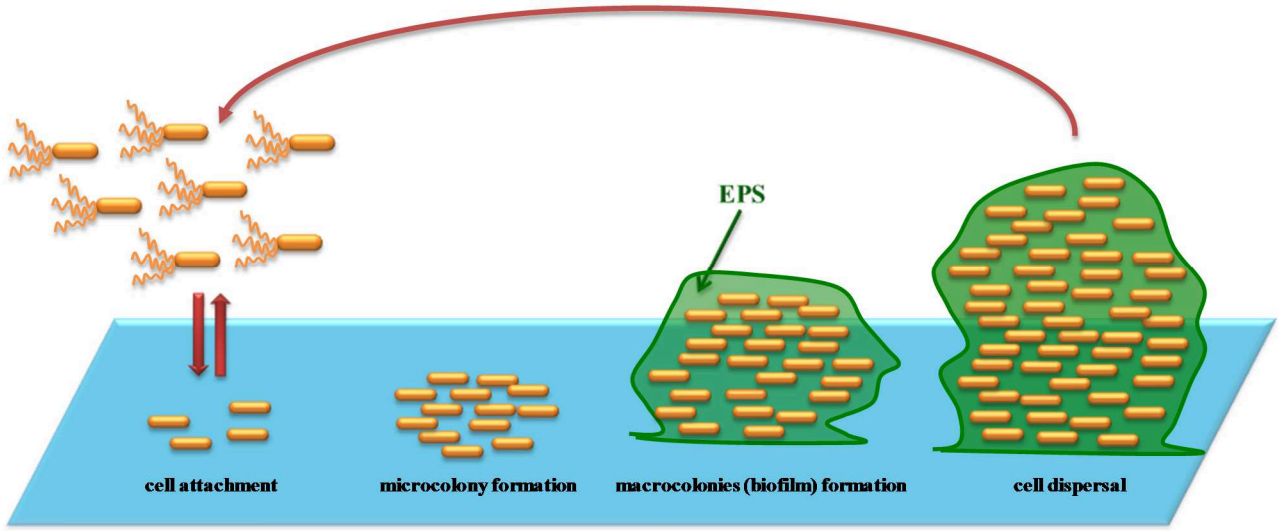

Figure 1.3 Biofilm formation phases

1.4.1 Attachment

Bacterial adhesion is a process that often occurs within 5 to 30 sec and can be divided into two stages: the primary or initial attachment and the secondary or irreversible attachment (Mittelman, 1998). An additional stage, the surface conditioning, can also be included to describe the interaction of the substratum with its environment. A material surface exposed in an aqueous medium inevitably and almost immediately becomes conditioned or coated by polymers from that medium. The properties of a conditioned surface are permanently altered and this resultant modification affects the rate and the extent of microbial attachment (Donlan, 2002).

The bacteria's initial attachment (reversible) can be active or passive, depending on their motility or the gravitational transportation of their planktonic, diffusion or shear force of the surrounding fluid phase. Once bacteria reach critical proximity to a surface, the adhesion depends on the predominant type of force, attractive or repulsive, which operates between the surface and the living cells. These include electrostatic forces, hydrophobic interactions, van der Waal's attractions and steric forces. At first, the adherent cells, those that originate biofilm formation on a surface, possess only a small quantity of EPS. This attachment is unstable and reversible and is characterized by a number of physiochemical variables that define the interaction between the bacterial cell surface and the conditioned surface of interest. If the environment is not favourable for the initial attachment, cells can detach from the surface (Singh et al., 2002; Liu et al., 2004).

The change from reversible to irreversible attachment is a shift from a weak interaction of the bacteria to a permanent bonding with the presence of EPS. Forces responsible for this type of attachment are dipole, ionic, hydrogen or hydrophobic interactions. Several studies indicate that irreversible attachment takes from 20 min to a maximum of 4 hours at 4 °C to 20 °C (Gilbert et al., 2001). Firm attachment of bacterial cells to the surface is assisted by bacterial motility structures (flagella, pili), bacterial surface structures (proteins, lipopolysaccharides (LPS) and exopolymers produced by bacteria. Flagella motility is important to overcome the forces that repel bacteria from reaching many abiotic materials. Once they reach the surface, the nonflagellar appendages as pili, curli, and outer membrane proteins (OMPs) are then required to achieve stable cell-to-cell and cell-to-surface attachments (Allison et al., 2000). Once the bacteria have attached irreversibly to the surface, they undergo genotypic and phenotypic changes to ensure the development and maturation of the biofilm. These changes result in the production of increased amounts of EPS, increased resistance to antibiotics, increased UV resistance and higher productions of secondary metabolites (O'Toole et al., 2000). After irreversible attachment, strong shear force or chemical breaking of the attachment forces by enzymes, detergents, surfactants, sanitizers and/or heat is needed for biofilm removal (Maukonen et al., 2003).

1.4.2 Microcolony formation and biofilm maturation

After the adherence of bacteria to the surface, the bacteria begin to multiply while sending out chemical signals that "intercommunicate" among the bacterial cells, through mechanisms belonging to the so called *quorum sensing*. As reported by several researchers, *quorum sensing* plays a role in cell attachment, biofilm maturation and cell detachment from biofilms (Parsek and Greenberg, 2005). After cell irreversible attachment, once *quorum sensing* signal intensity exceeds a certain level, the genetic mechanisms underlying exopolysaccharide production are activated. This way, the bacteria multiply within the embedded exopolysaccharide matrix, thus giving rise to the formation of a microcolony. Microcolonies further develop into macrocolonies that are divided by water channels and enclosed in an EPS matrix. Macrocolonies, compared to microcolonies, are composed of a large amount of cells, produce more EPS and have a higher metabolic and physiological heterogeneity (Ghannoum and O'Toole, 2004). Further increase in the size of biofilm takes place by the deposition or attachment of other organic and inorganic solutes and particulate matter to the biofilm from the surrounding liquid phase. Factors that control biofilm maturation include the availability of nutrients, the internal pH, oxygen, osmolarity, temperature, electrolyte concentration and the surface type (O'Toole and Kolter, 1998).

At some point, the biofilm reaches a critical mass and a dynamic equilibrium is reached at which the outermost layers of growth begin to generate planktonic organisms. These microorganisms are free to escape the biofilm and colonize other surfaces (Dunne, 2002).

1.4.3 Detachment and dispersal of cells from biofilms

As the biofilm ages, the attached bacteria, in order to survive and colonize new niches, must be able to detach and disperse from the biofilm. The bacteria from the biofilm, mainly the daughter cells, get detached individually or are sloughed off. Sloughing is a discrete process whereby periodic detachment of relatively large particles of biomass from the biofilm occurs. This process happens for mechanical reasons because some bacteria are shed from the colony due to the fluid dynamics and shear effects of the bulk fluid. Other bacterial cells stop producing EPS and are released into the surrounding medium, due to the presence of certain chemicals in the fluid environment or because of altered surface properties of the bacteria or substratum. The released bacteria can be transported to newer locations and again restart the biofilm process (Marshall, 1992).

1.5 PARAMETERS INFLUENCING BIOFILM FORMATION

The attachment of microorganisms to surfaces and the subsequent biofilm development are very complex processes, affected by several variables (Figure 1.4).

Adhesion surface	Bulk fluid	Cell
Texture or roughness	Flow velocity	Cell surface hydrophobicity
Hydrophobicity	pH	Extracellular appendages
Surface chemistry	Temperature	Extracellular polymeric substances
Charge	Cations	Signalling molecules
Conditioning film	Presence of antimicrobial products	
	Nutrient availability	

Figure 1.4 Important variables in cell attachment, biofilm formation and development (Simões et al., 2010)

Factors such as nutrients, environmental cues, substratum effect, conditioning film, availability of surface, velocity and turbulence and hydrodynamics regulate biofilm formation. Biofilms are more abundant, densely packed and thicker in environments with high nutrient levels. In fact, high nutrient concentrations promote the transition of bacterial cells from the planktonic to the biofilm state, while depletion of these nutrients has shown to cause detachment of biofilm cells from surfaces. However, nutrient concentrations too low to measure are still sufficient for biofilm growth (Prakash, 2003). Thus, it can be said that biofilms can form under diverse nutrient concentrations, ranging from high to almost non-detectable.

Other characteristics of the aqueous medium such as temperature, pH, oxygen ionic strength may also play a role in the rate of microbial attachment to a substratum. In a study, Fletcher (1988) found that an increase in the concentration of several cations (sodium, calcium, lanthanum, ferric iron) affected the attachment of *Pseudomonas fluorescens* to glass surfaces, presumably by reducing the repulsive forces between the negatively-charged bacterial cells and the glass surfaces. Regarding temperature values, small

changes in temperature are likely to produce substantial changes in biofilm growth, because microbial activity is very sensitive to temperature. For instance, studies have shown that biofilm thickness of *Escherichia coli* increased by 80% by raising the temperature from 30 to 35 °C (Melo and Bott, 1997).

The roughness and the physiochemical nature of the biotic or abiotic surface play an important role in the number of cells that will attach to a surface. Microbial colonization appears to increase as the surface roughness increases. This is because shear forces are less and surface area is more on rougher surfaces (Prakash et al., 2003). It has been shown that hydrophobic nonpolar surfaces, (like Teflon® and other plastics) are easier to colonize than hydrophilic surfaces (like glass and metals). This could be explained by the hydrophobic interaction which occurs between the cell surface and the substratum that would enable the cell to overcome the repulsive forces active within certain distance from the substratum surface and irreversibly attach (Prakash et al., 2003). Surface charge also affects the attachment of bacteria to surfaces. Pasmore et al. (2002) demonstrated that surface with neutral or small negative charges allowed for easy removal of biofilms, while surfaces with high charges (positive or negative) contained biofilms that were not easy to remove. In general, attachment occurs most readily on surfaces that are rougher, more hydrophobic, and coated by surface conditioning films (Donlan et al., 2002; Simões et al., 2008).

Hydrodynamic conditions can influence the formation, structure, EPS production, thickness, mass and metabolic activities of biofilms. Biofilms formed under turbulent flow can be described as "streamers" and these are typically formed by filamentous bacteria. The microcolonies formed under these conditions are stretched out in the direction of the current. Biofilms formed under low shear conditions (laminar flow conditions) are characterized by spherical microcolonies divided by water channels (Stoodley et al., 2002).

1.6 BIOFILMS IN THE FOOD INDUSTRY

Food processing environments are susceptible to biofilm formation, and biofilms can lead to serious hygienic problems and economic losses due to corrosion of equipment, reduction in heat transfer, obstruction of pipelines and loss of time when systems need to be stopped to remove biofilms (Mittelmann, 1998). In addition to that, several food spoilage and pathogenic bacteria, including *Pseudomonas* species, *Shewanella putrefaciens, Bacillus* species*, Listeria monocytogenes*, *Yersinia enetrocolitica*, *Campylobacter jejuni* and *Escherichia coli* O157:H7, have been reported to attach and form biofilms *in vitro* and on food contact surfaces, such as stainless steel, polystyrene or rubber (Chmielewski and Frank, 2003). The persistence of microorganisms in biofilms is a serious hygienic problem in food industries, causing processing and post-processing cross-contamination leading to reduced product shelf-life and effectiveness of sanitizing treatments as well as potentially affecting consumer health. Cross-contamination can occur in a number of ways when food passes over contaminated surfaces, such as via exposure to aerosols or condensation that originates from contaminated surfaces (Barnes et al., 1999). Improperly cleaned food contact surfaces, equipment and processing environments promote soil buildup, and, in the presence of water, contribute to the development of bacterial biofilms which may contain both pathogenic microorganisms and food spoilage bacteria.

Biofilms that develop in food processing environments are usually mixed-microbial communities, called "general biofilms", which frequently become thicker and more stable than monospecies biofilms. These biofilms are typically characterised by a multispecies microflora and a slow development, usually over several days or weeks (Marchand et al., 2012). Not only food contact surfaces, but also dead ends in pipes, crevices, corners, gaskets, joints, pumps and storage tanks, as well as walls, corners, drains and overhead structures with high humidity and condensation are particularly vulnerable to biofilm accumulation due to poor access during cleaning; such locations can then become a source of persistent potential food contamination.

Another type of biofilm that can form in food plants is called "process biofilms", often characterised by the presence of a selective pressure, mainly heat treatments that reduce competition from bacteria sensitive to different temperatures. Such biofilms typically consist of a single species and develop very rapidly, reaching concentrations of up to 10^6 CFU/cm^2 within 12 hours (Bouman *et al.*, 1982). Process biofilms can form in the regenerative sections of pasteurisers, where the cold incoming product and the hot pasteurised product are passed countercurrently on either side of the heat exchanger. The regenerative sections are often colonised by thermoduric bacteria that survive the heat treatment and can adhere and form biofilms. One microbial species that is commonly found in these sites is *S. thermophilus*, which can recontaminate the pasteurised product (Knight *et al.*, 2004).

1.6.1 Foodborne pathogens and spoilage organisms forming biofilm

Several bacterial pathogens as well as food spoilage bacteria can colonize several sites, and contaminate food products via cross-contamination (Table 1.1).

Table 1.1. Microorganisms forming biofilms in various food environments

Food industry	Microorganisms forming biofilm	References
Dairy processing plants	*Bacillus cereus*, *Escherichia coli*, Staphylococcus aureus	Sharma e Anand (2002)
Ice cream plants	*Listeria monocytogenes*, *Shigella* sp.	Gunduz and Tuncel (2006)
Fish industry	*Enterobacteriaceae*, *Serratia liquefaciens*, *Neisseriaceae*, *Pseudomonas* spp., *Vibrio* spp., *Listeria* spp.	Guobjornsdottier et al. (2005)
Caviar-processing unit	*Neisseriaceae*, *Pseudomonas* spp., *Vibrio* spp., *Listeria* spp., *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*	Bagge-Ravn et al. (2003)
Shrimp factory	*Pseudomonas* spp., *Pseudomonas fluorescens*, *Pseudomonas putida*, *Listeria monocytogenes*, *Shigella* spp.	Guobjornsdottier et al. (2005)
Meat industry	*Brochotrix thermosphacta*, *Carnobacterium* spp., *Enterobacteriaceae*, *Lactobacillus* spp., *Leuconostoc* spp., *Pseudomonas* spp., *Shewanella putrefaciens*	Borch et al. (1996)
Poultry industry	*Shewanella putrefaciens*, *Pseudomonas fluorescens*, *Pseudomonas fragi*	Russel et al. (1995)

Pseudomonas spp. are among the most common microorganisms implicated in food spoilage and are particularly important in chilled foods because many strains are psychrotolerant. They are found in food processing environments including drains and floor (Hood and Zottola, 1997). *Pseudomonas* spp. produces

copious amounts of EPS and has been shown to attach and form biofilms on stainless steel surface. Several studies relate the conditions of biofilm formation of *Pseudomonas* spp. to its antimicrobial susceptibility. It was observed that biofilms grown in laminar regime are quite thick, have a high number of protuberances and consequently are easily inactivated with biocides, while the biofilm formed in turbulent flow conditions has a quite strong EPS matrix that can resist the action of antimicrobial substances (Simões et al., 2003).

Listeria monocytogenes is known for its ubiquity and resistance to environmental stresses. Although *L. monocytogenes* is an environmental bacterium present on raw materials for food production, the immediate source of product contamination is often the processing environment itself (Lundèn et a., 2003). One of the major causes for concern about *L. monocytogenes* in food processing environments is its ability to attach to many different surfaces and form biofilms. In fact, *L. monocytogenes* can be found not only in food products, but can also be attached to food-processing facilities and equipment such as floors, walls, salt hoppers, brine containers, drain grids, store boxes, gaskets, conveyor belts, slicing, dicing and packaging machines, thereby increase the risk of food cross-contamination (Tresse et al., 2007). The ability of this pathogen to survive at low temperatures, colonize surfaces in the form of biofilm-like structures, and resist various food-related stresses is crucial for its persistence in the processing environments. Particularly, *L. monocytogenes* may adhere to and grow on processing surfaces, where food residues are accumulated and can persist also for years in food processing plants. Biofilms produced by *L. monocytogenes* are structurally simple in comparison to those produced by many other microorganisms, and a mature biofilm community can be established after 24 h making *L. monocytogenes* less susceptible to cleaning procedures (Rieu et al., 2008) .

Also *Staphylococcus aureus* can live in a wide variety of environments thanks to its ability to form biofilms on various materials and surfaces. This may contribute to the persistence of *S. aureus* in the food processing environments, consequently increasing cross contamination risks as well as subsequent economic losses due to recalls of contaminated food products. Several studies have shown attachment of *S. aureus* on work surfaces such as polypropylene, polystyrene, stainless steel and glass as well as in food products like poultry surfaces and meat (Pala and Sevilla, 2004; Marino et al., 2011).

1.7 BIOFILM CONTROL AND REMOVAL

1.7.1 Control

The first and most important thing to do against biofilm formation is to prevent it rather than treat it. However, nowadays there is no known technique that is able to successfully prevent or control the formation of biofilms without causing adverse side effects. The main strategy to prevent biofilm formation is to clean and disinfect regularly before bacteria attach firmly to contact surfaces. A prerequisite for an efficient sanitation programme is that the process equipment has been designed with high standards of hygiene. Dead ends, corners, cracks, crevices, gaskets, valves and joints are vulnerable points for biofilm accumulation (Chmielewski and Frank, 2004). The most effective sanitation programme cannot make up for basic

deficiencies in equipment design, and if design faults exist, sanitation can never be totally effective. The choice of materials used for contact surfaces must be made taking into account that different materials have different attitudes to the development of biofilms. A ranking of different materials with regard to supports for biofilm formation has been reported, although it can be asserted that, in fact, there is hardly any material that does not allow biofilm formation (Figure 1.5).

Support of biofilm growth	Material
Least	Glass
	Stainless steel
	Polypropylene
	Chlorinated PVC
	Unplasticised PVC
	Mild steel
	Polyethylene
	Ethylene–propylene
Greatest	Latex

Figure 1.5 Ranking of different materials with regard to support of biofilm growth (Meyer, 2003)

As a matter of fact, such rankings have to be evaluated with caution because biofilms may vary with microbial species and with test conditions. For example, it has been demonstrated that *L. monocytogenes* adhered much more on hydrophobic than hydrophilic surfaces (Cunliffe et al., 1995), while other authors reported that the adhesion force is greater on stainless steel than that one on polymers and rubber (Smoot and Pierson, 1998). The most practical material in processing equipment is nevertheless steel, which can be treated with mechanical grinding, brushing, lapping, and electrolytic or mechanical polishing.

Several attempts have been made to avoid biofilm formation by incorporation of antimicrobial products into surface materials, coating surfaces with antimicrobials or modifying the surfaces physicochemical properties. In a study of biofilm control, microparticles coated with benzyldimethyldodecylammonium chloride were found to effectively inactivate biofilm formation (Ferreira et al., 2011). Other authors reported that biofilm formation was inhibited by coating surfaces with silver (Knetsch and Koole, 2011). These studies focused on biomedical applications but the approaches may also be useful in the food industry if restricted to some parts of the process equipment such as valves, dead ends or where biofilms are more prone to formation and difficult to control. In fact, possible carryover of antimicrobials into food products is a concern when coatings release antimicrobial products. Finally, pre-conditioning the surface with a surfactant has also been reported to prevent bacterial adhesion. Cloete and Jacobs (2001) evaluated nonionic and anionic surfactants in preventing the adhesion of *Pseudomonas aeruginosa* to stainless steel and glass surfaces. The surfactants gave more than 90% inhibition of adhesion. Nevertheless, the application of such surface-active systems is restricted to some specific food contact materials, and their durability and application costs need to be carefully considered.

An efficient control programme evidently relies on adequate detection systems for biofilms. Several methods are commonly used like conventional total viable count, different microscopy and spectroscopy techniques, impedance measurement, ATP determination, colorimetry and flow cytometry techniques (Janknecht and Melo, 2003). The conventional methods include agar plate counting of product samples, swabbing or water flushes and contact plates, in order to indicate microbial contamination in the plant. In general these methods are inexpensive and easy to use. However conventional counting is too slow to be of practical use in food production. ATP bioluminescence test is a rapid biochemical method for estimating total ATP collected by swabbing a surface. Total ATP is related to the amount of food residues and microorganisms collected by the swab. ATP bioluminescence is a good method for rapid enumeration of cleaning effectiveness, since both food residues and microorganisms can be detected. Since the test is rapid, immediate corrective action can be taken. However, the ATP bioluminescence test cannot detect low levels of microorganisms (Griffiths, 1996). Impedance measurement, colorimetry and flow cytometry techniques applied to the product or process samples are more rapid methods but, with the exception of flow cytometry, they may still be too slow for process control or intervention purposes (Flint et al., 2001). All these techniques have been applied in the laboratory, but may be too delicate for industrial use. Lack of sensitivity may limit the ability to detect the early stages of development of biofilms that, nevertheless, have an impact on food production. Biosensor technologies may provide further solutions to the food industry to monitor biofilms. For example, a patented electrochemical probe can be installed in a line or tank to monitor biofilm activity in real time (Brooks and Flint, 2008). At any rate, it can be affirmed that all these techniques have advantages and constraints, and a well-chosen combination of detection methods guarantees the most efficient detection.

1.7.2 Removal and eradication

1.7.2.1 Cleaning process and chemical disinfectants

In the food industry, there is debris everywhere, which would promote the accumulation of microorganisms and encourage biofilm formation. Therefore, regular cleaning is required so as to prevent the contamination of food products. Adequate cleaning processes that break up and remove food residues deposited on the contact surfaces as well as biofilm matrix are important for the food processing industry, because incomplete removal facilitates the reattachment of bacteria to the surface and formation of a novel biofilm even if the bacteria from the previous biofilm are killed. Moreover, the disinfectants are less effective when food particles or dirt are present on the surfaces (Sinde and Carballo, 2000). The cleaning process can remove 90% or more of microorganisms associated with the surface, but cannot be relied upon to kill them. Bacteria can redeposit at other locations and, given time, water and nutrients can form a biofilm; therefore, sanitation in addition to cleaning must be implemented. Temperature, pH, water hardness, chemical inhibitors, concentration and contact time are important factors that affect the overall outcome of the

cleaning process (Bremer et al., 2002). The removal of biofilms is also significantly facilitated by the application of mechanical force (like brushing and scrubbing) to the surface during cleaning (Wirtanen et al., 1996).

A wide range of chemical disinfectants is used in the food industry, which can be divided into different groups according to their mode of action: (i) oxidizing agents including chlorine-based compounds, hydrogen peroxide, ozone and peracetic acetic, (ii) surface-active compounds including quaternary ammonium compounds and acid anionic compounds, and (iii) iodophores. In Figure 1.6 advantages and disadvantages of some disinfectants used in the food industry are reported.

Disinfectant type	Advantages	Disadvantages
Alcohols	Effective against vegetative cells, non-toxic, easy-to-use, colourless, harmless on skin, soluble in water, volatile	Microbistatic, ineffective against spores
Peracetic acid	Effective in low concentration, broad microbial spectrum, kills spores, penetrates biofilms, non-toxic (→ acetic acid and water)	Corrosive, unstable
Hydrogen peroxide	Decomposes to water and oxygen, relatively non-toxic, easy to use; weakens biofilms and supports detachment	High concentrations needed, corrosive
Chlorine	Effective in low concentration, broad microbial spectrum, easy to use, supports microbial detachment, cheap	Toxic by-products, resistance development, residues, corrosive, reacts with EPS, discolouration, explosive gas
Hypochlorite	Cheap, effective in a broad microbial spectrum, easy to use, supports detachment	Unstable, toxic, oxidative, corrosive, rapid regrowth, no prevention of adhesion, discolouration of products
Chlorine dioxide	Effective in low concentration, can be produced on-site, low dependency in pH	Toxic by-products, explosive gas
Quaternary ammonium agents	Effective, non-toxic, prevents regrowth, supports microbial detachment, non-irritating, non-corrosive, odourless, flavourless	Inactivated in low pH and by salts (Ca^{2+} and Mg^{2+}), resistance development, ineffective against Gram-negative bacteria
Iodophor	Non-corrosive, easy to use, non-irritating, broad activity spectrum	Expensive, flavour, odour, forms purple compounds with starch
Ozone	Similar effect as chlorine, decomposes to oxygen, no residues, decomposes biofilm	Corrosive, inactivated easily, reacts with organics (→ epoxides)
Glutaraldehyde	Effective in low concentrations, cheap, non-corrosive	Low penetration in biofilms, degrades to formic acid, increased DOC

Figure 1.6 Advantages and disadvantages of some disinfectants used in the food processes (Wirtanen and Salo, 2003)

Disinfectants should be chosen based on the process. The use of disinfectants in food plants depends on the material used and the adhering microbes. The efficiency of disinfection is influenced by water hardness, pH, temperature, concentration, contact time and interfering organic substances like food particles

and soil. Thus, cleaning agents like detergents and enzymes are frequently combined with disinfectants to synergistically enhance disinfection efficiency (Jacquelin et al., 1994).

In the selection of the disinfectant, it should be considered that cells within a biofilm are more resistant to biocides than their planktonic counterparts. For example, the antimicrobial efficacy of a widely used disinfectant product, benzalkonium chloride, is lower for biofilm-associated than for planktonic *Staphylococcus aureus* cells (Figure 1.7). Similarly, *Listeria monocytogenes* biofilms were more resistant to cleaning agents and disinfectants including trisodium phosphate, chlorine, ozone, hydrogen peroxide, peracetic acid and quaternary ammonium compounds as compared to planktonic cells (Robbins et al., 2005).

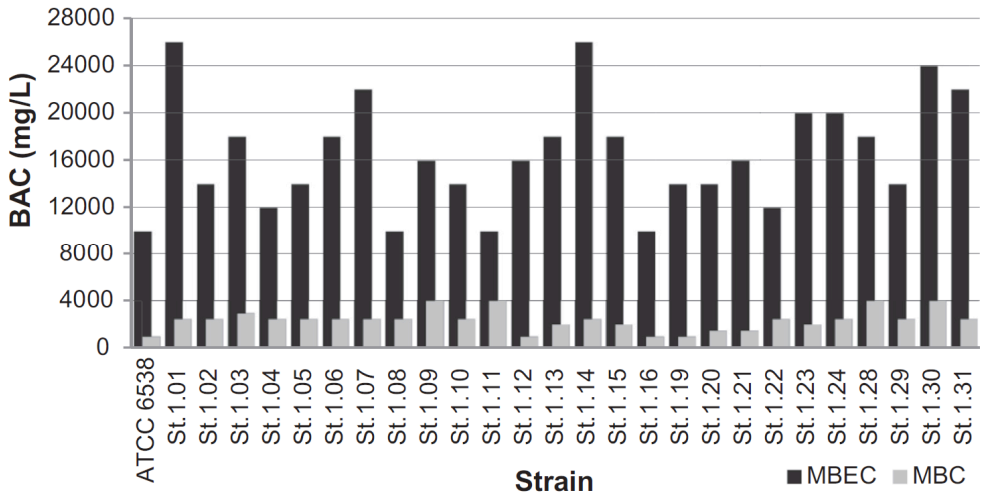

Figure 1.7 Effectiveness of benzalkonium chloride (BAC) against *Staphylococcus aureus* biofilms and planktonic cells after 48 h at 25 °C. MBEC: minimal biofilm eradication concentration; MBC: minimal bactericidal concentration (Vázquez-Sánchez et al., 2014)

Biofilm resistance to antimicrobial compounds is attributed to different mechanisms: a slow or incomplete penetration of the biocide into the biofilm, an altered physiology of the biofilm cells, expression of an adaptive stress response by some cells, or differentiation of a small subpopulation of cells into persister cells (Van Houdt and Michiels, 2010).

The slow or incomplete penetration of the biocide into the biofilm is partly due to the presence of the exopolymeric matrix, but primarily due to the neutralization of the active compound in the outermost regions of the matrix. Biofilm cells, especially those placed deep in the biofilm, exhibit decreased growth rates because of oxygen and nutrient gradients. The transition from exponential to slow or no growth is generally accompanied by an increase in resistance to biocides, so older biofilms appear to be more resistant against various disinfectants than younger biofilms. Another possible mechanism of biocide resistance is that some of the biofilm cells are able to sense the biocide challenge and actively respond to it by deploying protective stress responses more effectively than planktonic cells (Szomolay et al., 2005).

1.7.2.2 Physical methods

Physical treatments have been studied as alternatives to the use of chemical disinfectants in the food industry in particular for the sanitation of surfaces. Examples of technologies applied for disinfection are radiation with ultraviolet (UV) light and ionizing radiation. UV-C light treatment ($100 < \lambda < 280$ nm) has been widely used in the food industries and hospitals for air and surface sanitation (Sommers et al., 2010). One of the newest technologies proposed as a non-thermal treatment based on UV-C light is pulsed light treatment, which has been proven effective for killing a wide variety of microorganisms on foods and food contact materials (Ozen and Floros, 2001).

Ionizing radiation was tested on *Salmonella* biofilm and was observed that this technique was equally or more effective against biofilm cells than against planktonic cells of *Salmonella* spp. Therefore, it can be said that ionizing radiation could be a useful sanitation treatment on a variety of foods and contact surfaces (Figure 1.8).

Isolate (serovar)	Culture	CFU/ml ($\log_{10}$)[a]	Radiation D_{10} (kGy)[b]	R^2	5-$\log_{10}$ dose (kGy)[c]
Anatum	Planktonic	8.98	0.677 ab	0.94	3.385
	Biofilm	5.98	0.645 a	0.96	3.225
Enteritidis	Planktonic	9.15	0.535 c	0.98	2.675
	Biofilm	6.25	0.436 d	0.97	2.180
Stanley	Planktonic	9.08	0.591 b	0.99	2.955
	Biofilm	6.30	0.531 c	0.95	2.655

[a] Cell density in the nonirradiated control samples, $\log_{10}$ CFU/ml.
[b] D_{10} values among the six isolate-culture combinations followed by the same letter are not significantly different (analysis of covariance, $P < 0.05$).
[c] The dose required to achieve a 5-$\log_{10}$ (99.999%) reduction in population.

Figure 1.8 Radiation sensitivity of three *Salmonella* isolates in planktonic and biofilm-associated forms (Niemira and Solomon, 2005)

A relatively recent technique, called atmospheric plasma inactivation, makes use of reactive oxygen species and radicals generated by high voltage atmospheric pressure glow discharges to inactivate micro-organisms. The technique appears to be effective against both biofilm and planktonic microorganisms (Vleugels et al., 2005).

Ultrasonication is a well-known technique used in various food industry processes, namely freezing, cutting, drying, tempering, bleaching, sterilization and extraction. It was reported to be also used as an efficient biofilm removal method on food contact surfaces, especially when combined with other techniques like the use of ozone or enzyme preparations (Baumann et al., 2009).

1.7.2.3 Biological methods

The use of enzyme-based detergents as bio-cleaners, also known as “green chemicals”, can serve as a viable option to overcome the biofilm problem in the food industry. Since EPS is a heterogenic matrix,

a mixture of enzymes may be necessary in order to degrade the complex. The enzymes efficiency in biofilm removal may vary according to the species of bacteria, and it can also be enhanced in combination with surfactants (Lequette et al., 2010). However, formulation containing several different enzymes seems to be fundamental for a successful biofilm control strategy, like for example protease and polysaccharide hydrolyzing enzymes. Therefore, the specificity in the enzymes mode of action makes it a complex technique, increasing the difficulty of identifying enzymes that are effective against all the different types of biofilm. Another biological strategy is based on bacteriophages, which may provide a natural, highly specific, non-toxic, feasible approach for controlling several microorganisms involved in biofilm formation. This technology has not yet been successfully developed and relatively little information is available on the action of bacteriophages on biofilms. Moreover, the infection of biofilm cells by phages is extremely conditioned by their chemical composition and the environmental factors, such as temperature, growth stage, media and phage concentration (Sillankorva et al., 2008).

The negative consumer perception against artificial synthetic chemicals has shifted the research effort towards the development of alternatives that consumers perceive as "natural". Studies have indicated that essential oils and extracts of edible and medicinal plants, herbs and spices constitute a class of very potent antibacterial agents (Marino et al., 2001). Essential oils have been recently evaluated for their activity against biofilm formation, even if the literature examining their use in sanitizing solutions for biofilm control is currently limited. It has recently been observed that a contact time of 10 min of disinfectant solutions formulated with lemongrass (*Cymbopogon citrates*) and peppermint (*Mentha piperita*) essential oils significantly reduced adhered bacteria population of *Salmonella enterica* serovar Enteritidis attached to stainless steel AISI 304 (Figure 1.9).

Essential oil	Treatments/contact time (minutes)		
	10	20	40
Control	$7.7673 \pm (0.02)^{Aa}$	$6.7506 \pm (0.03)^{Ab}$	$5.1834 \pm (0.20)^{Ac}$
C. citratus	$3.5610 \pm (0.03)^{Ba}$	$0.0000 \pm (0.00)^{Bb}$	$0.0000 \pm (0.00)^{Bb}$
M. piperita	$3.7326 \pm (0.04)^{Ba}$	$0.0000 \pm (0.00)^{Bb}$	$0.0000 \pm (0.00)^{Bb}$

Sanitizing solution formulated with sodium hydroxide NaOH 1% added to Tween 80 0.5% and essential oils at 7.8 μL/mL[1]. Controls were run without essential oils.
Means values ± standard errors.
Values followed by the different small letter within the same line, and by the different capital letter within the same column, are significantly different ($p \leq 0.05$) according to Turkey's test.

Figure 1.9 Bacterial counts of *Salmonella enterica* serovar Enteritidis attached cells on stainless steel coupons AISI 304 after treatment with sanitizing solutions with lemongrass (*Cymbopogon citrates*) or peppermint (*Mentha piperita*) essential oils, expressed as Log CFU/cm^2, after 240 h of biofilm formation (Valeriano et al., 2012)

Moreover, nowadays many researchers are studying biofilm disinfection, or rather the development of molecules that interfere with quorum sensing mechanisms and acting as biocides with either a wide action spectrum or a more specific action against particular pathogenic and spoilage bacteria (Girennevar et al., 2008; Lebert et al. 2007).

Finally, it can be said that probably the best technology to obtain biofilm cells eradication is the combination of two or more different control techniques which have been proven to be effective. This combination summarizes different obstacles to be administered to biofilms in order to provide a synergistic effects. For example, DeQueiroz and Day (2007) studied the antimicrobial activity and effectiveness of a combination of sodium hypochlorite and hydrogen peroxide in killing and removing *Pseudomonas aeruginosa* biofilms from surfaces. The synergistic effect of ozone and ultrasound was also shown to be efficient for biofilm cell reduction (Patil, 2010).

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Chapter 2. EVALUATION OF CULTURE CONDITIONS AND METHODS IN STUDYING MICROBIAL BIOFILM

2.1 INTRODUCTION

Studies of biofilm development have the purpose to collect all possible information to better understand the bacterial ability to adhere to and to form biofilm on different surfaces. Over the last decades, a broad range of model systems has been described for the *in vitro* study of biofilm formation and development, as microtiter plate-based methods, drip-flow methods and batch-biofilm reactors. In all of them, subsequently biofilm growth sessile bacteria are enumerated after detachment from the surface by scraping, vortexing, sonication and plate counting, or the biomass quantified using the microtiter plates assay and microscopy techniques (epifluorescence, confocal laser scanning microscopy, transmission electron and scanning electron microscopy) (Coenye and Nelis, 2010).

Microtiter plate-based assays are among the most frequently used model systems to screen the ability to form biofilm by microbial strains. As a matter of fact, these methods can be used as rapid and simple techniques to screen for differences in biofilm production between strains, or for evaluating of the efficacy of biocides in killing sessile cells (Peeters et al., 2008). A microtiter plate is a polystyrene flat-bottom plate with multiple wells, each of which holds a few hundred microlitres of liquid or culture medium. In each plate several biofilms can be formed under different conditions. The microtiter plate assay is superior to other static tests, e.g. tube test, in terms of objectivity, accuracy, simplicity and indirect measurement of bacteria attached to the walls of the wells (Stepanović et al., 2000). The microtiter plate techniques allow the quantification of matrix and both living and dead cells using the crystal violet staining, as well as the viable cells and the matrix quantification, using for example ruthenium red staining of EPS (Borucki et al., 2003).

Other *in vitro* systems for growing and testing biofilms include simple batch/static systems, batch systems with introduced shear, flow cells and systems that can be operated under continuous-flow conditions (Mittelman et al., 1992; Ceri et al., 1999). These systems generally provide a surface that can be removed and examined once it is colonized to assess biofilm formation. Donlan et al. (2004) developed a reactor (CDC Biofilm Reactor, CBR) that incorporated 24 removable biofilm growth surfaces made of different materials (e.g stainless steel, rubber, glass, …) allowing biofilm formation under moderate to high shear in batch or continuous-flow conditions. Studies that utilized this reactor showed that it could be used for detecting biofilm formation, characterizing biofilm structure and assessing the effect of antimicrobial agents on the biofilm. According to the results obtained by Goeres et al. (2005), the CBR system is a reliable experimental tool for growing a standard biofilm in the laboratory and it can be adapted in order to study several different microorganisms under a wide range of controllable conditions. For this reason, this system must be set up for each strain since in literature there is CBR setting for only few microbial species.

In this study, preliminary experiments were performed to generate and to examine biofilms of food relevant spoilage and pathogen microorganisms using different techniques such as the microtiter plate assay and the CBR system. These methods were evaluated in order to study their applicability and repeatability

for the formation and quantification of bacterial biofilms. Moreover, the drop plate counting method was tested and compared to the traditional plate counting.

2.2 MATERIALS AND METHODS

2.2.1 Microtiter plate assay

Thirteen *L. monocytogenes* strains belonging to different serotypes (see List of Strains) were used in the microtiter plate assay (Lm_1, Lm_4, Lm_5, Lm_7, Lm_23, Lm_29, Lm_174, Lm_254, Lm_288, Lm_SB, Lm_1E, Lm_5E and Lm_Scott A). All strains were cultured overnight at 37 °C (~ 10^8 CFU/mL).

Three different broth cultures were used to grow biofilms on microtiter plates, Brain Heart Infusion (BHI, Oxoid, Milan, Italy), Tryptone Soya Broth (TSB, Oxoid, Milan, Italy) and Luria Bertani broth (LB, 10 g/L tryptone, 5 g/L NaCl, 5 g/L yeast extract), in order to determine the influence of the growth medium on biofilm formation by *L. monocytogenes* strains.

The test was carried out in three biological replicates in 96-well polystyrene flat-bottom microtiter plates (Corning Life Science, Amsterdam, the Netherlands). Each well was filled with 200 µL of each broth and inoculated with 10 µL of the overnight culture of each *L. monocytogenes* strain. The microtiter plates were incubated at 37 °C for 7 days. After 48, 96 and 144 hours the microtiter plates were subjected to refreshing by replacing of 150 µL of exhausted broth with an equal volume of sterile broth. For each experiment, eight wells were used as negative controls and filled with 200 µL of not inoculated broth. At the end of the incubation, the content of the plate was poured off and the wells were rinsed twice with 220 µL sterile saline solution (0.9% NaCl). After fixation (60 °C, 1 hour), each well was stained with 200 µL of a 2% crystal violet solution (Merck, Darmstadt, Germany) for 15 min at room temperature. The wells were then washed under running tap water and the microtiter plates were dried at room temperature. The wells were filled with 200 µL of 95% ethanol and incubated for 30 min at room temperature. The optical density at 570 nm (OD_{570}) of each well was measured in a microplate reader (Sunrise, Tecan, Männedorf, Switzerland). For each replicate and each culture medium, the data of eight wells were averaged. The strains were classified for their biofilm-forming ability as described by Stepanović et al. (2000) (Table 2.1).

Table 2.1 Biofilm-forming ability classification; *ODc = the mean of the negative control + 3 x SD

Optical density	Biofilm-forming ability
OD ≤ ODc*	non-adherent
ODc < OD ≤ 2xODc	weakly adherent
2ODc < OD ≤ 4xODc	moderately adherent
4xODc < OD	strongly adherent

2.2.2 CBR assay

The CDC Biofilm Reactor (CBR, BioSurface Technologies, Bozeman, MT, USA) consisted of a one-litre glass vessel, in which a polyethylene top supports eight independent and removable polypropylene rods, a medium-inlet port, and a gas-exchange port. Each rod holds three removable coupons (biofilm growth surfaces; 1.27 cm diameter, 0.3 cm thick) for a total of 24 sampling opportunities (Figure 2.1). In this step of the study stainless steel AISI 304 (food stainless steel) was used as the surface for biofilm growth, and *P. fluorescens* Ps_019 as the microbial model.

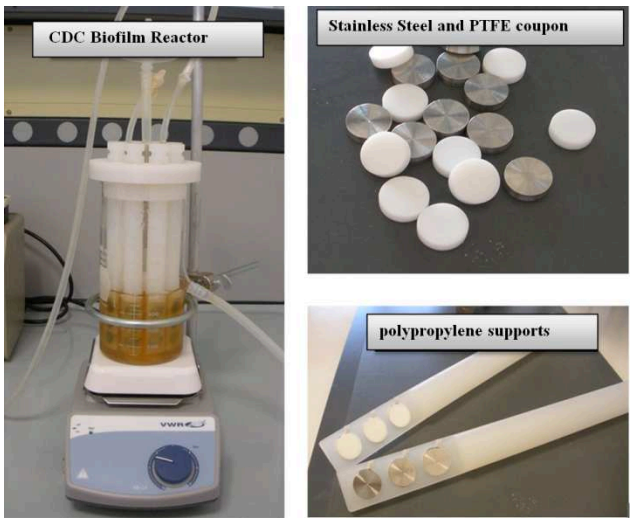

Figure 2.1 CDC Biofilm Reactor, removable coupons and removable polypropylene rods

One milliliter of an overnight culture (~ 10^8 CFU/mL) was used to inoculate 500 mL of TSB in the CBR. The incubation of the CBR was performed for 5 days in dynamic conditions (in batch with rotation of the magnetic stir bar to 125 rpm) both at 4 °C and 15 °C in a thermostatic chamber. Each trial was performed in two biological replicates.

At the end of incubation, the coupons were aseptically removed from each rod of the CBR, and each coupon was rinsed twice with 10 mL of a sterile saline solution, in order to eliminate the non-adherent cells. The biofilm detachment from each coupon was carried out by using a sterile cell scraper (StarLab, Milan, Italy) and detached cells were suspended in 1 mL of Maximum Recovery Diluent (MRD, Oxoid, Milan, Italy). In order to break the cell clumps, the microbial suspension was subjected to two cycles of sonication in an ultrasonic bath LBS2 (Falc Instruments, Treviglio, Italy) at 25 °C for 30 sec at 59 KHz interspersed by 30 sec vortexing. Each microbial suspension was then subjected to microbial count by serial dilutions on Pseudomonas Agar Base added of Pseudomonas CFC Supplement (PSA, Oxoid, Milan, Italy). The plates were incubated at 30 °C for 24-48 hours.

In order to study the repeatability of the position of the coupons in the reactor, the rods and rod position were labelled with the same letters. Moreover, the coupon position in each rod was labelled as A, B, C from the top to the bottom of the rod (Figure 2.2).

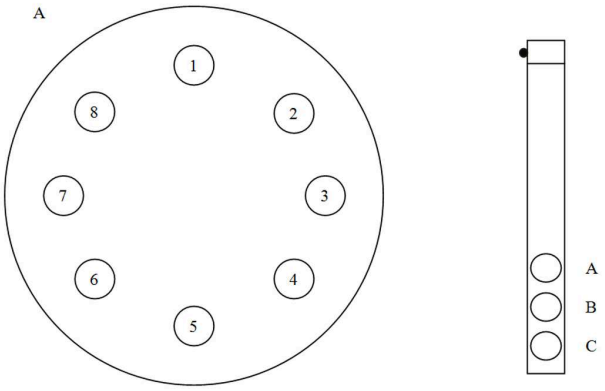

Figure 2.2 Labelled positions of rods (A) and coupons (B) within CBR

2.2.3 Drop plate method

In this step the use of the drop plate (DP) method in counting biofilm cells was evaluated and compared to the more common spread plate (SP) method in terms of accuracy, costs and practical advantages. Both *L. monocytogenes* biofilms grown on microtiter plates (strains Lm_1, Lm_278 and Lm_Scott A) and in CBR (strain Lm_278) were analyzed. Tests were performed in three biological replicates in TSB for microtiter plate assay, and in two biological replicates in TSB for CBR assay. For each assay, three samples were tested for each biological replicate (i.e., three wells of a microtiter plate and three coupons for CBR).

The biofilm cells from the CBR assay were obtained as described above (paragraph 2.2.2). As for the biofilms grown on microtiter plates, after removing loosely the attached bacteria by washing each well twice with 200 µL with a sterile saline solution, the sessile cells were manually scraped from the sides of the wells using a pipette tip and resuspended in 200 µL of MRD.

The microbial suspensions obtained from each well and coupon were subjected to two cycles of sonication (paragraph 2.2.2) and to a ten-fold dilution in MRD. Each dilution was then subjected to a microbial count on BHI agar plates using both the SP method (100 µL of suspension per plate) and DP method. For DP method, each agar plate was divided into four quadrants and each quadrant was reserved for one dilution in the series. 50 µL of each bacterial suspension and dilution were dispensed in five evenly spaced 10-µL drops onto a designated quadrant of the plate (Herigstad et al., 2001). After the drops on the agar dried, the plates were incubated at 37 °C for 48 hours.

For the SP method, the countable dilution was the one that gave 30 to 300 colonies for 100-µL sample dispensed. Regarding the DP method, the countable dilution was the one that gave 3 to 30 colonies for 10-µL drop of the dispensed sample. In both cases, the total count was scaled up and the viable cell counts were expressed as CFU/cm^2 (mean of three samples for each biological replicate).

2.2.4 Statistical analysis

The data were statistically analysed using the analysis of variance and the means separated according to Tukey's HSD test with a significant level (*p* value) of 0.05 using Statistica 8.0 (StatSoft, Tulsa, OK, USA).

2.3 RESULTS AND DISCUSSION

2.3.1 Microtiter plate assay

The microtiter plate assay is a well-known method that allows to quantify the microbial biomass within a microbial biofilm. Although this method has been applied to a wide range of microbial species, different experimental conditions and protocols make result comparisons difficult. Regarding *L. monocytogenes*, there is a general agreement that it is capable of biofilm formation on plastic surfaces. However, different results were obtained for strains belonging to different serotypes and with different origin (Borucki et al., 2003).

In this study thirteen *L. monocytogenes* strains of different serotypes were characterized for their ability to form biofilm in polystyrene microtiter plates in the presence of different nutrient media. BHI was chosen as one of the nutrient-rich laboratory media, TSB is a less-rich culture media but frequently used in biofilm investigation, and LB was chosen as it does not provide as many nutrients compared to rich laboratory media, i.e. it does not contain carbohydrates. The data revealed that all the tested strains produced biofilm on polystyrene surfaces (Figure 2.3), although the biofilm-forming ability of the strain collection was significantly higher ($p<0.05$) in BHI, followed by TSB and LB medium. A possible explanation can be the quantity of carbohydrates present in the medium. In fact, it has been observed that the addition of increasing concentrations of glucose promoted the density of biofilms formed by *L. monocytogenes* at 37 °C (Pan et al., 2010). The mean OD_{570} data were much higher than those reported by other authors (Djordjevic et al., 2002; Borucki et al., 2003). It has to be highlighted that the incubation conditions used in this study (seven-day growth) are more similar to the environmental conditions in a food plant, where it is possible that some sites are subjected only to weekly or biweekly cleaning (Gibson et al., 1999).

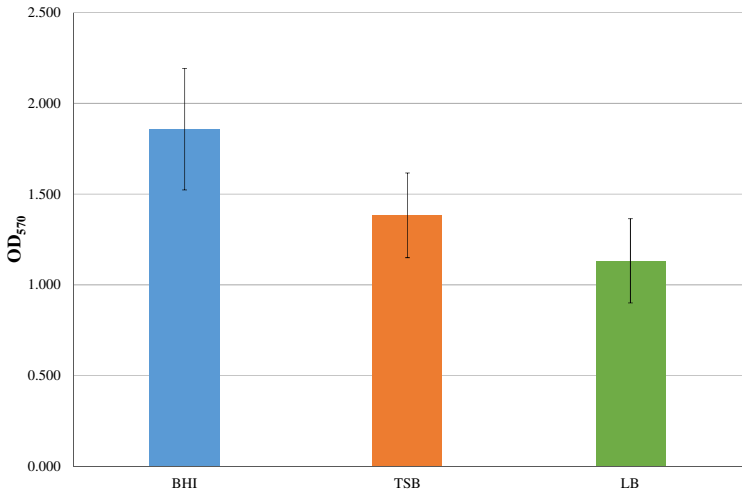

Figure 2.3 Biofilm biomass (mean OD_{570} ± SD; n=13) production by *L. monocytogenes* strains in different culture media

The strains tested belonged to lineage I or II and to different serotypes, and only weak correlations were observed between cell adherence and biofilm formation and serotype. For example, strains Lm_5E, Lm_Scott A, Lm_SB and Lm_7, whose serotype was 4b (lineage I) were generally low biofilm-producers, whereas most lineage II strains (e.g. Lm_254 and Lm_1E, serotypes 2a and 1/2a respectively) were found to produce more dense biofilms on plastic surfaces. However this observation was not supported by results obtained in other studies (Norwood and Gilmour, 1999). As a matter of fact, conflicting results were obtained on possible correlations between biofilm formation and genetic lineage (Folsom et al., 2006). The composition of the medium did not have the same influence on all *L. monocytogenes* strains (Figure 2.4). For example, while most of the *L. monocytogenes* strains formed the highest amount of biofilm in BHI medium, there are few strains (e.g. Lm_1 and Lm_29) whose biofilm forming-ability in LB, a nutrient-poor medium, was similar to that in BHI. Bacteria in a food processing environment may be exposed to different levels of nutrients, depending upon the location in the plant (Djordjevic et al., 2002). It is well known that diverse environmental factors, including the growth medium richness, can regulate biofilm formation (Donlan, 2002), which is still a poorly understood process. One possible explanation for different response of microorganisms to environmental conditions could be the results of mutations in genes that control biofilm formation (Römling et al., 1998).

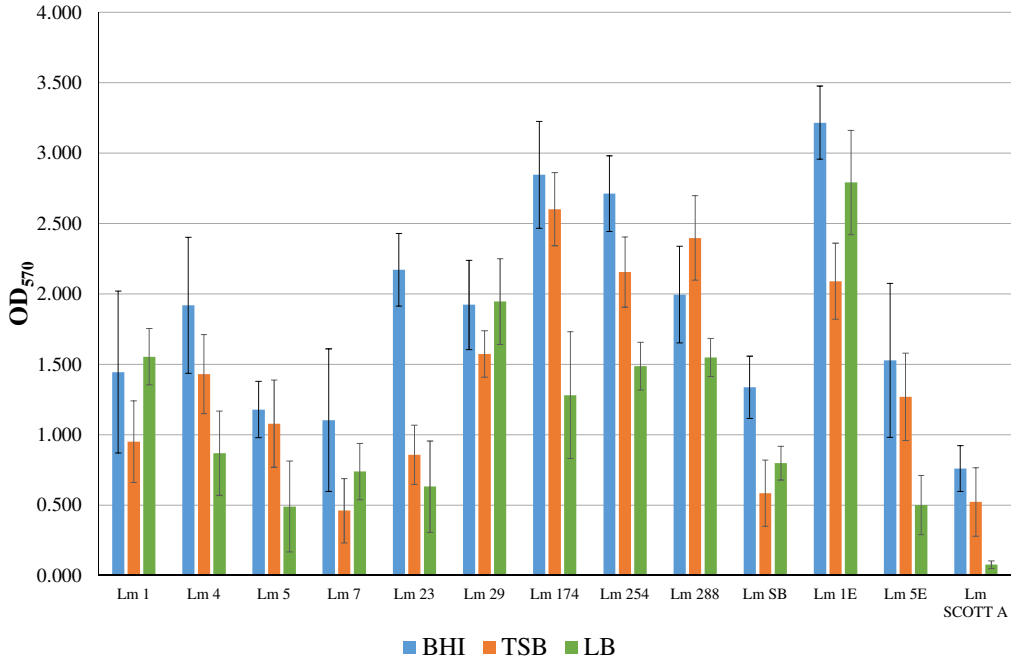

Figure 2.4 Biofilm biomass (mean OD_{570} ± SD; n=3) production by each *L. monocytogenes* strain in BHI, TSB and LB

These results are in agreement with previous findings and remark the high variability in biofilm formation among *L. monocytogenes* strains (Chae and Schraft, 2000; Borucki et al., 2003). The varying ability of different *L. monocytogenes* strains to form biofilm may be an important factor in the development of persistent strains within food processing environments. Further studies are warranted to understand the mechanism of different biofilm growth among strains of *L. monocytogenes*. Understanding this mechanism is an important step towards prevention of biofilms in food processing environments.

According to the ranking of biofilm formation ability (Table 2.1), almost all strains presented moderate to strong ability to form biofilm in all culture media (Figure 2.5).

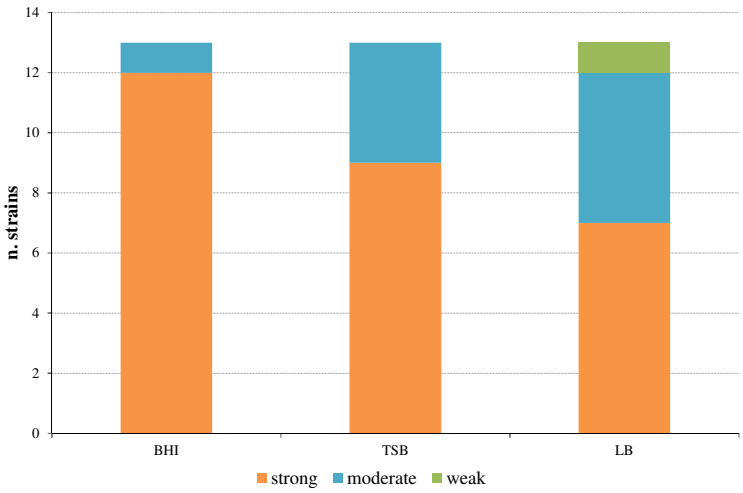

Figure 2.5 Biofilm formation ability of *L. monocytogenes* strains on polystyrene

LB seemed to be the best growth medium for the differentiation of the biofilm ability between *L. monocytogenes* strains, probably due to the lowest nutrient content. Interestingly strain Lm_Scott A, which was isolated during a listeriosis outbreak in the USA (Fleming et al., 1985), resulted as a weak biofilm producer in LB. It should be noted that in this study the biofilm formation on microtiter plates was performed at 37 °C, which is a temperature that significantly affects the flagellin expression in *L. monocytogenes*. In fact, Peel et al. (1988) showed that flagellin production is a less marked feature of organisms grown at 37 °C as compared to 20 °C. Moreover, it has been demonstrated that biofilm formation is significantly influenced by temperature, probably modifying cell surface hydrophobicity (Di Bonaventura et al., 2008).

In this study polystyrene microtiter plates were used as a model surface for *L. monocytogenes* adhesion and biofilm formation under laboratory conditions. The results are in agreement with other studies in which polystyrene microtiter plates are commonly used as standard bioreactor systems for adhesion and biofilm formation of bacteria isolated from many different environments, providing reliable comparative data (Djordjevic et al., 2002; Cotter et al., 2009). This study demonstrated that *L. monocytogenes*, a foodborne pathogen, readily forms biofilm on polystyrene surfaces, which has physico-chemical surface properties (hydrophobicity) similar to those of other materials used in food processing environments, such as stainless steel (Simões et al. 2007). Moreover, this study confirms that crystal violet microtiter assay is a rapid and simple method to screen differences in biofilm production between *L. monocytogenes* strains.

2.3.2 CBR assay

In food plants microbial biofilms can grow in different fluid dynamic conditions, i.e. in absence of shear stress like on floors or walls (a condition well simulated by the microtiter biofilm assay), or in conditions of moderate to high shear stress as in the case of tubes and pipes. It is expected that the biofilm biomass is different in these two conditions both quantitatively and qualitatively. Hence, in this study pre-

liminary experiments were performed to generate biofilms in dynamic conditions using CBR. *P. fluorescens* biofilms grown at 4 °C and 15 °C on stainless steel surfaces were used as model systems in this step of the study.

CBR allows for the simultaneous growth of 24 biofilms on 1.27 cm^2 surfaces, placed in eight polypropylene rods in vertical alignment at three different distances from the bottom of the 1000 mL vessel. Viable counts evaluated in two independent experiments showed that there were no significant differences between coupons with different positions within the CBR both at 4 °C (p=0.6732; Table 2.2) and at 15 °C (p=0.3015; Table 2.3).

Table 2.2 Cell viable counts (mean Log CFU/cm^2 ± SD; n=2) of biofilm formed on stainless steel at 4 °C in CBR

Rod	Coupon position		
	A	B	C
1	5.30 ± 0.10	5.39 ± 0.08	5.68 ± 0.00
2	5.71 ± 0.02	5.73 ± 0.03	5.14 ± 0.03
3	5.21 ± 0.01	5.74 ± 0.10	4.95 ± 0.37
4	5.87 ± 0.01	5.05 ± 0.01	5.93 ± 0.05
5	5.05 ± 0.04	5.42 ± 0.30	5.40 ± 0.04
6	5.18 ± 0.01	5.07 ± 0.02	5.18 ± 0.19
7	5.32 ± 0.05	5.58 ± 0.01	5.94 ± 0.02
8	5.11 ± 0.06	5.40 ± 0.06	5.26 ± 0.16

Table 2.3 Cell viable counts (mean Log CFU/cm^2 ± SD; n=2) of biofilm formed on stainless steel at 15 °C in CBR

Rod	Coupon position		
	A	B	C
1	4.68 ± 0.29	4.38 ± 0.39	4.65 ± 0.37
2	4.38 ± 0.00	4.47 ± 0.04	4.56 ± 0.02
3	4.44 ± 0.21	4.85 ± 0.36	4.88 ± 0.05
4	4.80 ± 0.08	4.30 ± 0.13	4.59 ± 0.01
5	4.67 ± 0.36	4.89 ± 0.25	4.92 ± 0.09
6	4.62 ± 0.21	4.31 ± 0.06	4.67 ± 0.15
7	4.60 ± 0.43	4.79 ± 0.36	4.66 ± 0.19
8	4.73 ± 0.58	4.56 ± 0.24	4.36 ± 0.03

CBR is engineered to emulate a specific real-world environment even if it is, in any case, a laboratory biofilm growth system. Because the choice of reactor affects the laboratory biofilm formation, it is important for researchers to choose the appropriate reactor and growth conditions. The results clearly showed that CBR system is a reliable experimental tool for growing a standard biofilm in the laboratory. The high number of biofilm samples possible to obtain under the same experimental conditions could supply several opportunities in research aimed at studying biofilm growth kinetics, comparison of different biofilm killing strategies, as well as the qualitative and quantitative characterization of biofilms grown in different environmental and culture conditions.

Regarding the study of biofilm cell density, the results demonstrated that CBR, when operated in dynamic conditions (i.e. in batch with rotation of the magnetic stir bar to 125 rpm), was capable of generating dense biofilms of *P. fluorescens* Pfl_019 on replicate stainless steel AISI 304 surfaces. According to previous studies, *P. fluorescens* strain has a high ability to form biofilms on stainless steel (Somers and

Wong, 2004). The incubation temperature statistically influenced the quantity of biofilm formed by *P. fluorescens* ($p<0.05$), with an increased production at the lowest temperature value (Table 2.4), which is quite common in refrigerated sites of food plants.

Table 2.4 Cell viable counts (mean Log CFU/cm^2 ± SD; n=48) of biofilms grown for seven days on stainless steel at 4 °C and 15 °C. Mean values with a different letter indicate statistically different values ($p<0.05$)

Temperature	Log CFU/cm^2 ± SD
4 °C	$5.46^a \pm 0.36$
15 °C	$4.62^b \pm 0.28$

In conclusion, this preliminary study showed that CBR can be used to grow a standard biofilm for addressing diverse research questions. It is important to emphasize that to obtain a rough statistical evaluation, the working conditions of the reactor should be standardized and multiple experiments must be performed on the microorganisms and the culture conditions to be tested.

2.3.3 Drop plate method

Studying microbial biofilms frequently require the quantification of viable counts grown on surfaces, and the "gold standard" method to obtain these data is the spread plate (SP) technique, in which a series of decimal dilutions of the suspension containing biofilm cells is evenly distributed on at least duplicate agar plates. This method requires the use of a high number of disposable consumables (e.g. dilution tubes, pipette tips, spatulas, Petri dishes), culture media and is also time-consuming. These circumstances greatly affect studies on microbial biofilms, both economically and in terms of time, where numerous surfaces have to be tested in order to obtain reliable information. Thus, alternative methods to enumerate biofilm microrganisms are strongly appreciated. Among them, the drop plate (DP) method exhibits many positive characteristics, allowing easy execution of the plating process without sacrificing the accuracy of the results.

In this step the DP and the SP methods were used to evaluate viability of *L. monocytogenes* biofilms grown on microtiter plates and on stainless steel coupons in CBR. The results of viable cell counts of the biofilms grown in microtiter plates and in CBR are shown in Figure 2.6.

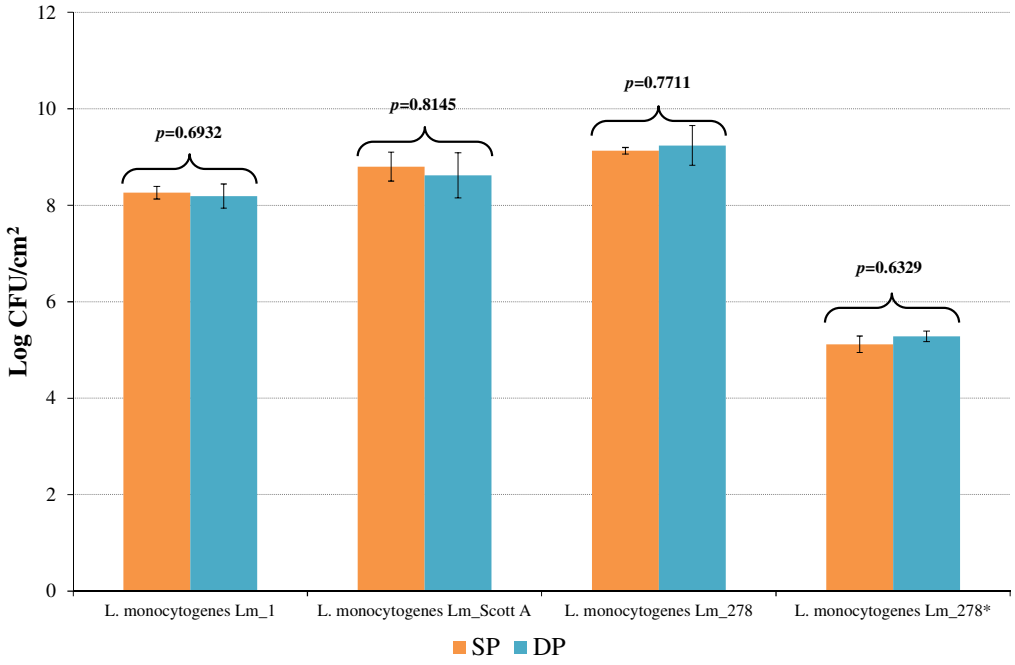

Figure 2.6 Mean viable counts (Log CFU/cm^2) ± SD of *L. monocytogenes* biofilms grown in microtiter plates (Lm_1, Lm_Scott A and Lm_278) (n=3) and in CBR (Lm_278*) (n=2) as evaluated by SP and DP methods

The analysis of variance performed on mean viable counts evaluated by SP and DP showed that no significant statistical difference between the two methods of bacterial count for each strain existed, not even for biofilms with very different densities formed on different surfaces. This was the case of the strain Lm_278, which was tested both on microtiter plate, where the mean viable count was 9.18 Log CFU/cm^2, and in CBR, where the mean viable count was 5.20 Log CFU/cm^2.

In addition to providing results not statistically different from the SP technique, the DP technique offered a lot of operational and economic advantages. First of all, less time and effort are required to dispense an equivalent volume of microbial suspension in drops on agar plates than to spread the same volume using a spatula. This advantage can be much greater if an electronic micropipette with repetitive dispensing is used. Furthermore, the sample volume dispensed in each drop and the number of drops for each dilution can be modulated as a function of microbiological consideration (e.g. mean size of the colonies) and the aim of the study. For example, in disinfection killing tests, it would be useful to dispense a high number of drops (and consequently a high volume of suspension) to reduce the limit of detection of the method. Another significant advantage of DP is that, for the same decimal dilution, less time is required for counting drop plates colonies compared to spread plates, because usually a lower suspension volume is sampled. The colonies grown from DP plates cover a smaller area than SP plates, so the colony count can be done more accurately and it is less tiring for the technician performing the counting (Figure 2.7).

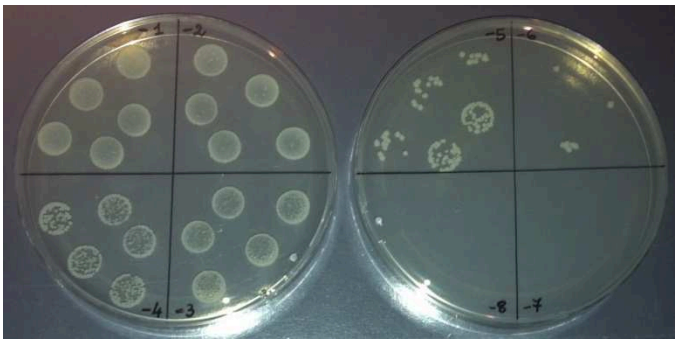

Figure 2.7 Drop plate method. Agar plate divided into four quadrants (for each dilution). In each quadrant five evenly spaced "drops" of each bacterial growth are evident

The DP method expends relatively few supplies with respect to SP method. For example, for plating four dilutions in duplicate using the DP method only two Petri plates would be necessary instead of eight, and at least four spatulas for the SP method. In Table 2.5 the disposables and materials, and their rough costs according to the current year price list, required to perform SP and DP methods on the same sample are reported. Not only the cost of the disposables and the culture media needs to be considered, but also the additional time and, incubator space, to handle more plates. The use of DP method for counting viable cells in biofilm is particularly useful, because the viable counts are high enough to justify a considerable time saving compared to SP.

Table 2.5 Disposables and materials needed to perform SP and DP methods for an eight-fold diluted sample in duplicate

	SP	DP
Petri plates	16	4
pipette tips	8	8
spatulas	8	0
mL of culture medium (ca. 20 mL/plate)	320	80
rough cost (euro)	8.39	1.53

2.4 CONCLUSIONS

To study microbial biofilms of food interest, it is necessary to be able to work under different environmental conditions, both in terms of culture conditions and extrinsic parameters (for example, nutrient concentrations, pH, temperature ...). Furthermore, it is important to be able to create, in the shortest time possible, a number of the most probable biofilm conditions, standardized and similar to the reality of the food industry. Furthermore, due to the high microbial biodiversity, an in-depth study of biofilms in very large collections of strains is necessary, resulting in an increase in the number of samples to be processed. It is therefore necessary to have a reliable, rapid and standardized system for the production and study of microbial biofilms. The results of this first phase of the experiment indicate that the microtiter plate assay and the CDC biofilm reactor assay are sufficiently reliable and repeatable tools to produce, under different operating conditions, a number of samples large enough to provide sufficient information on the ability of food microorganisms to produce biofilms. Finally, the drop plate method has proved particularly suitable,

sufficiently accurate and reliable, as well as advantageous from the economic point of view, for the quantification of viable cells present in the biofilm.

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Chapter 3. BIOFILM FORMATION OF FOOD PATHOGENS AND SPOILERS AS AFFECTED BY TEMPERATURE, pH, GLUCOSE AND SODIUM CHLORIDE

3.1 INTRODUCTION

Bacterial attachment and biofilm formation on surfaces is influenced by physico-chemical properties of the environment, surface and microorganism, as well as by other factors, including the medium in which bacteria are grown, presence of organic residues, and cell-to-cell communication mechanisms (Chmielewski and Frank, 2003). The environmental factors can influence biofilm formation through alterations of the bacterial cell surface. For instance, curli expression and attachment to plastic surfaces by enterotoxin-producing *E. coli* strains was found to be higher at 30 °C than at 37 °C (Szabo et al., 2005). Likewise, expression of fimbriae in *Salmonella* Thyphimurium and in *Aeromonas veronii* strains isolated from food was affected by temperature (28 and 20 °C, respectively) favouring expression and consequent attachment (Romling et al., 1998; Kirov et al., 1995). Production of these outer surface structures at lower temperatures could enhance surfaces attachment, and hence facilitate persistence and survival in food-processing environments. The adhesion of *L. monocytogenes* to polystyrene after growth at pH 5 was lower than at pH 7, and this could be attributed to the down-regulation of flagellin-synthesis (Tresse et al., 2006).

Regarding both planktonic and sessile growth conditions, the different environmental parameters act, in synergy with each other. However, studies related to biofilm formation by food pathogens and/or food spoilers only rarely report data about the synergy of different variables. For example, Smoot and Pierson (1998) studied attachment of *L. monocytogenes* Scott A to Buna-N rubber and stainless steel, and showed that exposing cells to sublethal levels of environmental stress, such as pH and temperature, can affect the ability of this pathogen to attach to common food contact surfaces. Hamanaka et al. (2012) showed that biofilm development of vegetable-related *Pseudomonas* cells was considerably affected by incubation temperatures and nutrient conditions, and physically weak biofilms were developed under high nutrient conditions, especially at low temperature. Rode et al. (2007) studied biofilm formation by *S. aureus* under different conditions relevant in food production, and the phenotypic and genotypic results showed highly diverse and complex patterns of biofilm formation in *S. aureus*. Food environment is characterized by several areas with significant micro-environmental differences (in temperature, pH, nutrient level and salt concentration), therefore it is important to identify the conditions under which microorganisms are able to survive, multiply and attach to surfaces, with regard to food processing, storage and distribution, in order to prevent biofilm formation.

The aim of this study was to analyze the effects of pH, concentration of glucose, concentration of NaCl and temperature on the biofilm formation by *L. monocytogenes*, *S. aureus*, *P. fluorescens*, *P. fragi* and *P. putida*. The combined effects of environmental parameters on biofilm formation were studied through a 5 levels-4 variables central composite design (CCD). The quantification of the biofilm formation was carried out by using the crystal violet assay and, in the case of *L. monocytogenes*, the viable count assay was also used, as well.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and culture conditions

Eight *L. monocytogenes* strains (Lm_1, Lm_4, Lm_278, Lm_287, Lm_288, Lm_SB, Lm_6E, and Lm_Scott A), eight *S. aureus* strains (St_037, St_059, St_117, St_132, St_137, St_174 and St_231) and three *Pseudomonas* strains (*P. fluorescens* Ps_019, *P. fragi* Ps_053 and *P. putida* Ps_071) were used as test organisms (see List of Strains). In a preliminary phase the strains were classified based on their biofilm-forming ability in TSB broth in microtiter plates as described by Stepanović et al. (2000) (Table 2.1).

The biofilm formation tests for CCD were carried out in three biological replicates in 96-well polystyrene flat bottom microtiter plates in culture broth modified in composition according to the established concentrations of glucose and NaCl and pH. In order to avoid any alteration of the modified culture media following to autoclave sterilization, sterile modified TSB or LB broths were obtained by 0.2 µm filtration.

Each well was filled with 200 µL of modified TSB for *L. monocytogenes* and *S. aureus* strains and modified LB broth for *Pseudomonas* spp. strains, and inoculated with 10 µL of each overnight culture (~ 10^8 CFU/mL). For each experiment, eight wells were filled with each strain. Eight wells were used as controls and filled with not inoculated 200 µL of each condition. The microtiter plates were incubated for 48 hours at the requested temperature. At the end of the incubation, the microtiter plates were treated and the wells stained, as reported in paragraph 2.2.1. For *L. monocytogenes*, which is a foodborne pathogen that can cause a severe disease with a high case fatality rate, the quantification of the biofilm cells after incubation was also carried out using the Drop Plate method (paragraph 2.2.3). For each condition, three wells were used to quantify the biofilm cells.

3.2.2 Central Composite Design and statistical analysis

The combined effects of temperature, pH, concentration of NaCl and concentration of glucose (factors) on biofilm formation were studied through a 5 levels-4 variables Central Composite Design (CCD) planned for each species tested (Table 3.1). For each strain a total of 30 conditions were tested, each of which was performed three times.

Table 3.1 Coded levels of experimental design

Coded levels	Temperature (° C)	pH	% NaCl (wt/vol)	% glucose (wt/vol)
		L. monocytogenes		
-2	4	4.5	0.50	0.25
-1	7	4.9	2.25	2.25
0	10	5.3	4.00	4.25
+1	13	5.7	5.75	6.25
+2	16	6.1	7.50	8.25
		S. aureus		
-2	17	5.0	0.50	0.25
-1	22	5.5	1.50	1.25
0	27	6.0	2.50	2.25
+1	32	6.5	3.50	3.25
+2	37	7.0	4.50	4.25
		Pseudomonas spp.		
-2	8	4.5	0.00	0.00
-1	11	5.0	1.00	1.00
0	15	5.5	2.00	2.00
+1	18	6.0	3.00	3.00
+2	20	6.5	4.00	4.00

3.2.3 Statistical analysis

The data were statistically analysed using Statistica 8.0 (StatSoft, Tulsa, Oklahoma, USA).

3.3 RESULTS AND DISCUSSION

Environmental factors including temperature, salt, pH and nutrients, which are common in foods and food-processing environments can have a big impact on microbial adhesion to surfaces and biofilm formation. These environmental factors, e.g. temperature, can exist in a wide range of food plants, so it could be very useful to obtain information about the behaviour of microorganisms in food plants with very different environmental conditions, for example in cooling areas as opposed to sites near thermal treatment plants, or sites near brines as opposed to areas with a very low presence of NaCl. For these reasons, the effect of pH, temperature, % glucose and % NaCl on biofilm formation by well-known food pathogens and food spoilers was investigated. For each microbial species, a different range of the tested variables was chosen, based on considerations linked to the physiological features of microorganisms and to their potential development in specific sites of the food plants.

In a preliminary phase, *L. monocytogenes*, *S. aureus* and *Pseudomonas* spp. strains were classified according to their ability to form biofilm (Table 3.2).

Table 3.2 Biofilm-forming ability of *L. monocytogenes* (Lm), *S. aureus* (St) and *Pseudomonas* spp. (Ps) strains. Biofilm-forming ability: +, weak; ++, moderate; +++, strong

Strain	Biofilm-forming ability
Lm_1	+++
Lm_4	+++
Lm_278	+++
Lm_287	+++
Lm_288	+++
Lm_SB	+++
Lm_6E	+++
Lm_Scott A	+++
St_037	+++
St_059	+++
St_117	+
St_132	++
St_137	+++
St_174	++
St_231	++
St_DSMZ 20231	++
Ps_019	+++
Ps_053	++
Ps_071	+

All the strains tested were able in forming biofilm on plastic surfaces, though at different levels. For example, all *L. monocytogenes* showed to be strong biofilm-producers, while within *S. aureus* and *Pseudomonas* spp. moderate and weak biofilm producers were present, as well. These results, obtained under optimal cultural conditions, confirm previous findings, which showed that *L. monocytogenes*, *S. aureus* and *Pseudomonas* spp. are able to form biofilm on plastic surfaces (Stepanović et al., 2000; Djordjevic et al., 2002; Simões et al., 2003). Polystyrene, the material used to produce microtiter plates, is a hydrophobic material, and it has been shown that most microorganisms adhere in high numbers to more hydrophobic materials (Donlan, 2002). The microtiter plate assay is widely used to screen the biofilm forming ability of microbial strains. It has to be underlined that, if some environmental parameter is modified, for example temperature or incubation time, it is possible to obtain different results. This is the case of strain Lm_ScottA, which resulted a strong biofilm former, while under different conditions previously used it resulted a moderate former (see Figure 2.4).

The experimental plan used to study the combined effects of environmental and nutritional parameters on the biofilm formation was a CCD, which could completely describe the influence of these factors and their interactions on the ability to produce biofilm by *L. monocytogenes*, *S. aureus* and *Pseudomonas* spp. The advantage of using a CCD is to obtain the highest amount of information while limiting the number of experiments to be carried out. The CCD can describe the whole influence of the applied conditions and their interactions on the ability to produce biofilm by the tested microorganisms. It has to be noted that only little information is available in the literature about the synergistic effects of the environmental parameters on biofilm formation regarding the food area. In fact, most authors tested only two or three combined variables for a limited number of strains (Rode et al., 2007; Nilsson et al., 2011).

The results obtained were statistically treated in order to identify the most significant factor that affects biofilm formation in polystyrene plates. For each species the Pareto charts and 3-D response surface plots are reported. Pareto charts report the statistical significance and the size (expressed as standardized effect estimate) of each factor on the dependent variable considered (OD_{570} or Log CFU/cm^2). The size of the standardized effect estimate is a measure of the factor influence intensity, while the positive or negative sign indicates whether the dependent variable is positively or negatively affected by the factor. The 3-D surfaces illustrate the effects of two factors on the variable; a constant value is set for the 3rd and the 4th factor.

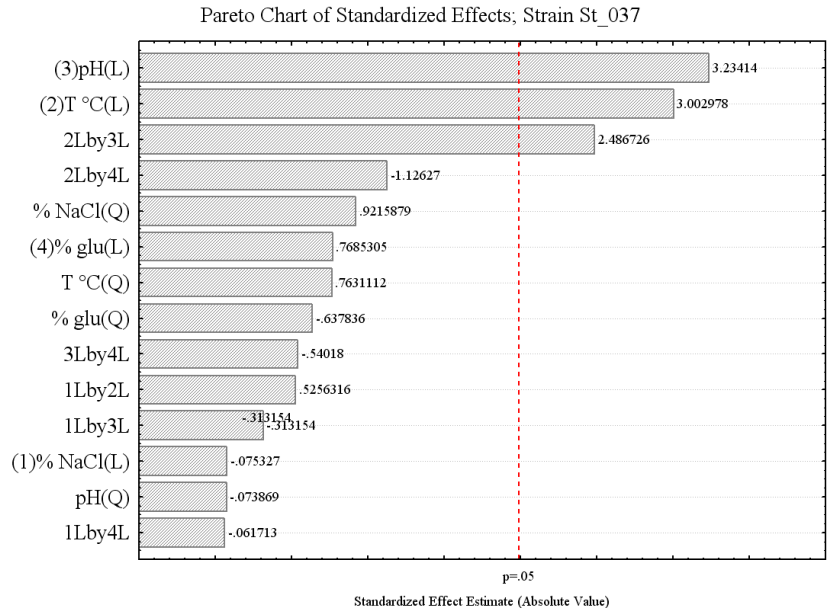
3.3.1 *Staphylococcus aureus*

S. aureus is an opportunistic human pathogen that can cause a foodborne intoxication reported as one of the most common bacterial foodborne diseases in several countries (Balaban and Rasooly, 2000). This microbial pathogen is very adaptable and can live in a wide variety of environments, including food plant surfaces, thanks to its ability to adhere on surfaces and to form biofilm. This may contribute to the persistence of *S. aureus* in the food processing environments, consequently increasing cross contamination risks. Indeed, it is quite commonly isolated from surfaces (Marino et al., 2011).

The data obtained showed that temperature strongly influenced (p<0.05) the formation of biofilm by all strains (Figure 3.1 to Figure 3.8). It should be noted that a commonly used temperature in biofilm experiments with *S. aureus* is 37 °C, its optimum temperature of growth. However, in the food production environment, temperatures below 37 °C are relevant: this is why in this study different temperatures ranging from 17 to 37 °C were tested. Within this range, the highest amounts of biofilm formed were observed at the highest temperatures, regardless of biofilm forming ability. This observation is opposite from those made by other authors. In particular, Rode et al. (2007) and Pagedar et al. (2010) evidenced that biofilm production is highest at suboptimal growth conditions. However, recently it has been reported that, within a collection of twenty-eight strains isolated from seafood, most of the strains had a higher biofilm production at 37 °C than at 25 °C (Vázquez-Sánchez et al. 2014). This disparity might be attributed to different experimental setups, strain specific behaviour and other factors like enhanced biofilm/EPS production and altered cell surface hydrophobicity, which has been observed for other pathogens under stressful conditions (Costerton et al., 1995). In any case, despite the temperatures used to prolong the shelf-life of foods are considerably lower than those tested in our study, it has to be highlighted that in food processing plants there are areas where temperatures can be much higher, considering geographical and seasonal variations, as well.

S. aureus is a poor competitor in foods and presents the lowest risk in fermented foods, where safety is assured by low pH values granted by lactic acid bacteria metabolism. In fact, survival times generally increased with increased pH (Whiting et al., 1996). Unexpectedly, as far as biofilm formation is concerned, the effect of pH levels of the growth medium has received only little attention. The only data available reported the influence of both acidic and alkaline pH on biofilm formation by clinical strains (Zmantar et

al., 2001). The data obtained in this study showed that biofilm formation seems to be inhibited by lowering the pH in the range from pH 7.0 to pH 5.0. This factor was significant ($p<0.05$) for four out of eight strains. Interestingly, for strains St_037 (Figure 3.1) and St_137 (Figure 3.5) an interactive effect between temperature and pH was observed. In particular, for strain St_037 at 37 °C the biofilm formation was highest at pH 7, whereas at 17 °C the biofilm formation appeared stimulated by the lowest pH tested. A similar behaviour was observed by Rode et al. (2007). Strain St_137, instead, at the highest temperatures produced more biofilm at pH 7, whilst at 17 °C the biofilm formation appeared stimulated at intermediate pH levels (i.e. pH 6.0).



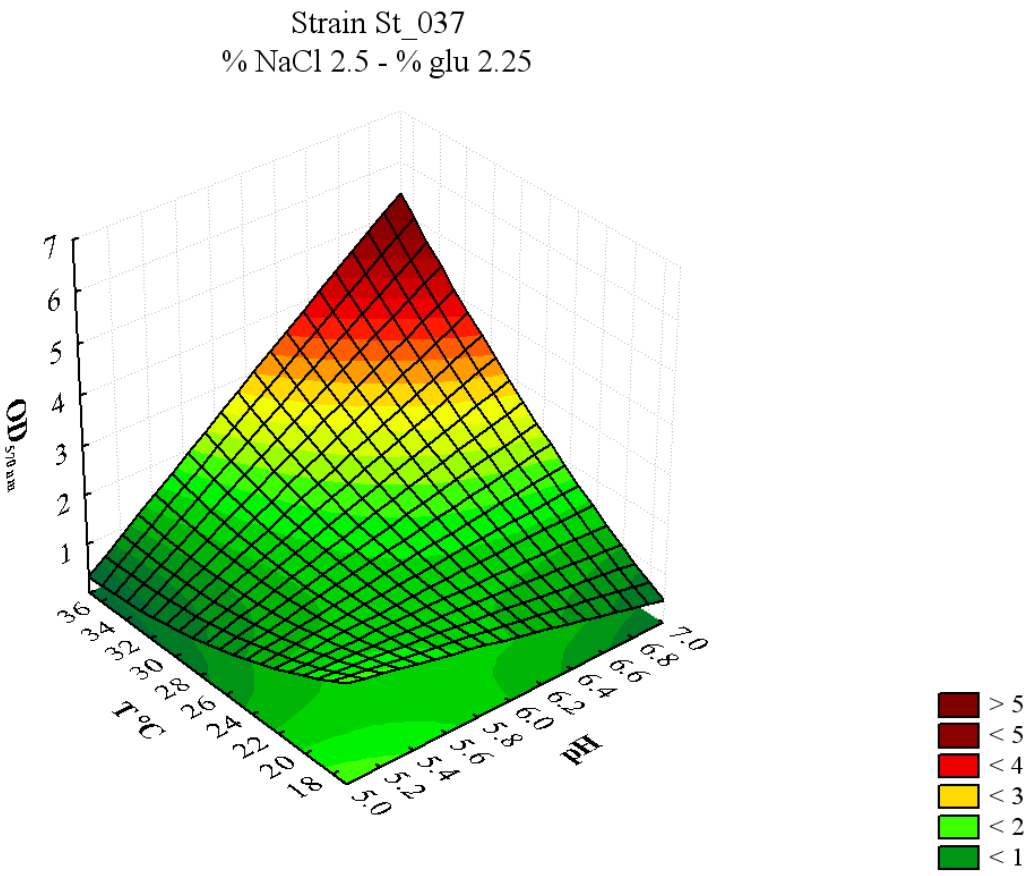
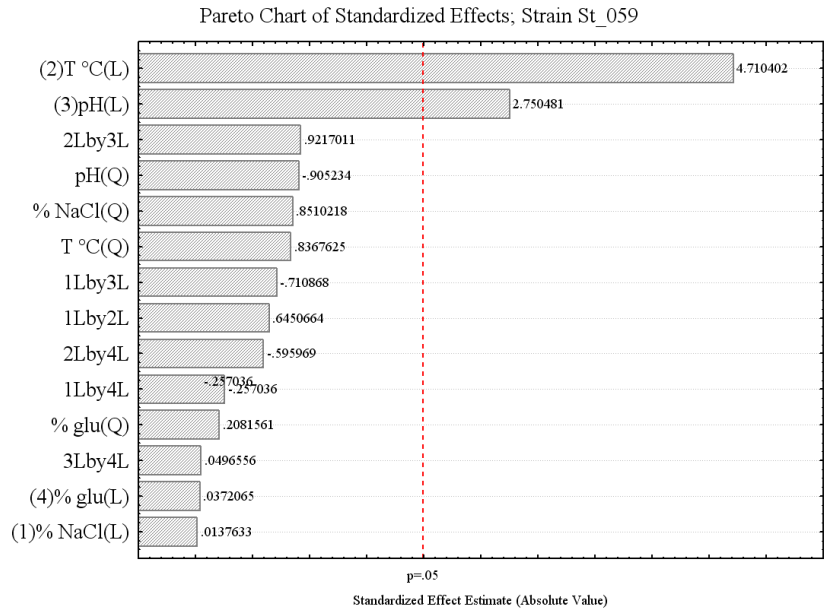


Figure 3.1 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation by *S. aureus* St_037 strain



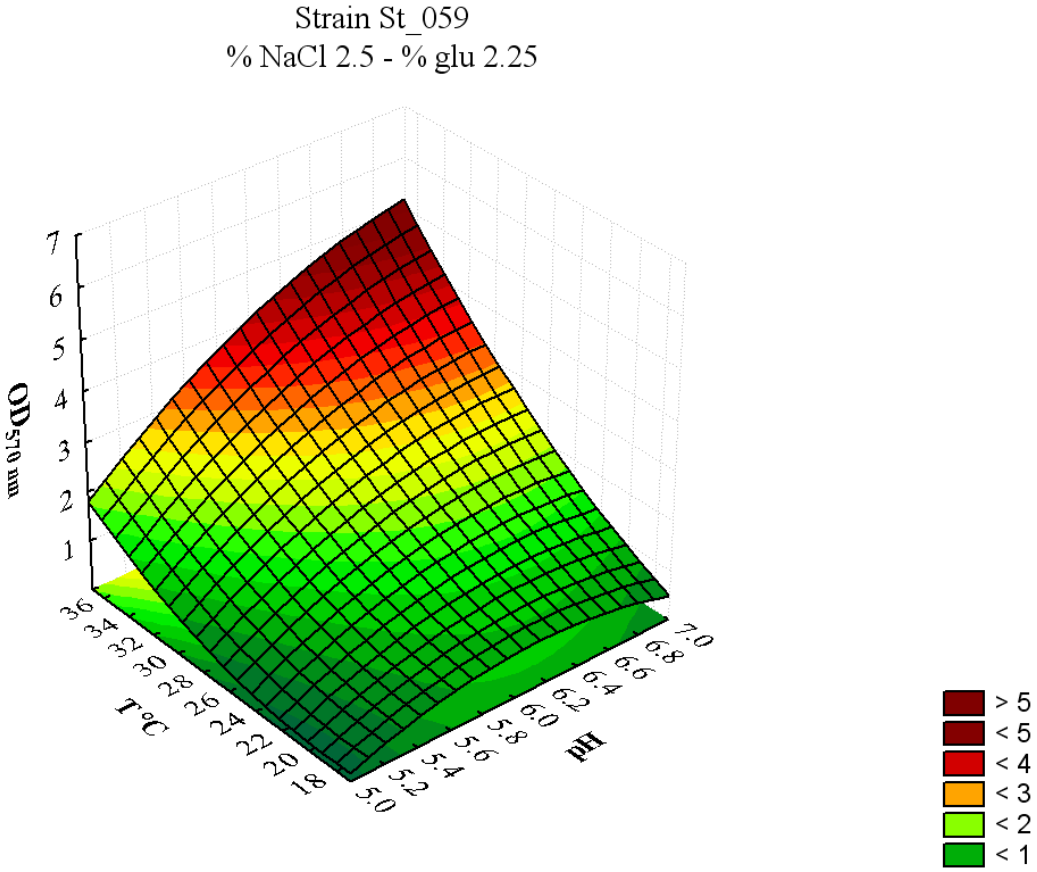
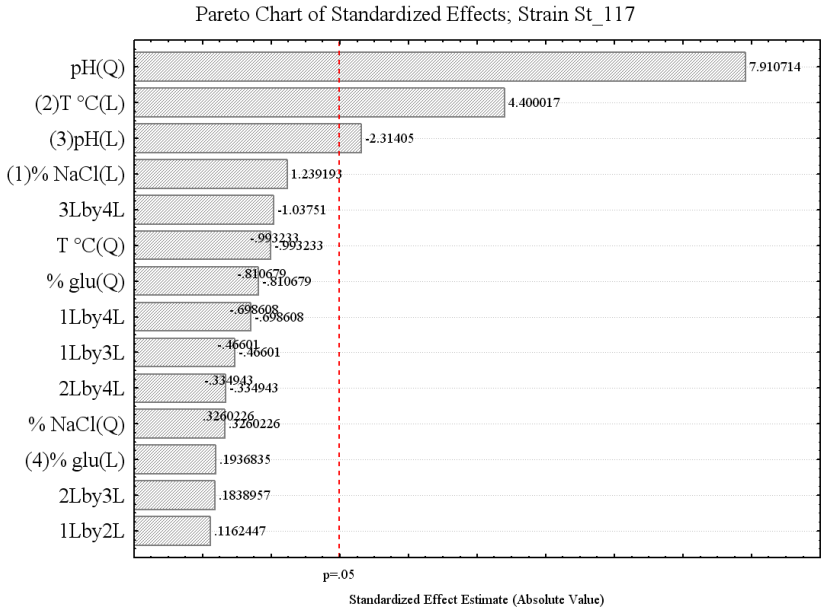


Figure 3.2 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation by *S. aureus* St_059 strain



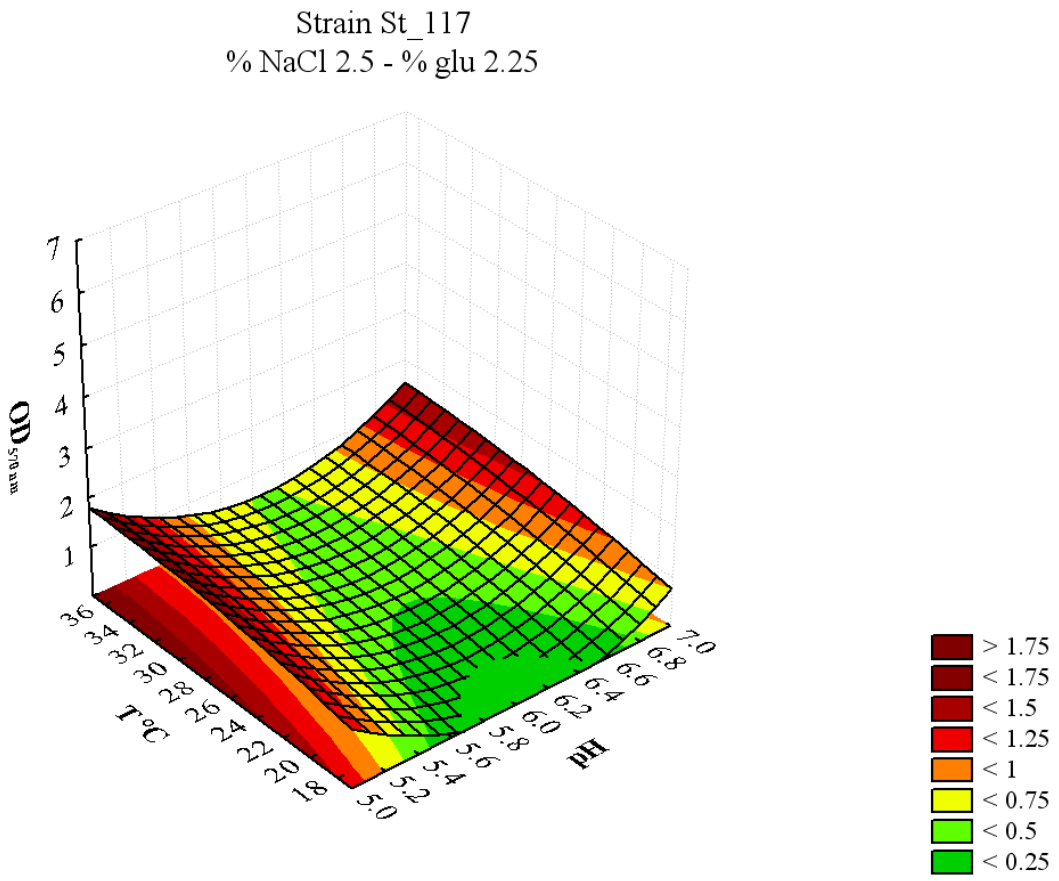
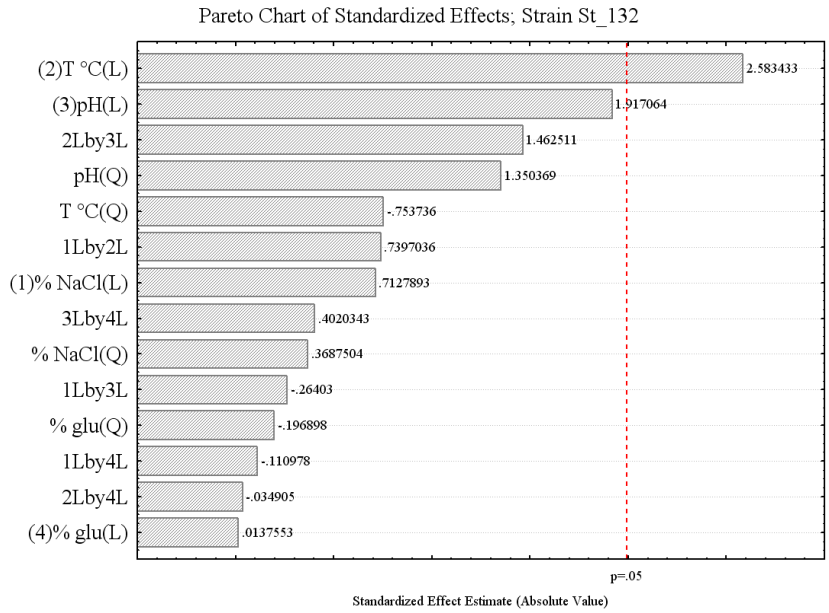


Figure 3.3 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation by *S. aureus* St_117 strain



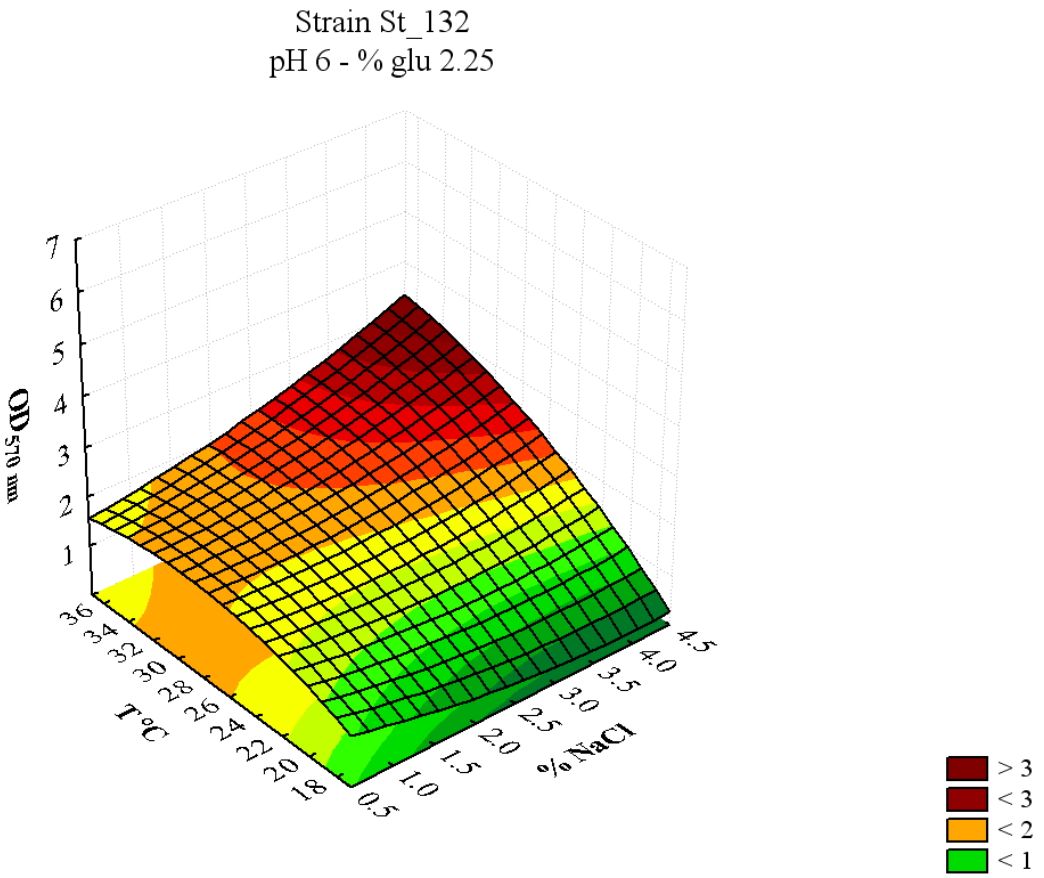
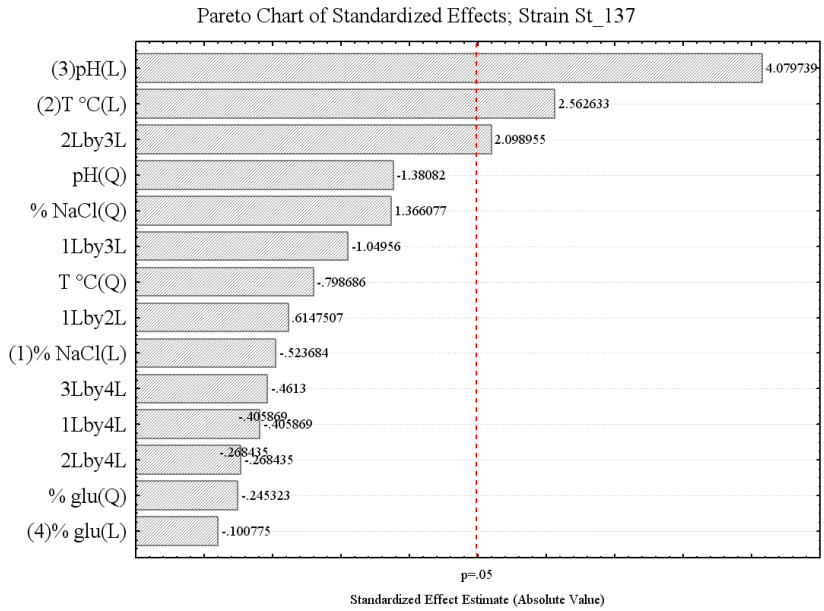


Figure 3.4 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation by *S. aureus* St_132 strain



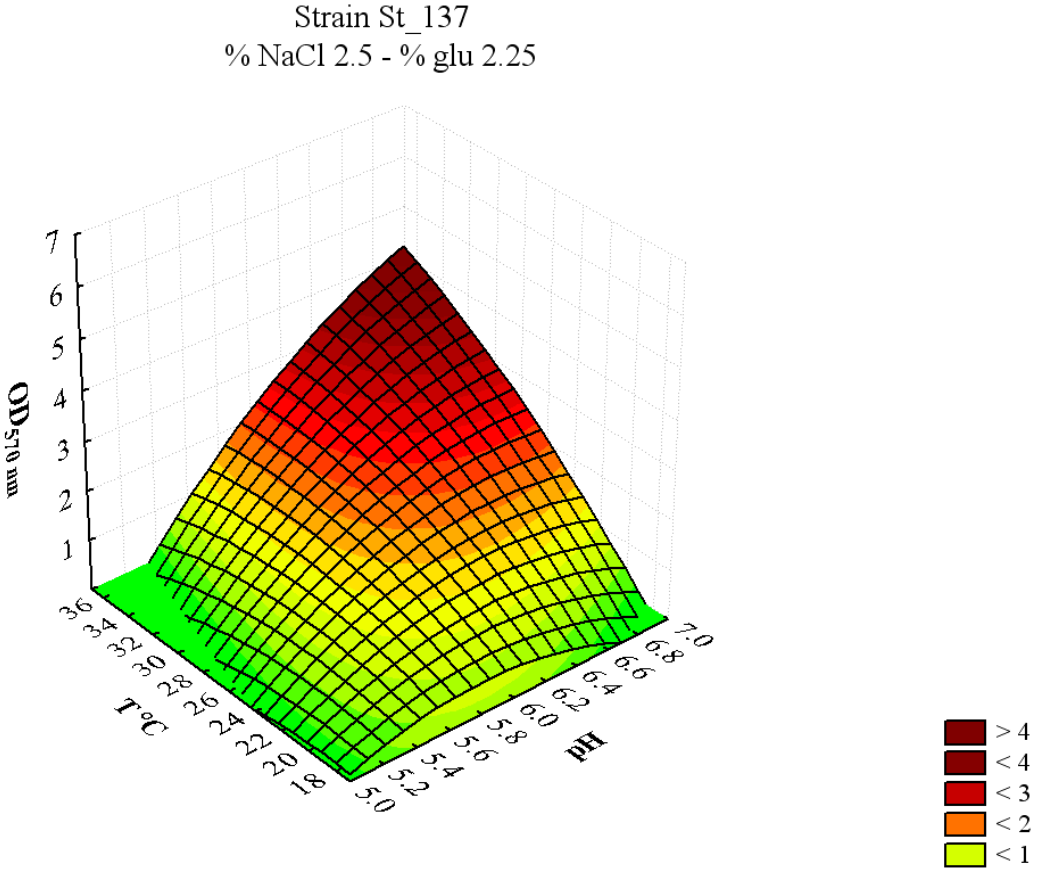
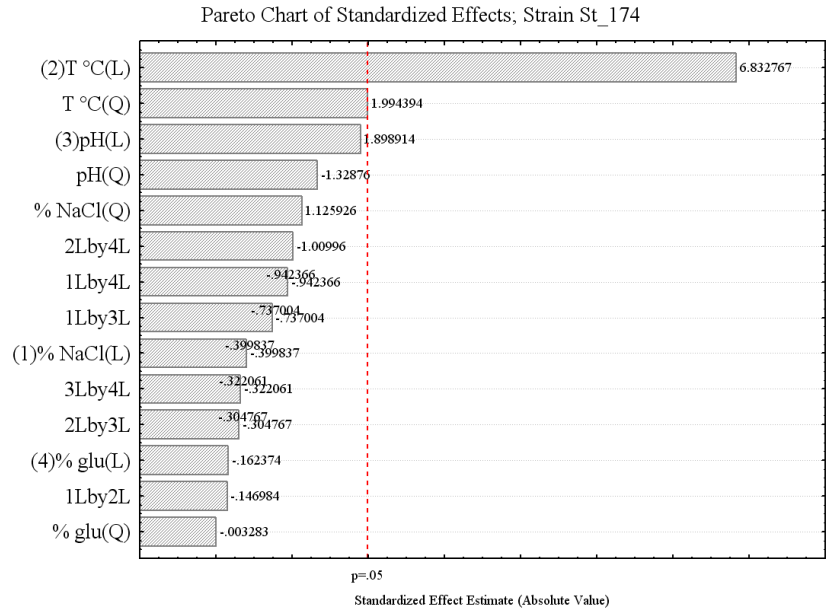


Figure 3.5 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation by *S. aureus* St_137 strain



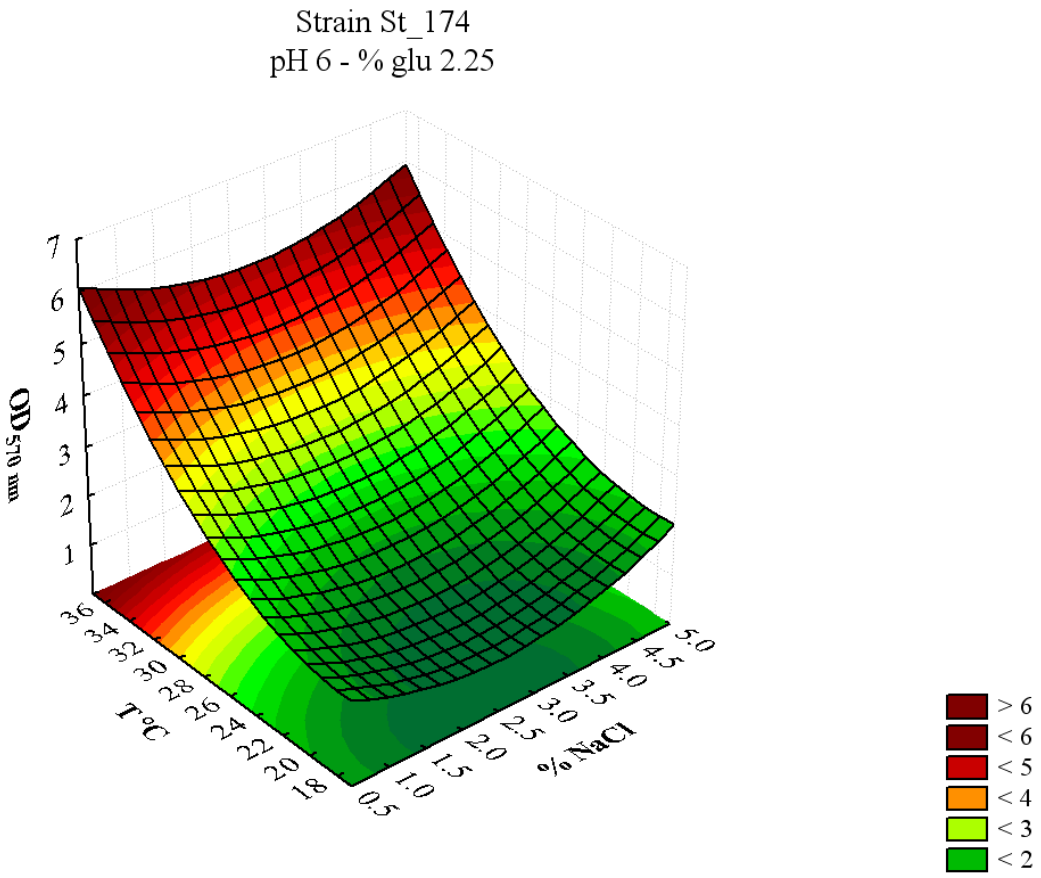
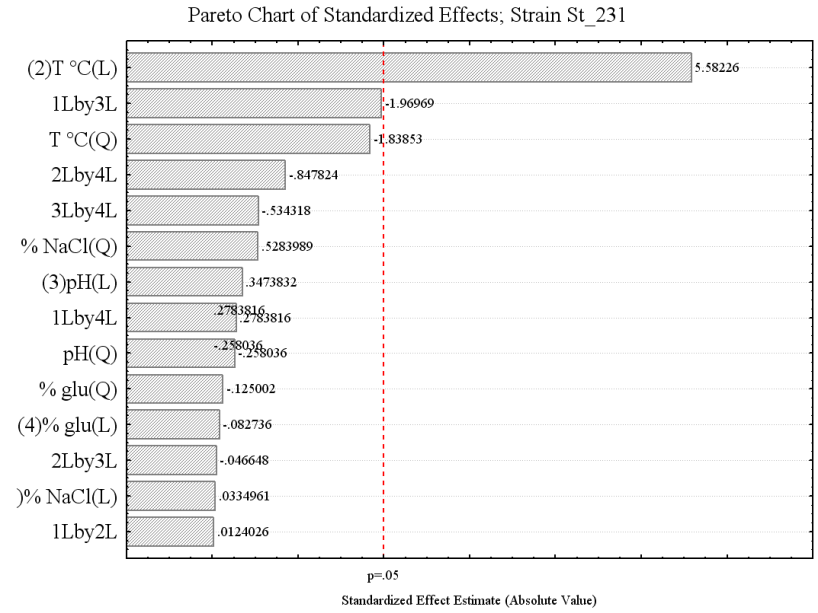


Figure 3.6 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation by *S. aureus* St_174 strain



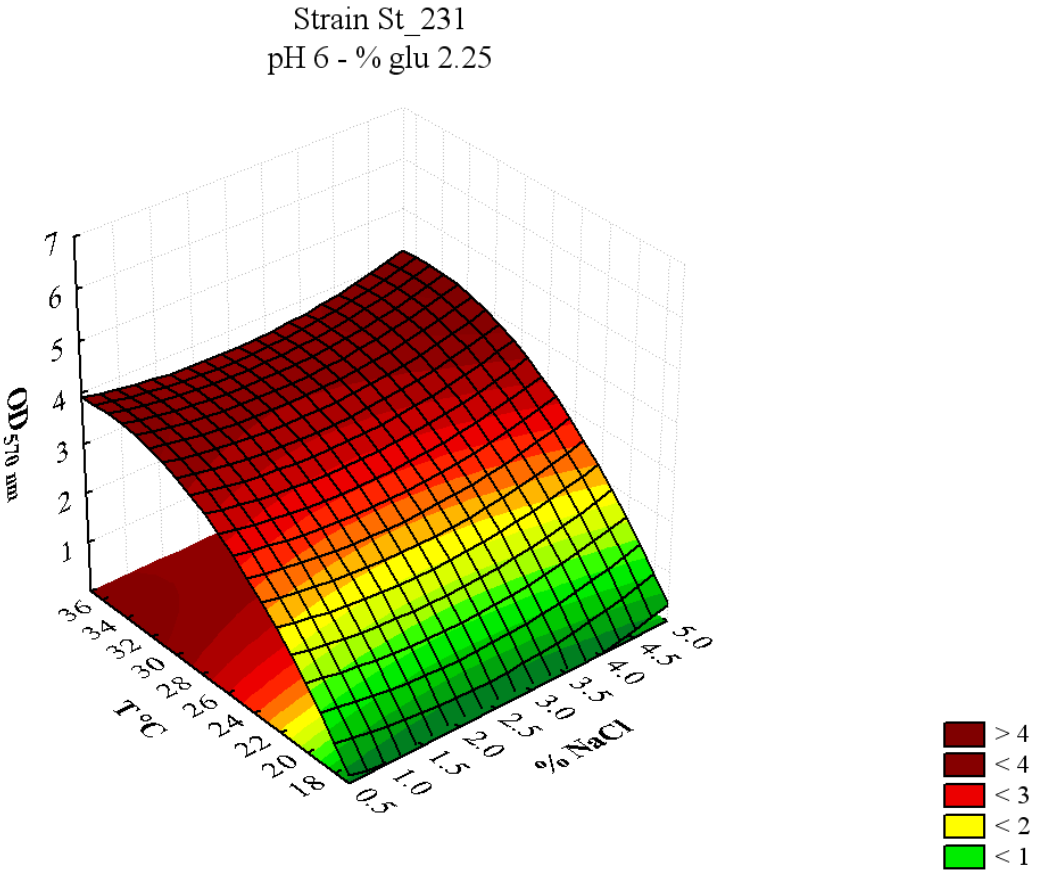
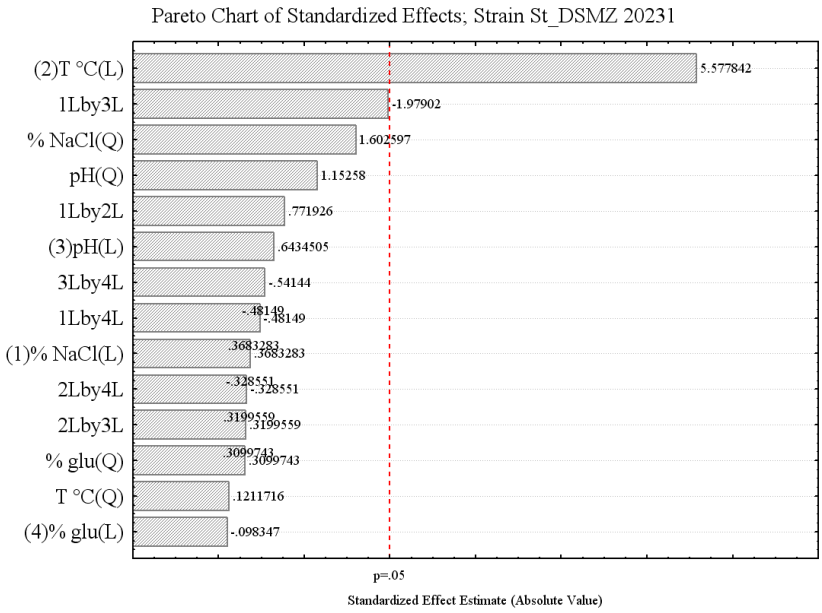


Figure 3.7 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation by *S. aureus* St_231 strain



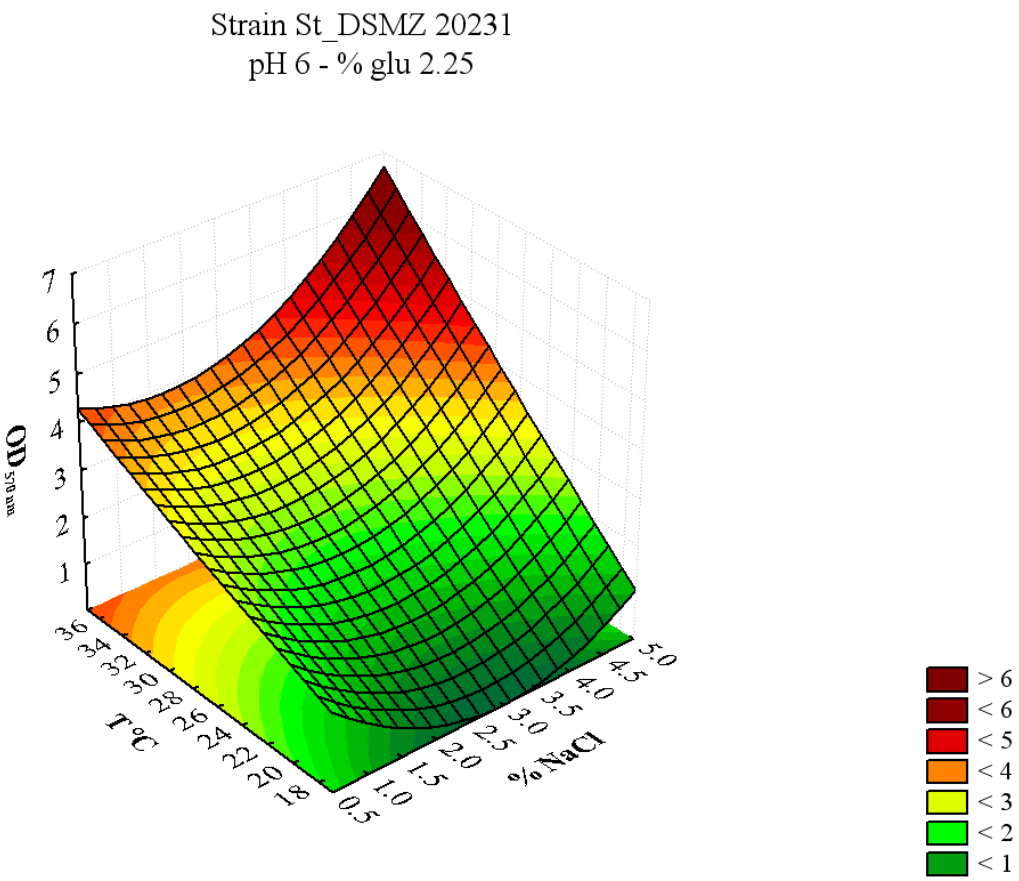


Figure 3.8 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation by *S. aureus* St_DSMZ 20231 strain

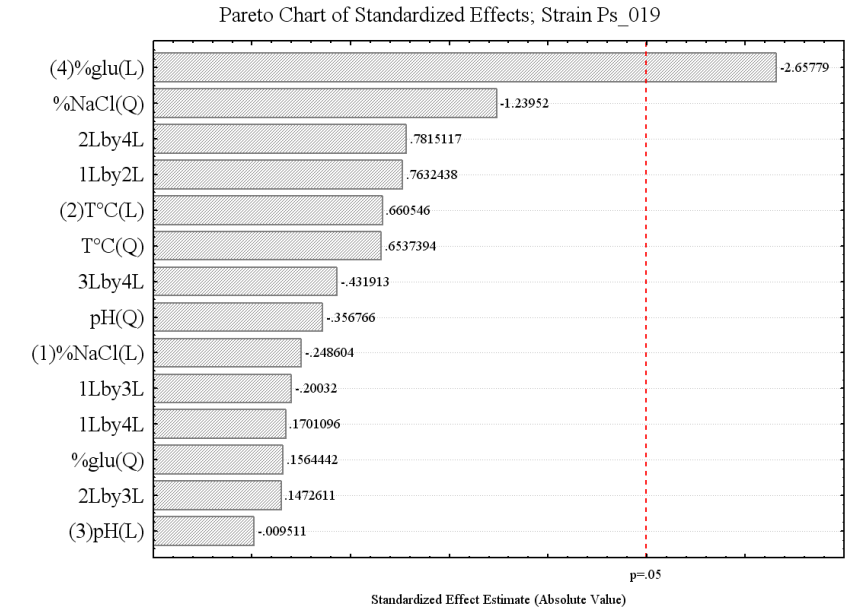
In the experimental model used in this study, glucose (0.25-4.25%) and NaCl (0.5-4.5%) concentration did not significantly influence the biofilm formation by *S. aureus*, either considered separately or in combination. As regards glucose, the highest attachment rates were observed at high sugar levels, even if the OD_{570} values remain quite low. Rode et al. (2007) found that adding 5% of glucose to TSB caused a

more dense biofilm to be produced, and the combination of glucose and NaCl enhanced much more the attachment rate. A similar observation was made by Vázquez-Sánchez et al. (2013), probably due to the sugar requirement during the EPS components production. *S. aureus* is highly salt tolerant and has been reported to grow in NaCl concentrations up to 25% (Stewart et al., 2002). In our study *S. aureus* was differently affected by NaCl concentrations, i.e. some strains produced the highest biofilm levels at low concentrations, whereas others did so at the highest. Different authors showed that NaCl could promote bacterial aggregation, and enhance the stability of biofilm on polystyrene (Rode et al., 2007; Møretrø et al., 2003). For some strains the effect of salt on biofilm formation was markedly affected by incubation temperatures. Thus, a positive correlation between biofilm formation and salt concentration was observed at 37 °C, while at 17 °C strains appeared stimulated by the lowest concentrations. The presence of high concentrations of NaCl is relevant in food industry, for example in the case of brines used in cheesemaking practices, so investigating the effect of this factor could be considered valuable.

3.3.2 *Pseudomonas* spp.

Pseudomonas spp. are ubiquitously present in nature, they are easily spread through food production systems, and contamination with this microbial genus is almost inevitable. The genus *Pseudomonas* is of great concern in the food industry, because it produces proteases, lipases, pectinases as well as pigments and slimes, which can result in food spoilage, mostly in refrigerated foods (Rajmohan et al., 2002). Members of the genus *Pseudomonas* have frequently been reported to produce exopolymers, and they have the ability to attach rapidly to surfaces in the food industry, where they are frequently found (Leriche et al., 2004). Within the *Pseudomonas* genus, *P. aeruginosa* is known for its ability to form biofilms on abiotic surfaces (Giltner et al., 2006), but little is known about the biofilm-forming capacity of *Pseudomonas* spp. isolated from food environments. Moreover, there are only few reports about the effect of environmental factors on biofilm formation by *Pseudomonas* spp. and in particular about the interaction between the environmental stresses on biofilm production.

Pseudomonas strains tested in this study belonged to different species (*P. fluorescens*, *P. fragi* and *P. putida*), and they were differently affected by the environmental factors (Figure 3.9 to Figure 3.11).



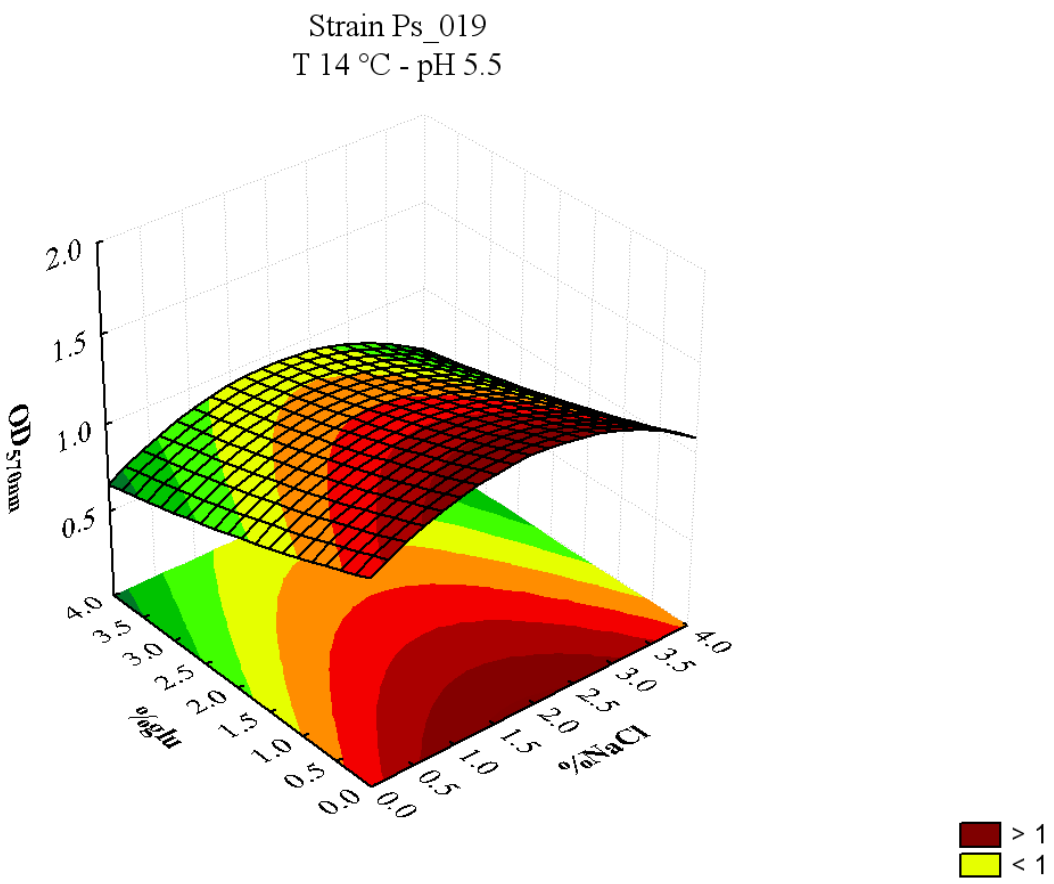
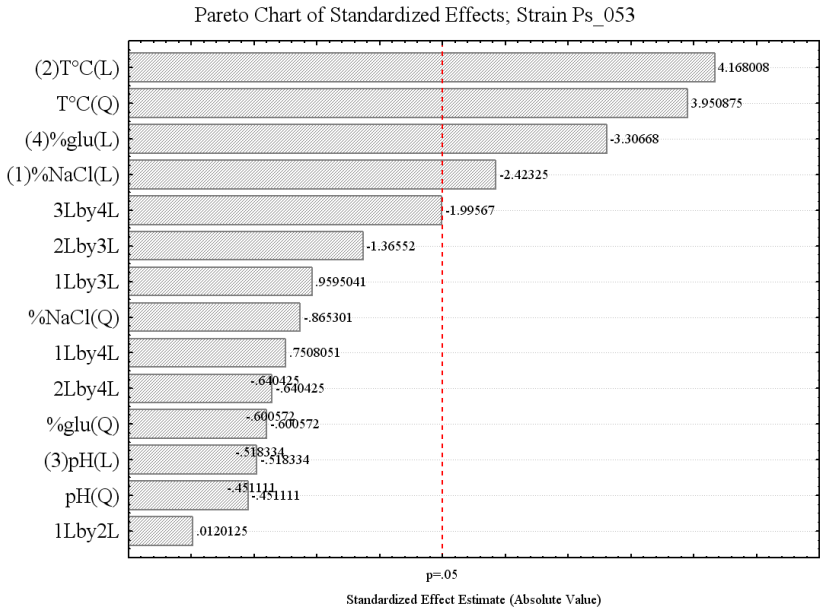


Figure 3.9 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation by *P. fluorescens* Ps_019



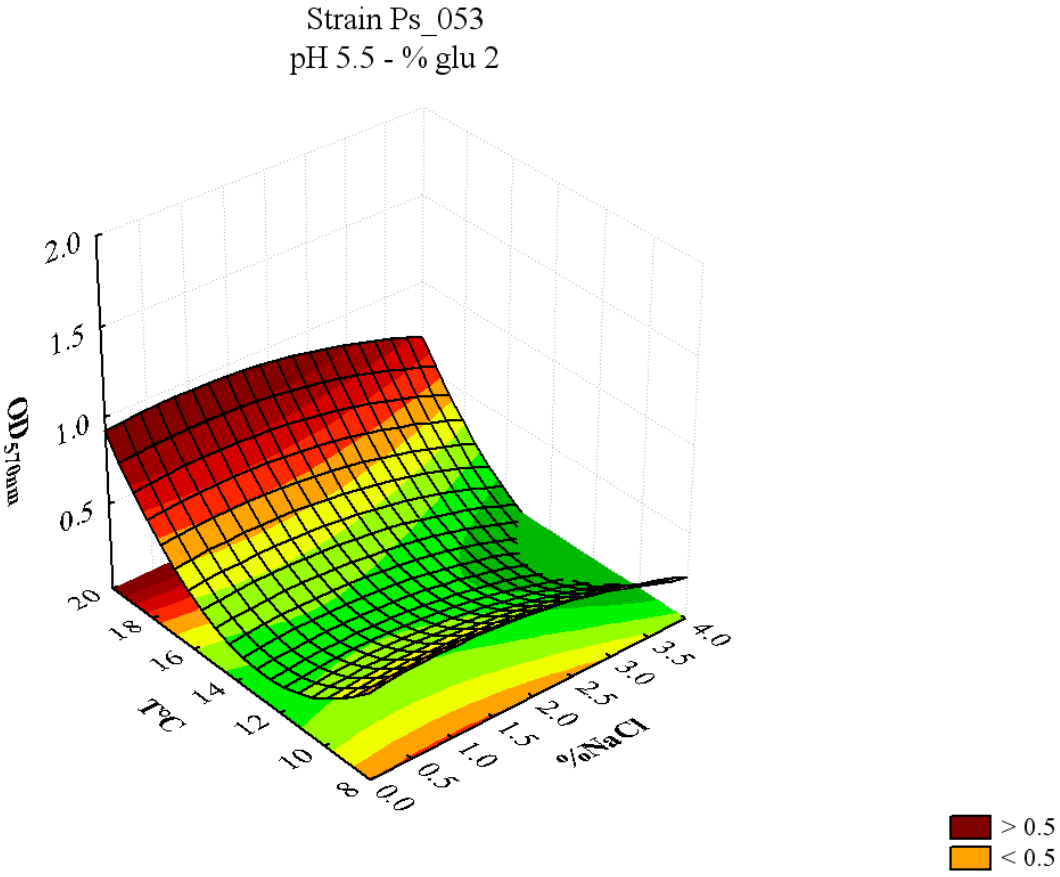
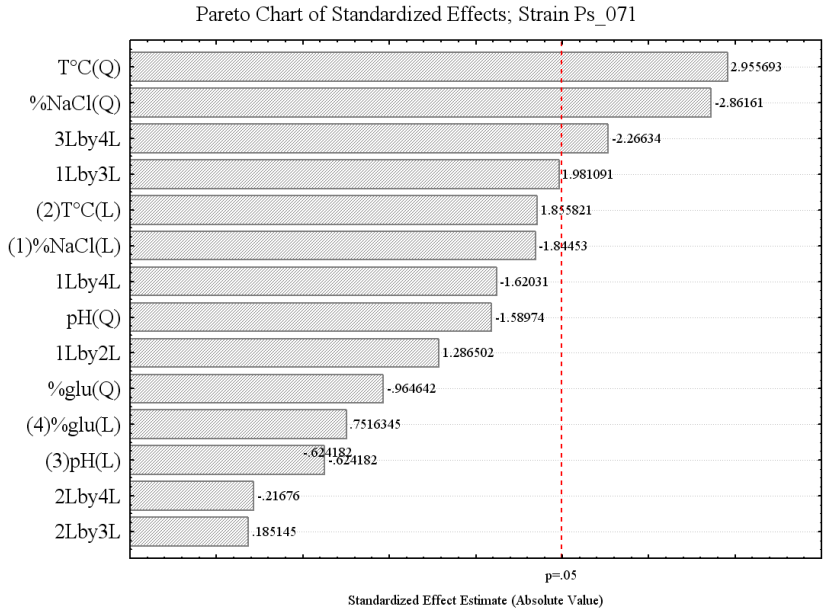


Figure 3.10 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation by *P. fragi* Ps_053



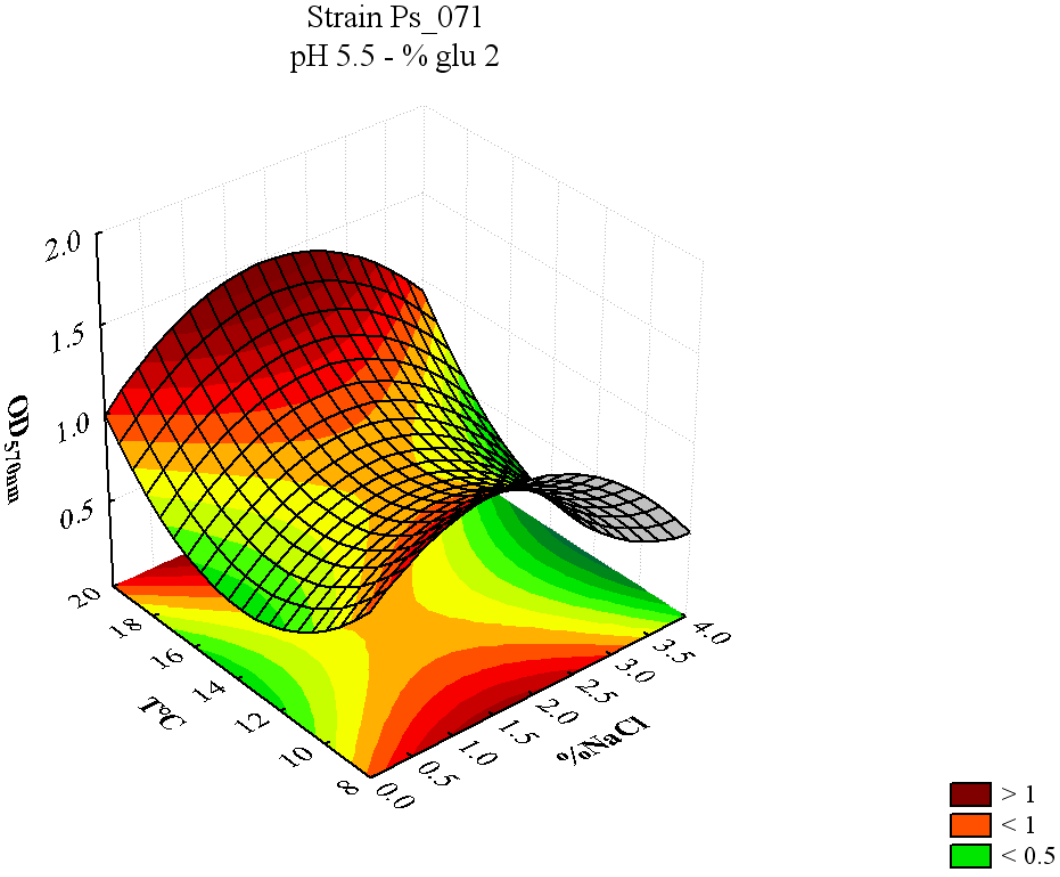


Figure 3.11 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation by *P. putida* Ps_071

P. fluorescens Ps_019 produced high amounts of biofilm in the entire range of T, pH and NaCl % tested (Figure 3.9), so its behaviour was not affected by those environmental factors. This microbial species is isolated quite frequently from diverse food products, such as vegetables, fish, meat and dairy products, and this behaviour could be regarded as a high metabolic flexibility. Indeed, essentially any habitat with a

temperature range of 4-42°C, a pH between 4 and 8 and containing simple or complex organic compounds is a potential habitat for *P. fluorescens* (Gennari and Dragotto, 1992; Arslan et al., 2011). This adaptability, observed in this study also for the sessile state, might be one reason for the ubiquitous character of this species in the food field. Interestingly, enhancing the glucose concentration in the tested range (0-4%) resulted in an inhibition of surface colonization. From a hygienic point of view, it could be hypothesized that such a strain could efficiently colonize food contact surfaces free of soil after the application of a routine sanitation program. To our knowledge, this is the first report on the effect of glucose concentration on the biofilm formation by *P. fluorescens*. The ability to form a more dense biofilm in deprived conditions of glucose can be due to a bacterial survival strategy in nutritionally limited environments. For other *Pseudomonadaceae*, i.e. *P. aeruginosa* and *P. putida*, it is reported that glucose could function as a promoter rather than a repressor of biofilm formation (Huang et al., 2009). A repressing effect of glucose on biofilm formation by inhibiting the expression of a gene that is involved in surface colonization was only reported for *Bacillus subtilis* (Stanley et al., 2003). *P. fluorescens* strain Ps_019, which was isolated from raw milk, formed high amounts of biofilm both at low (8 °C) and high temperatures (20 °C): this behaviour might contribute to its persistence in food plants, such as dairy-processing environments, where temperatures can be 10 °C or less (e.g. brining and ripening areas), or more than 15 °C (cheesemaking area).

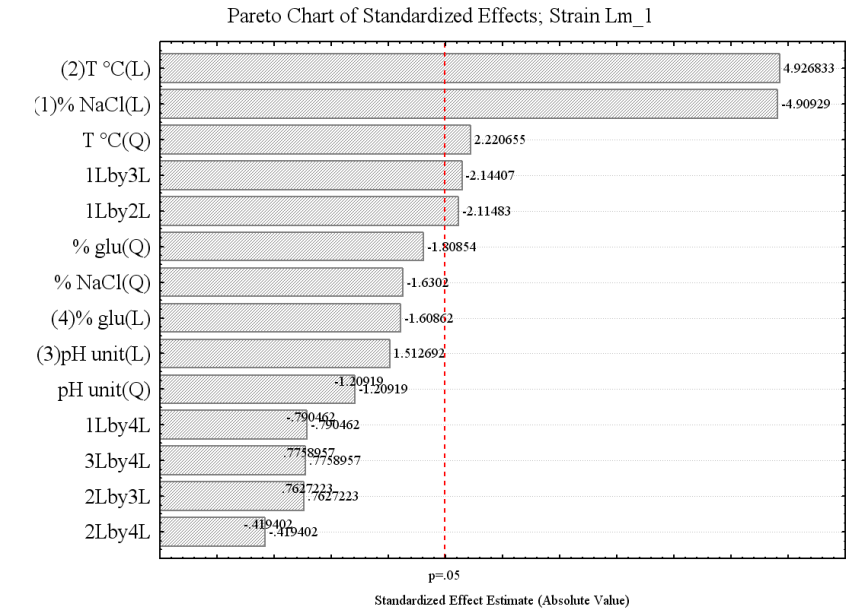
P. fragi Ps_053 was a less efficient surface-colonizer than *P. fluorescens* Ps_019 (Figure 3.10), and its biofilm formation was affected by temperature, glucose and NaCl concentrations. The highest biofilm production was limited to a narrow temperature range, between 18 °C and 20 °C, therefore this strain could be considered less hazardous for cooling areas of the food plants. As *P. fluorescens* Ps_019, *P. fragi* Ps_053 showed enhanced biofilm formation at a low glucose concentration, and it was inhibited by high salt amounts. A quite similar behaviour regarding the effect of temperature was observed for *P. putida* Ps_071, which showed a high biofilm-forming ability at temperatures above 18 °C (Figure 3.11). However, this strain resulted less affected by NaCl concentrations up to 2.5%, regardless of the pH value. A synergistic effect ($p<0.05$) of pH and glucose concentration was also observed for *P. putida* Ps_071, which produced the highest amounts of biofilm in the intermediate values of both factors, and the lowest at the extreme values.

Usually acidic pH resulted in strong inhibition of *Pseudomonadaceae*, such as in fermented foods (Carraro et al., 2011). However, according to the results of this study the sessile growth seemed to be quite unaffected by pH solely. This behaviour is suggestive for an indication of the potential risk related to the ability to colonize surfaces in a rather indiscriminate way, regardless of environmental conditions, and it could be linked to a protective effect of the high amounts of EPS produced by this microbial group. The ability to form biofilm on the surfaces of the food industry by *Pseudomonas* spp. is considered a potential risk of cross-contamination, which can cause the spoilage of food products. Furthermore, it has been shown that many psychrophilic microorganisms including *P. putida*, *P. fragi*, *P. fluorescens* and *Flavobacterium* can enhance the adhesion, colonization and the formation of biofilms by *L. monocytogenes*, by protecting the pathogen from desiccation (Daneshvar Alavi and Truelstrup Hansen, 2013).

3.3.3 *Listeria monocytogenes*

L. monocytogenes is a foodborne pathogen of particular concern to the food processing industry because of its ability to grow at refrigeration temperatures and its tolerance to environmental stresses, such as acidic pH and high salt concentration. In almost all cases of contaminated food, this pathogen can be isolated from the environment of the food plants from which the products originated, in particular in drains, floors and food-contact surfaces, wet and refrigerated food processing plants (Cox et al., 1989). This is explained by its ability to adhere to surfaces and to form biofilms that become less susceptible to cleaning procedures (Cloete, 2003). The biofilm eventually constitutes a reservoir of dissemination and cross-contamination in foods. It is hypothesized that environmental factors such as pH, water activity, temperature and nutrient composition of the food soil can be important for the phenotypic transition of planktonic cells to sessile form and for the consequent initial attachment to a surface. Therefore it is important to identify factors that influence the colonization of surfaces, in order to better understand the implication of biofilm formation to food safety.

As can be seen from the Pareto charts, in the range from 4 °C to 16 °C the temperature was the parameter that significantly affected the quantity of biofilm ($p<0.05$) in all the strains. In fact the standardized effects estimated were the highest for all the strains (Figure 3.12 to Figure 3.19 and Figure 3.27). In particular, increasing the temperature resulted in higher Log CFU/cm^2 and OD_{570} values, which is a measure of the total biomass (cells and EPS matrix) adhered to the plastic surface. It has been demonstrated that *L. monocytogenes* is flagellated and motile at temperatures below 30 °C, and generally non-flagellated and non-motile at temperatures above 30 °C. At low temperatures flagella production by *L. monocytogenes* may increase and it could be correlated to its adhesion ability to surfaces (Tresse et al., 2009). Moreover, biofilm formation is significantly influenced by temperature, probably modifying cell surface hydrophobicity (Di Bonaventura et al., 2008).



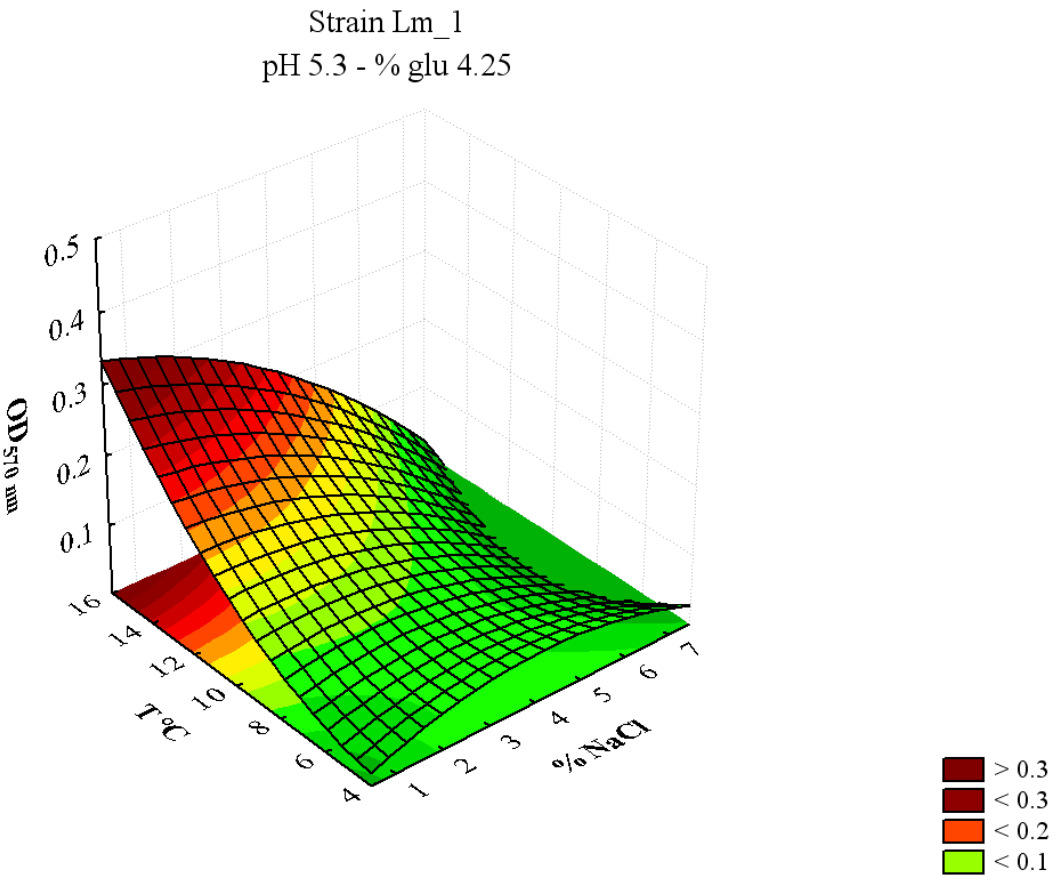
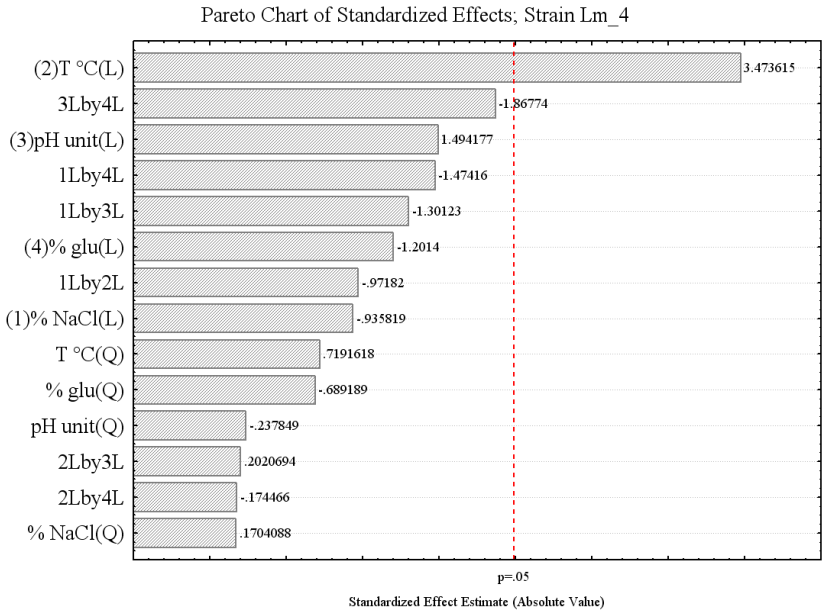


Figure 3.12 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation (OD_{570} by *L. monocytogenes* Lm_1 strain



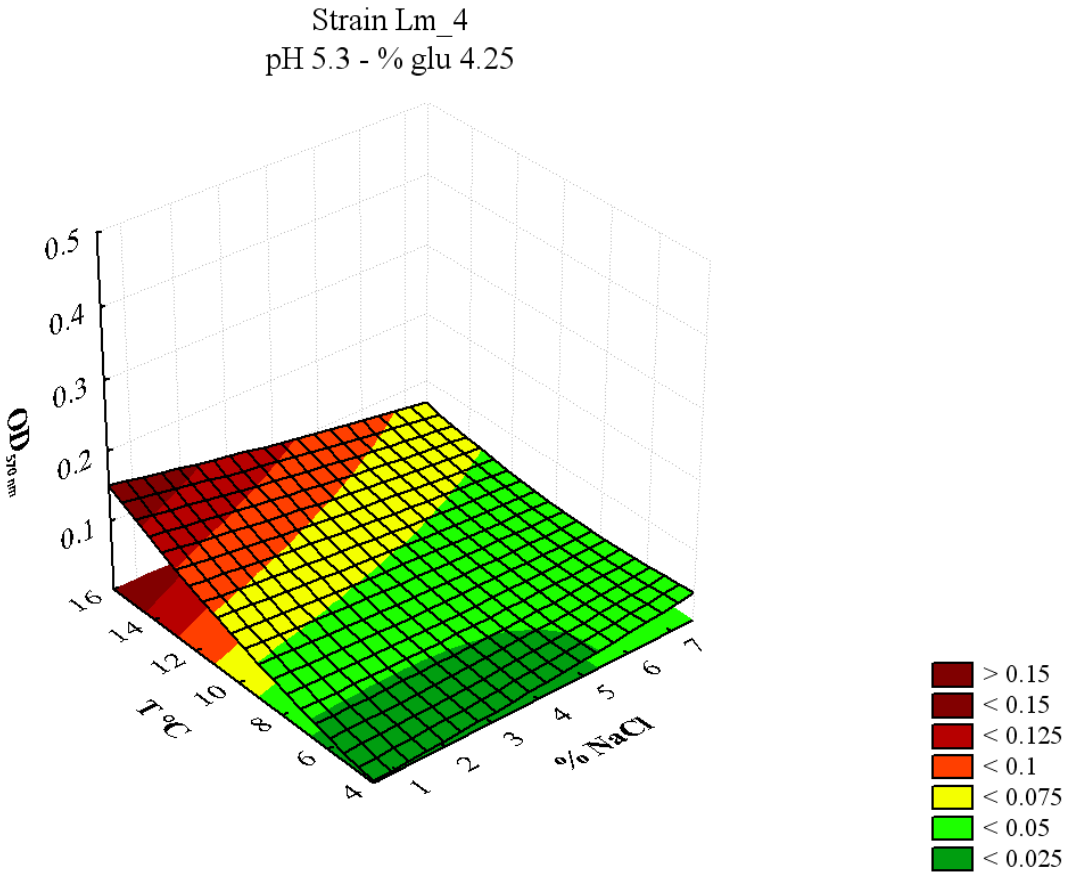


Figure 3.13 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation (OD_{570} by *L. monocytogenes* Lm_4 strain

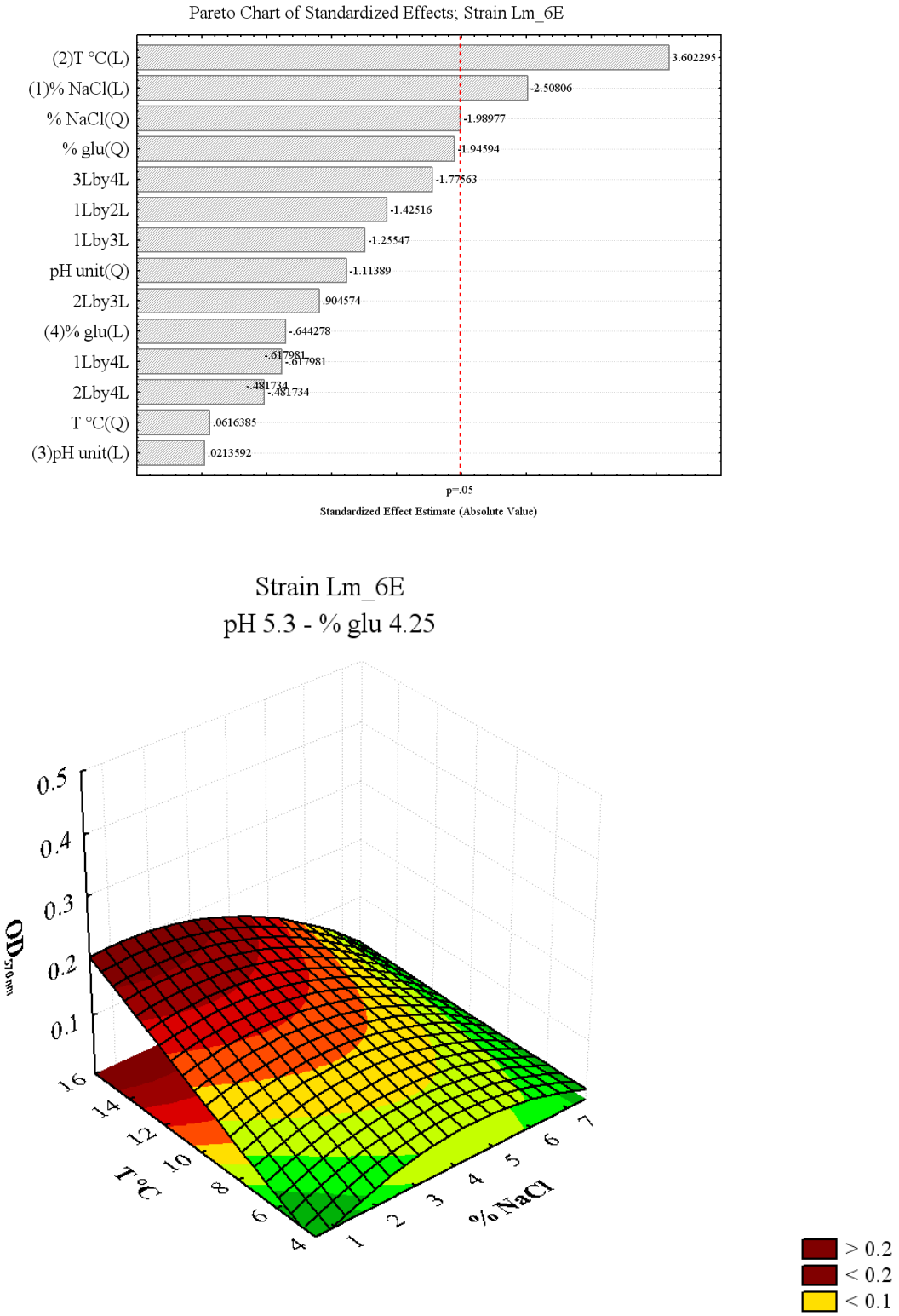
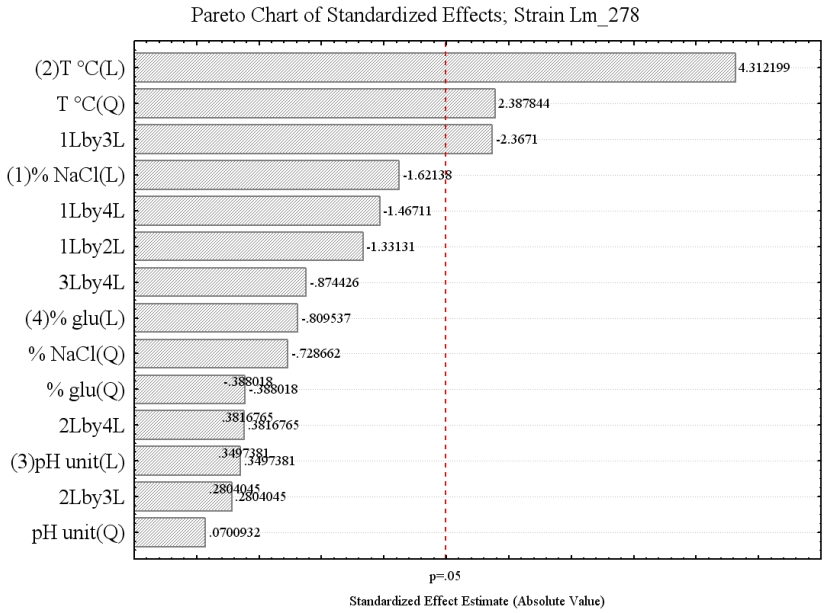


Figure 3.14 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation (OD_{570} by *L. monocytogenes* Lm_6E strain



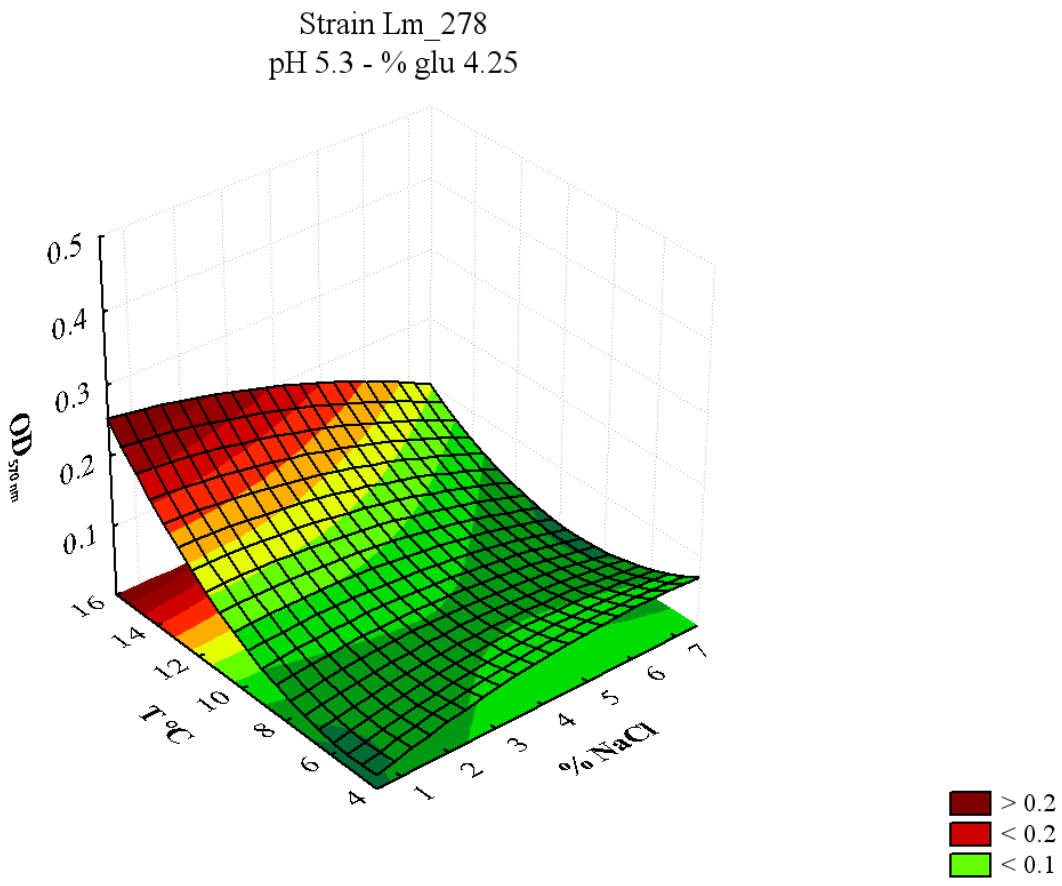
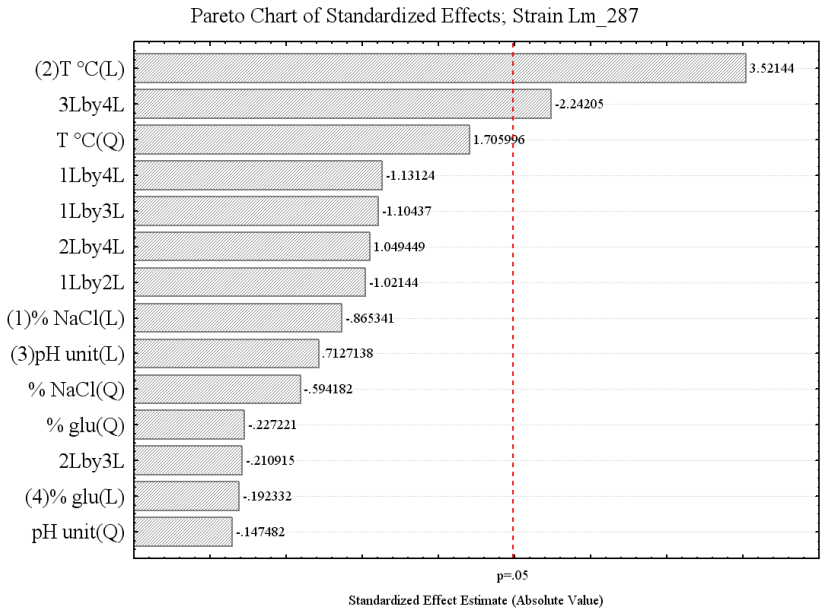


Figure 3.15 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation (OD_{570} by *L. monocytogenes* Lm_278 strain



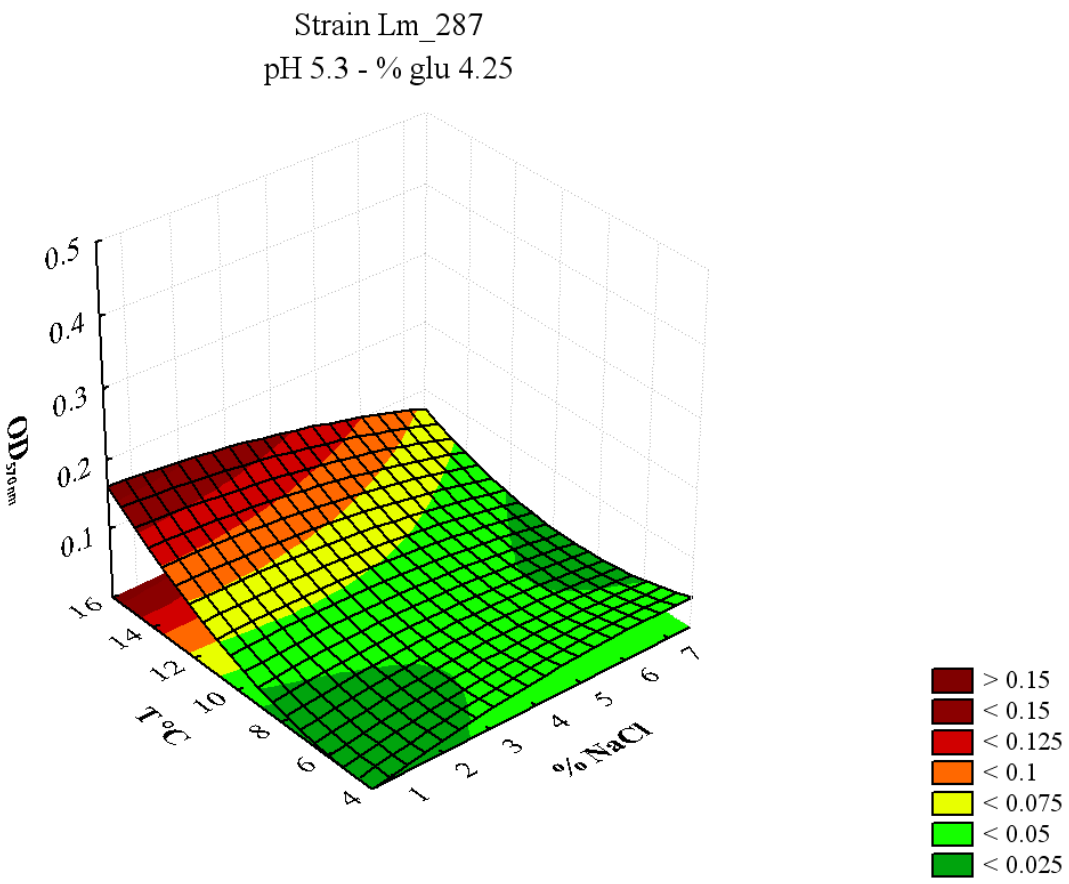
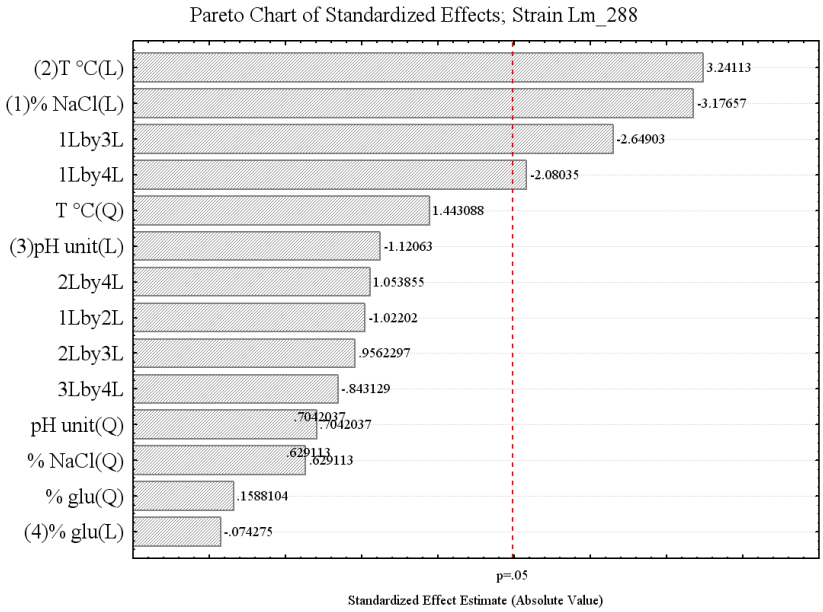


Figure 3.16 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation (OD_{570} by *L. monocytogenes* Lm_287 strain



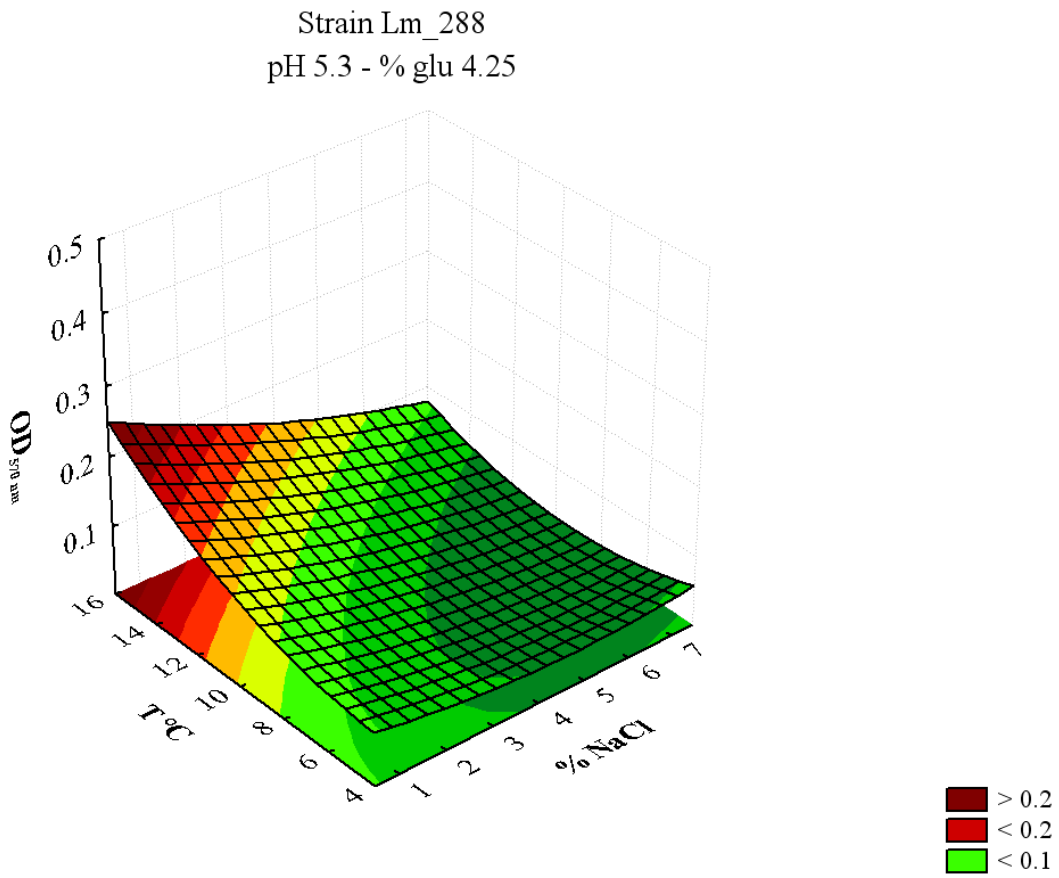
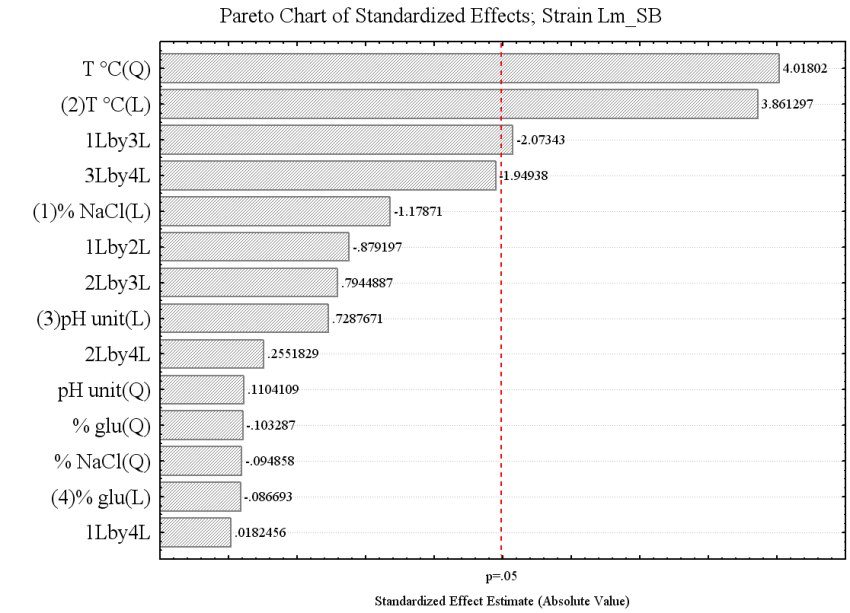


Figure 3.17 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation (OD_{570} by *L. monocytogenes* Lm_288 strain



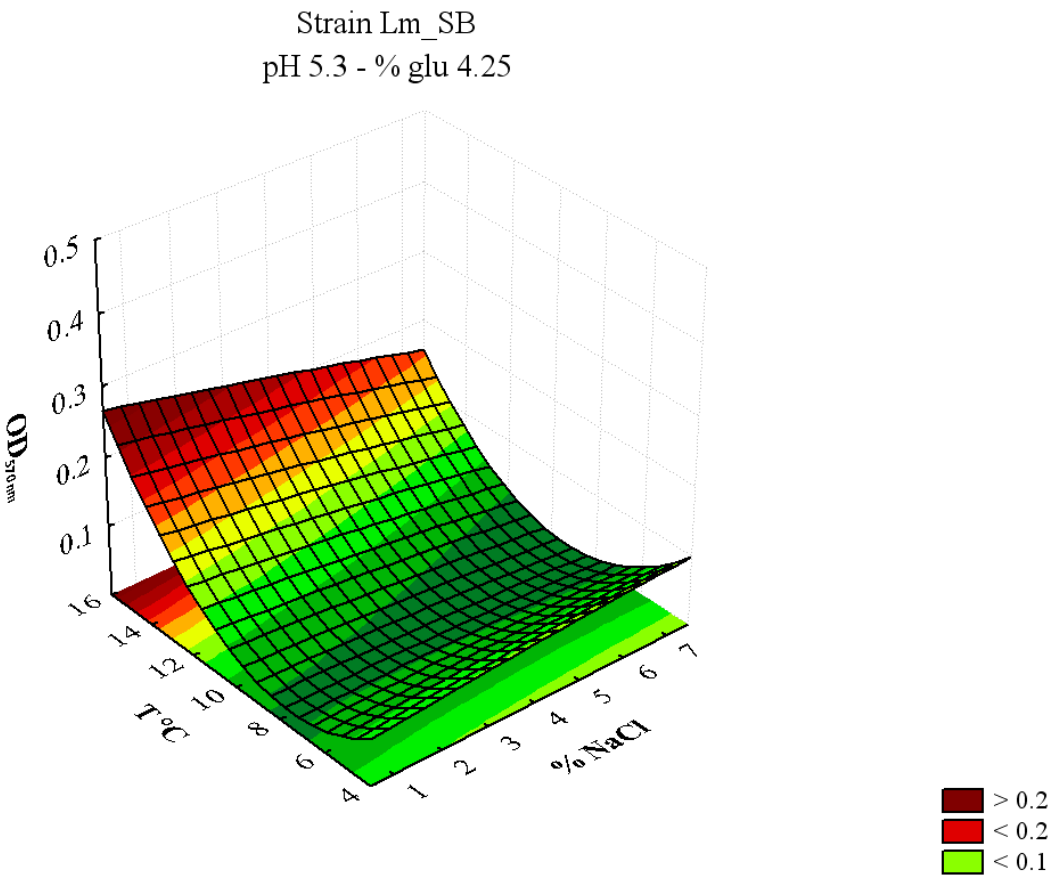
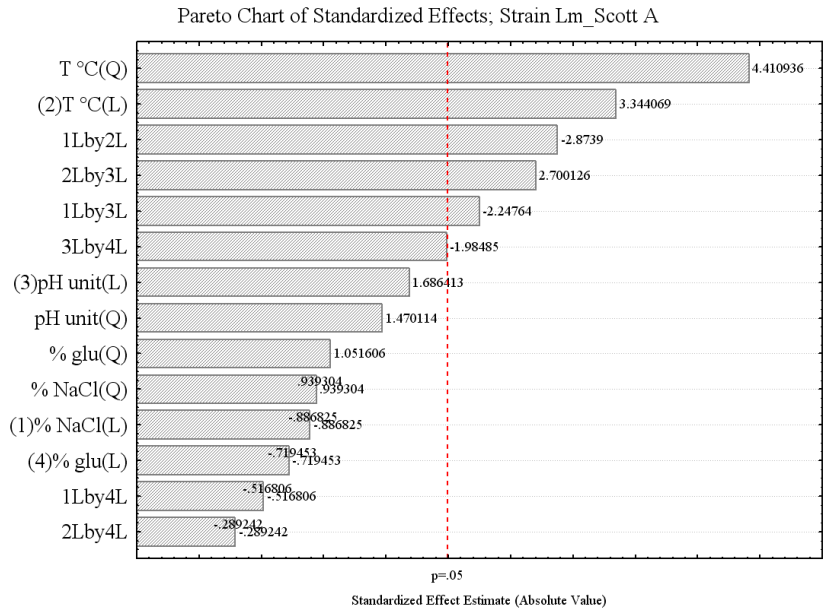


Figure 3.18 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation (OD_{570} by *L. monocytogenes* Lm_SB strain



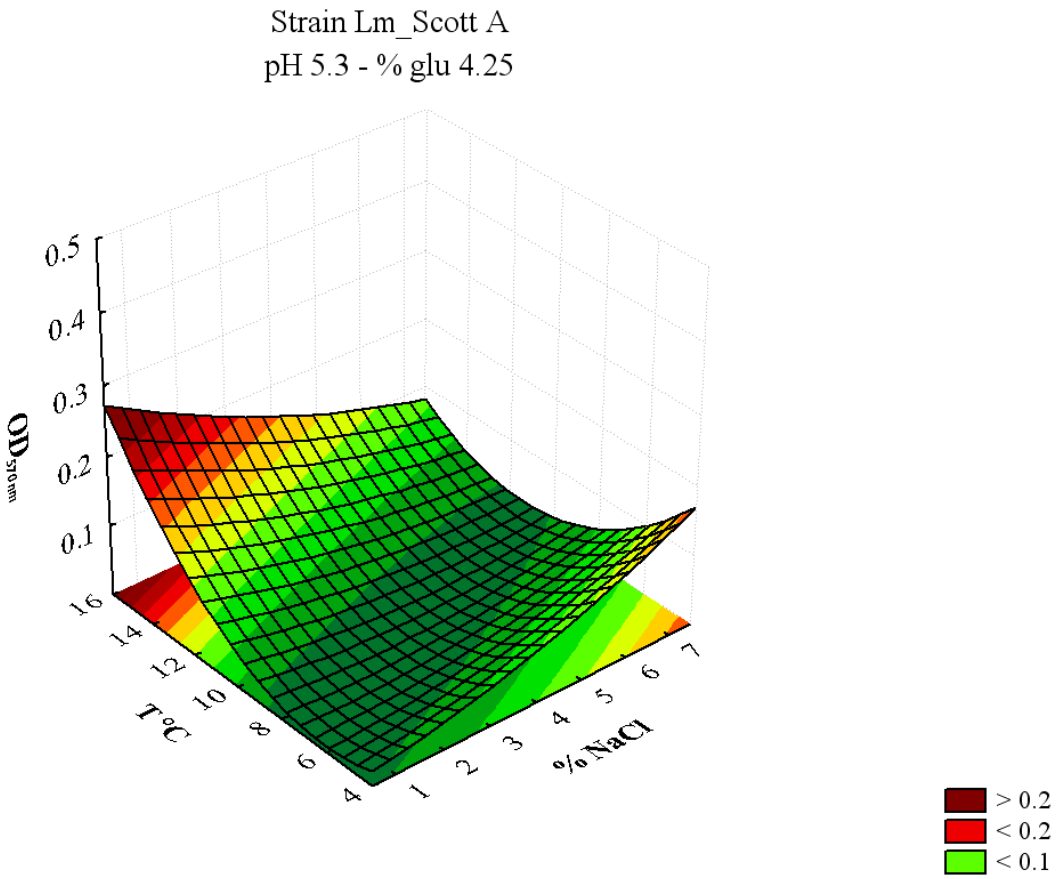
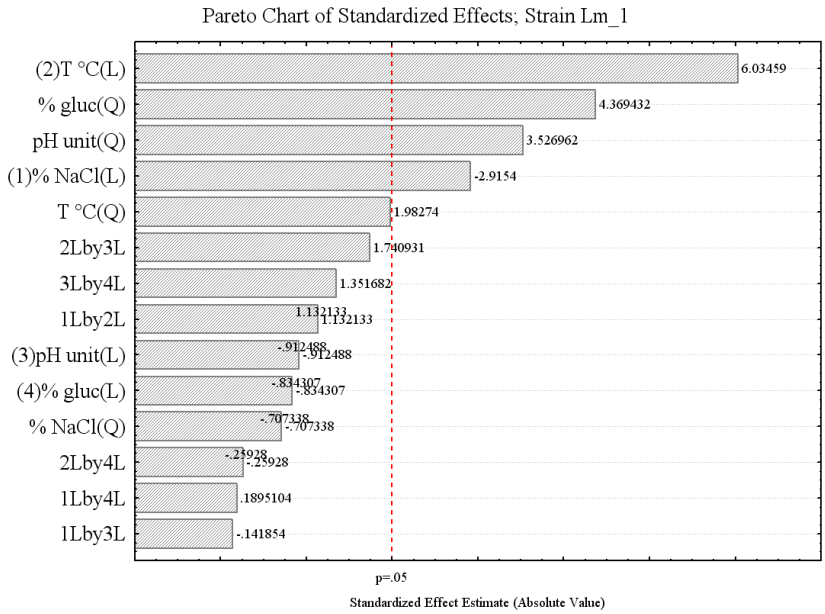


Figure 3.19 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm formation (OD_{570} by *L. monocytogenes* Lm_Scott A strain

For all *L. monocytogenes* strains the lowest values of OD_{570} were evidenced at 4 °C, which is the most commonly used refrigeration temperature. However, even if at 16 °C the highest biofilm formation was observed for all strains, substantial adherence of *L. monocytogenes* still occurred at 4 °C. This observation was supported also by viable counts (Figure 3.20 to Figure 3.27).



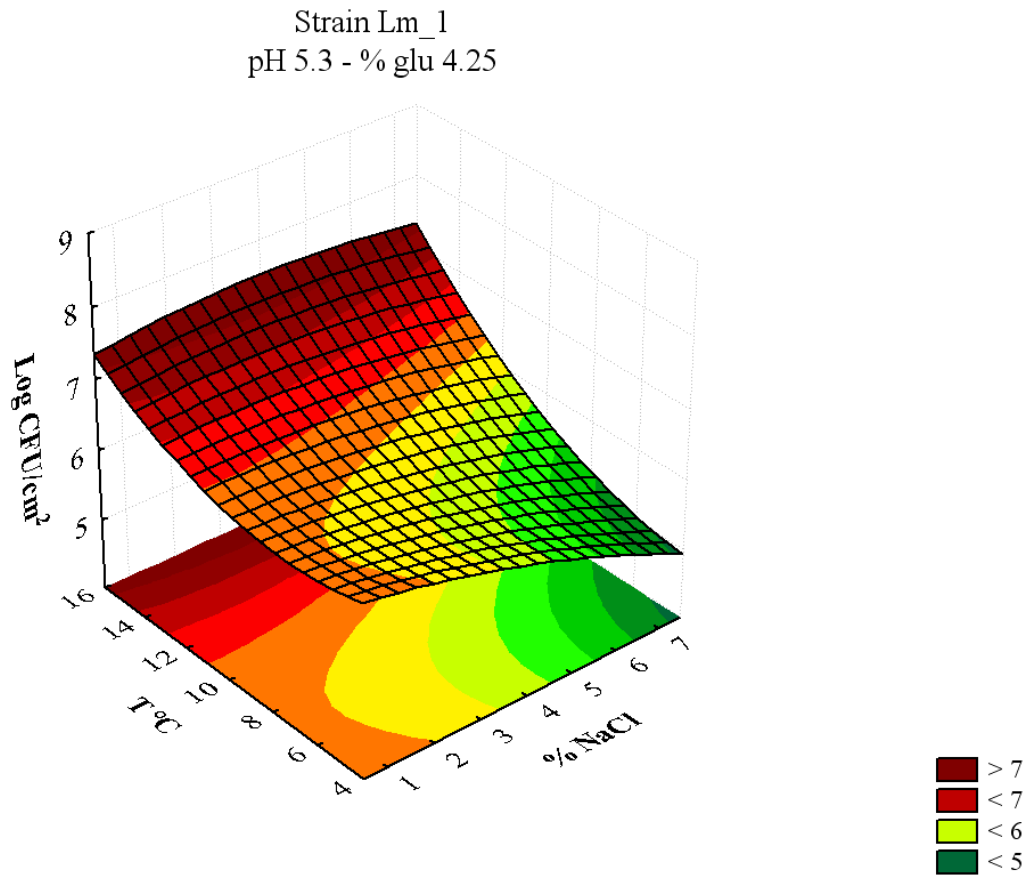
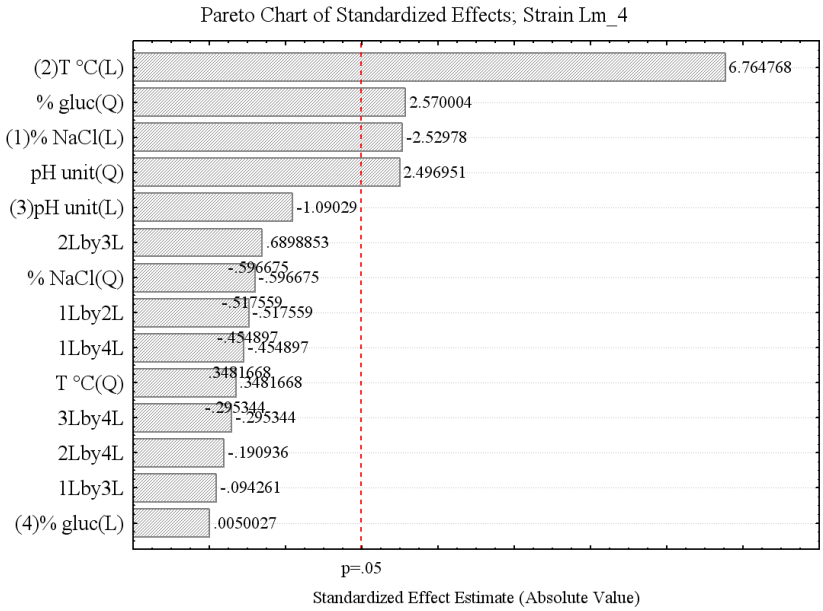


Figure 3.20 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm viable cells (Log CFU/cm^2) of *L. monocytogenes* Lm_1 strain



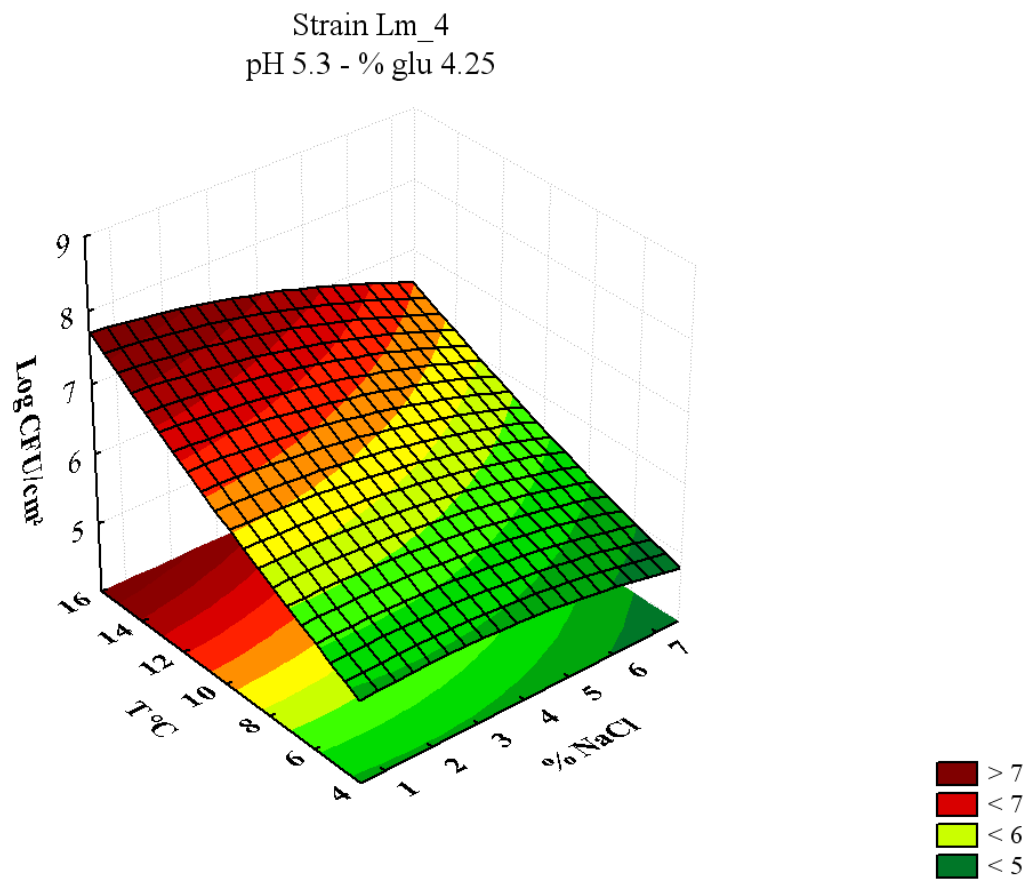
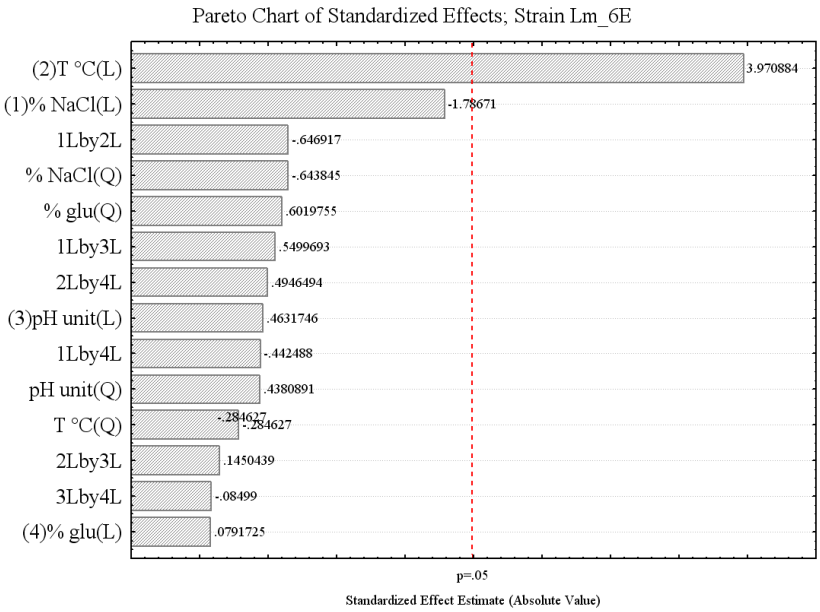


Figure 3.21 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm viable cells (Log CFU/cm^2) of *L. monocytogenes* Lm_4 strain



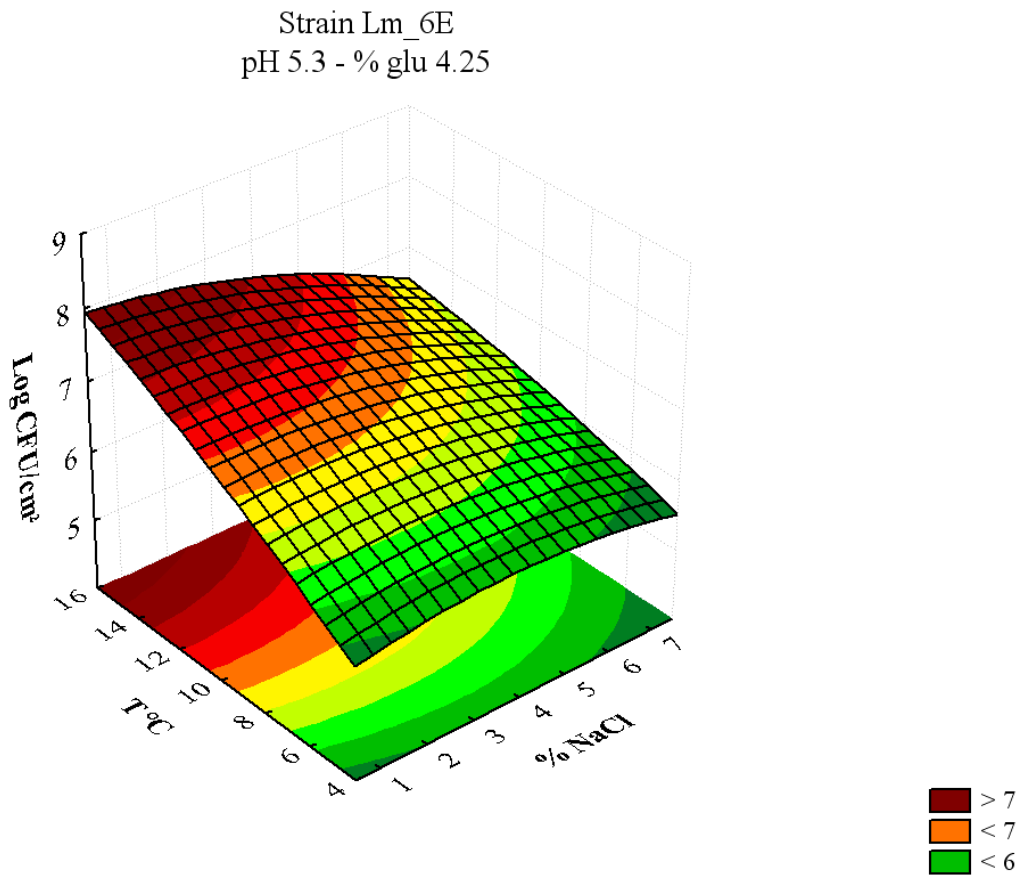
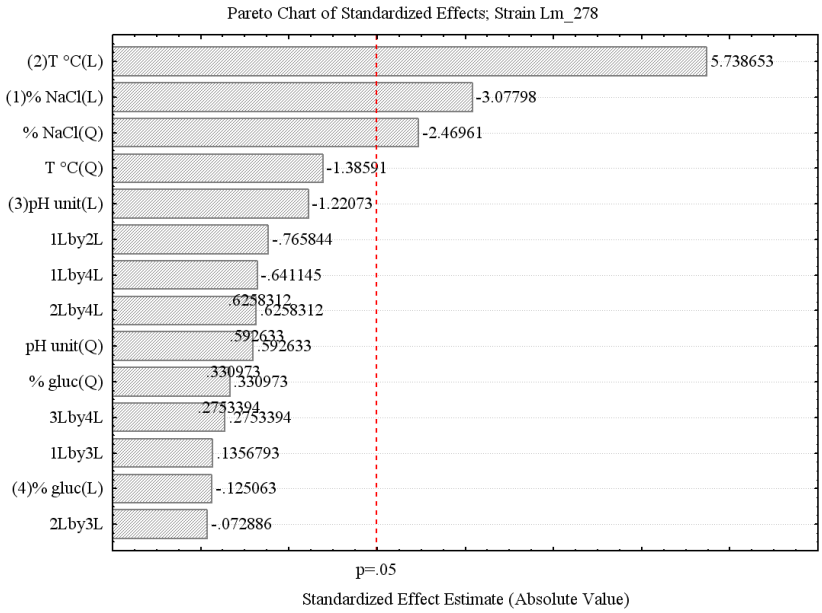


Figure 3.22 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm viable cells (Log CFU/cm^2) of *L. monocytogenes* Lm_6E strain



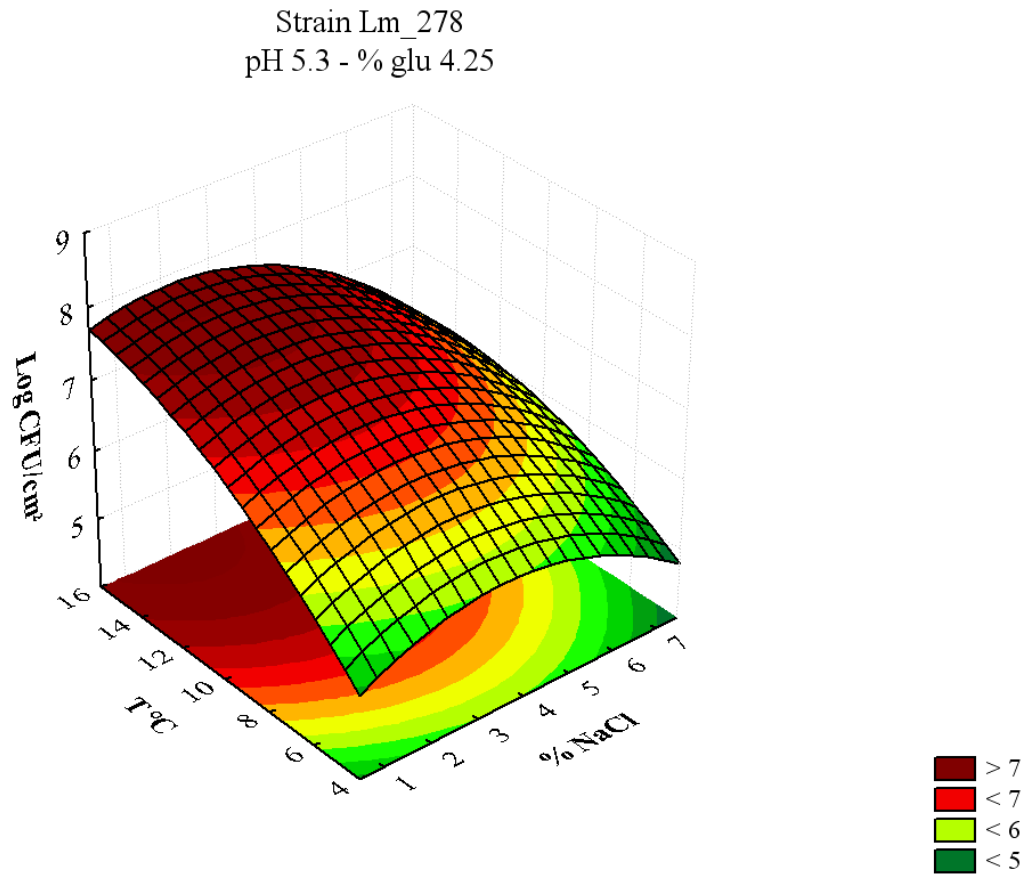
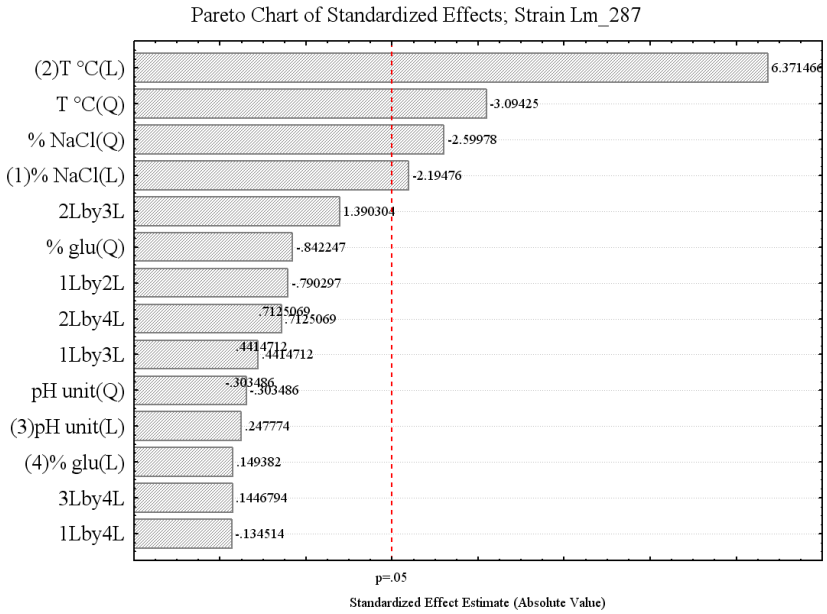


Figure 3.23 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm viable cells (Log CFU/cm^2) of *L. monocytogenes* Lm_278 strain



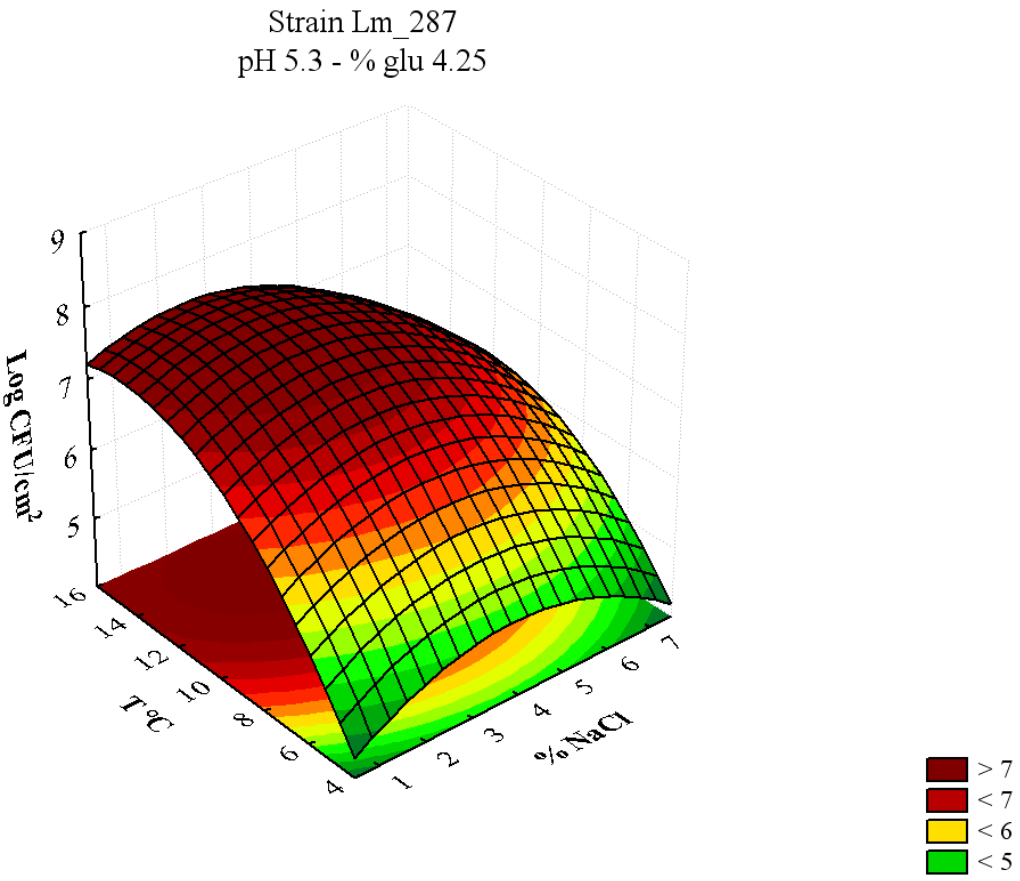
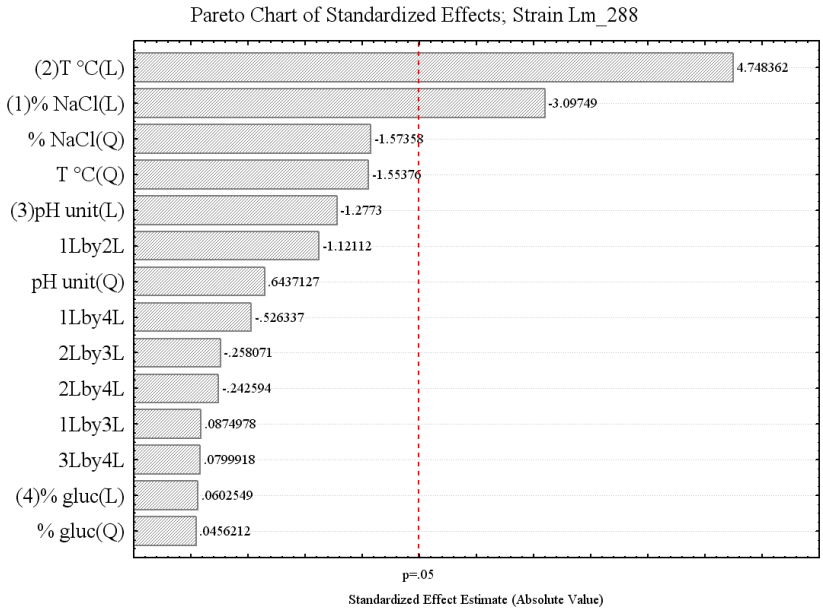


Figure 3.24 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm viable cells (Log CFU/cm^2) of *L. monocytogenes* Lm_287 strain



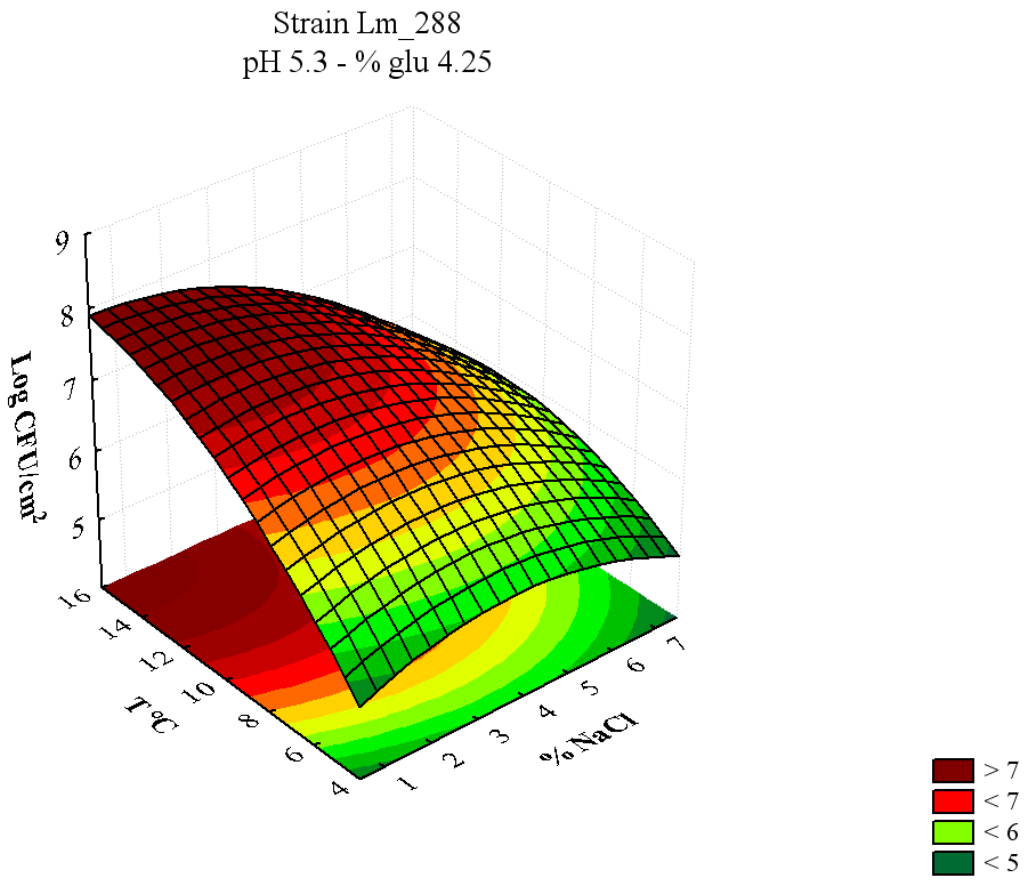
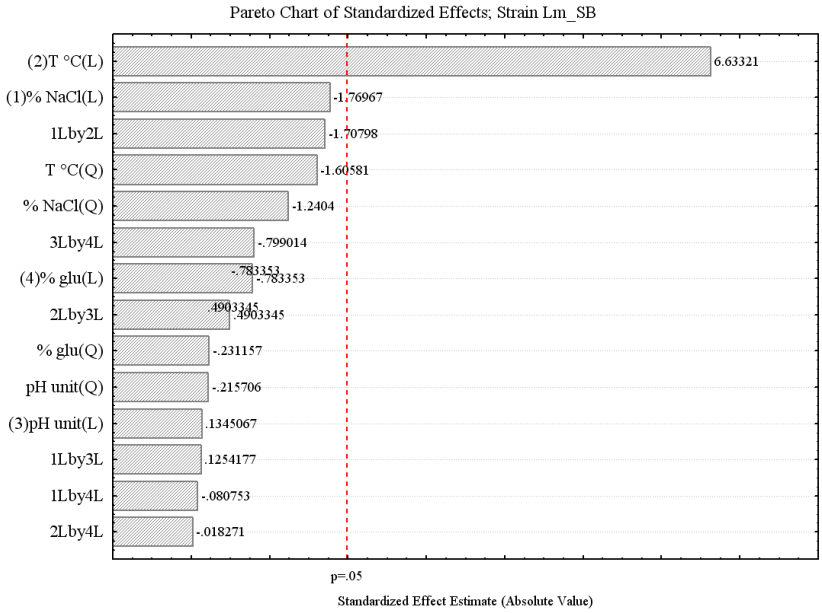


Figure 3.25 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm viable cells (Log CFU/cm^2) of *L. monocytogenes* Lm_288 strain



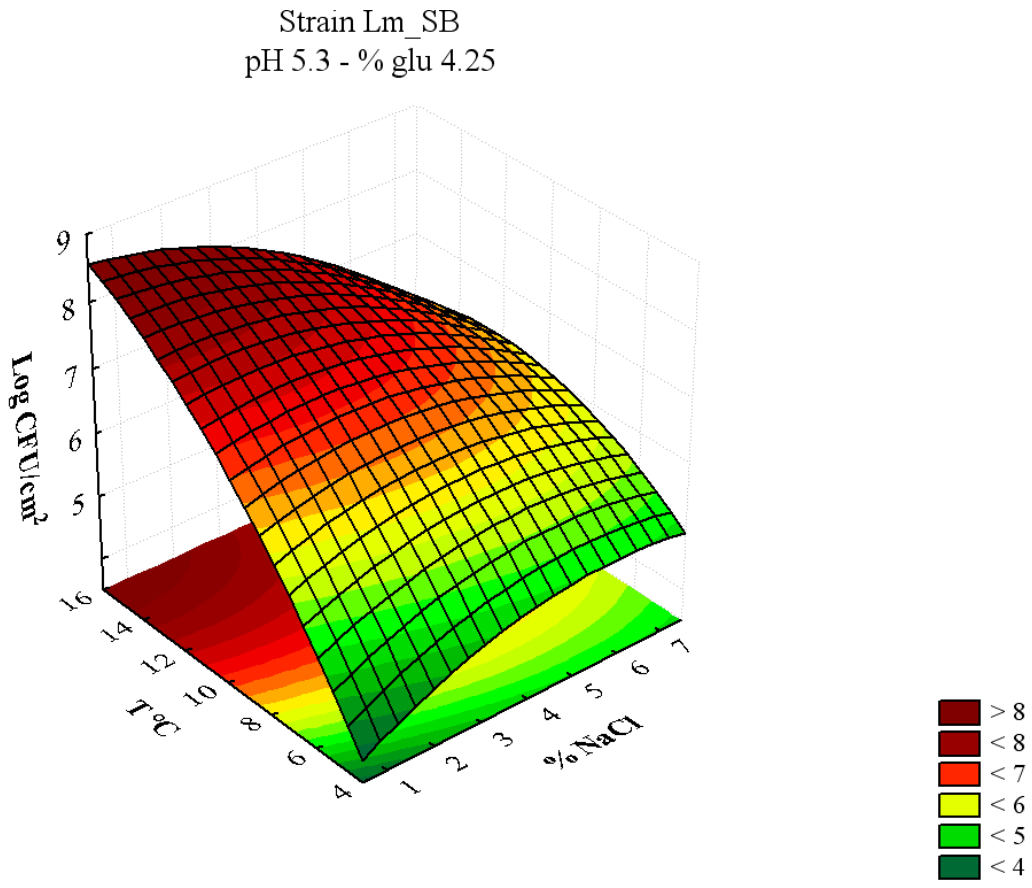
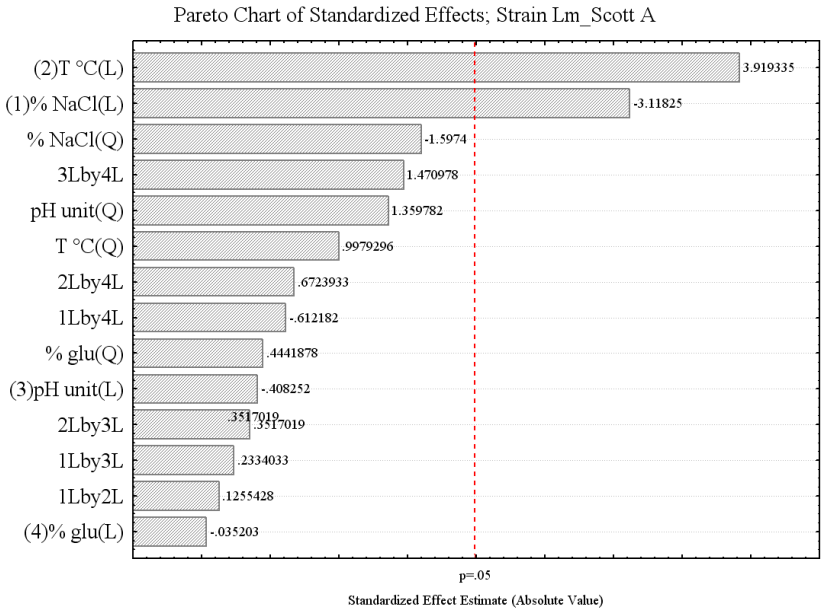


Figure 3.26 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm viable cells (Log CFU/cm^2) of *L. monocytogenes* Lm_SB strain



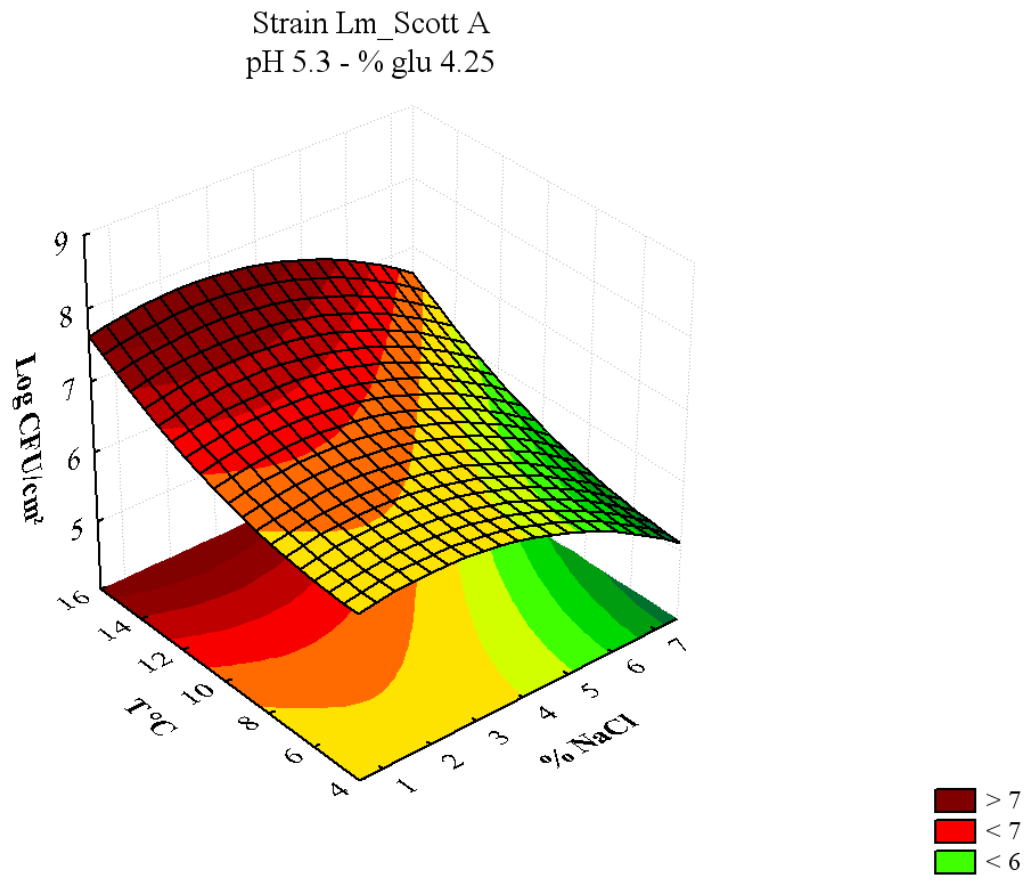


Figure 3.27 Pareto charts and 3D-plots of the effect of the interaction of environmental parameters on biofilm viable cells (Log CFU/cm^2) of *L. monocytogenes* Lm_Scott A strain

In fact, the mean value of cell counts of biofilms grown at 4 °C, regardless of the other parameters tested, was 6.02 Log CFU/cm^2, which is a quite high level. Indeed, the biofilms formed on microtiter plates are expected to be more dense than those formed on materials widely used in the food industry (e.g. stainless steel), because the material used for attachment assays is treated so as to maximize the microbial adherence.

Despite the lower adherence rates expected onto a real surface, the cell count in microbial biofilms formed at low temperatures can be a great hygienic issue, considering the clinical importance of *L. monocytogenes* as a foodborne pathogen. In fact, low temperatures tested in this study are comparable to those found in the cooling areas of many food-processing plants, and refrigeration is one of the most common ways to increase the shelf-life of foods. Therefore, biofilm produced by *L. monocytogenes* strains at refrigeration temperatures must be taken into account, because it may be the rationale for the persistence of *L. monocytogenes* in the food industries.

The presence of NaCl solely was significantly relevant ($p<0.05$) only for selected *L. monocytogenes* strains. In particular, six out of eight strains were negatively influenced by the presence of NaCl in terms of viable counts, even though the mean count at the highest values of salt was 5.74 Log CFU/cm^2. In fact, increasing salt presence resulted in a lower number of adhered cells, even though it is well known that *L. monocytogenes* can survive and grow over a wide range of environmental conditions such as high salt concentrations (Gandhi and Chikindas, 2007). The formation of biofilms in *L. monocytogenes* could be stimulated in a medium supplemented with up to 5% of salt, which is in agreement with the findings from previous studies (Pan et al., 2010; Caly et al., 2009). As regards OD_{570} values, the presence of NaCl was statistically significant in a lower number of strains (three out of eight), which formed a less dense biomass with increased NaCl concentrations. This is not surprising given that OD_{570} evaluates both viable cells and EPS matrix, while the data obtained from DP method only quantify viable cells within the biofilm. It is therefore conceivable that the expression of genes coding for EPS production is affected by salt concentration in a different manner as compared to cell replication within a biofilm. Another possible explanation of decreasing biofilm formation is the repression of flagella expression at high salt concentrations, thus decreasing the adhesion capability of *L. monocytogenes* (Caly et al., 2009). An interesting observation made within the study was that in six out of eight strains tested, a synergistic effect ($p<0.05$) of salt with another factor (pH, temperature and glucose) was observed. These synergistic effects were observed only in the case of OD_{570} values, and not for viable biofilm counts. It is therefore conceivable that *L. monocytogenes* responses to environmental stresses mainly by modulating the production of biofilm matrix than replicating itself, which can allow the persistence of the pathogen in the food processing lines.

OD_{570} values of *L. monocytogenes* were not affected by pH solely, whereas the opposite occurred in viable cells for only two strains (Lm_1 and Lm_4, Figure 3.20 and Figure 3.21, respectively). In the temperature range considered, *L. monocytogenes* produced approximately the same amount of biomass irrespective of the pH values in a range between pH 6.25 and pH 4.25. Similar observations were made by Smoot and Pierson (1998), who evidenced that maximum levels of attached *L. monocytogenes* obtained on Buna-N rubber after a 120-min exposure period were not affected by altering the pH within a range of 4 to 9. However, when cells were exposed to the test surfaces under alkaline conditions, lower numbers of attached cells were observed when compared to neutral or acidic conditions. It is known that *L. monocytogenes* is a quite adaptable microorganism to stressful environmental conditions, able to overcome growth obstacles also by transitioning from the planktonic to sessile form. It has been shown that exposing cells to

sublethal levels of environmental stress, such as pH and temperature, can affect the ability of this pathogen to attach to common food contact surfaces (Smoot and Pierson, 1998). The mechanisms related to the acidic stress resistance and to the biofilm formation at low pH in *L. monocytogenes* could be related to variations in the surface protein composition, as well as the downregulation of the flagellin synthesis under the acidic conditions (Tresse et al., 2006). Moreover, stressful environmental conditions can activate the expression of a general stress response in *L. monocytogenes* controlled by σ^B, an alternative sigma factor, which provides the organism with multiple, non-specific resistance to stress and thereby promotes growth and survival in adverse conditions (Wemekamp-Kamphuis et al., 2004).

Increasing glucose concentration resulted in a decrease of OD_{570} values for all tested strains, even if this parameter proved to not statistically significant. In fact, the highest OD_{570} values were observed at the lowest glucose concentrations. Regarding the effect of nutrients on *L. monocytogenes* biofilm formation, conflicting results have been reported. For example, Pan et al. (2010) showed that the addition of glucose stimulated bacterial cells to produce more EPS matrix material. On the contrary, Kim and Frank (1995) evidenced that glucose levels did not affect biofilm development. These results suggest that the mechanisms involved in the stimulation of biofilm formation by glucose for *L. monocytogenes* may be strain-specific. Regarding the biofilm viable cell counts, the glucose concentration was statistically significant ($p<0.05$) for two out of eight strains, and interestingly the viable counts of all strains were positively affected by glucose, i.e. increasing nutrient concentration resulted in higher viable counts. It could be hypothesized that the presence of high concentrations of nutrients promotes cell replication, while limited concentrations of glucose would create stress for the microorganism and therefore favour the production of EPS, which has the function to protect the biofilm cells.

The results obtained regarding *L. monocytogenes* clearly show that this pathogen is able to adapt and form biofilm in a wide range of conditions including low temperatures, low pH, high salt concentrations and low nutrient concentrations, although the mechanisms involved in surfaces colonization in stressful conditions may depend on strain considered. The ability of *L. monocytogenes* to colonize surfaces in the presence of the stressful conditions used in the food industry during processing and storing, may contribute to the persistence of *L. monocytogenes* in the food processing lines and increase the probability of cross-contamination with the consequent hazard for the consumer health.

3.4 CONCLUSIONS

The results of the present study showed that environmental stresses differently influenced the biofilm formation by *L. monocytogenes*, *S. aureus* and *Pseudomonas* spp. strains. The increase in biofilm production in stressful environments represents a form of survival response, and has largely been attributed to stress-induced physiological adjustment in the cells resulting in an increased ability of the organism to attach to surfaces. The use of a CCD allowed to mimic the real environmental conditions of a food environment and to obtain the greatest amount of information while limiting the number of experiments to be carried out. Therefore, useful data were obtained, increasing information available in the literature about

the synergistic effects of environmental parameters on biofilm formation regarding the food sector. In fact, both for *L. monocytogenes* and *S. aureus*, which are food pathogens, there is a need for more information about them; for *Pseudomonas* spp., which are food spoilage bacteria, there are only few studies in literature.

Even if all the tested strains were able to produce biofilms in a wide range of the environmental factors, this study pointed out a high variability among the bacterial species and mainly among the strains belonging to the same species. The different behaviour within the same species subjected to several environmental conditions highlighted that biofilms are dynamic structural entities in which detachment, growth and microcolonies formation take place. This dynamic may be at the origin of dissemination of microorganisms and contamination of surfaces in food industries. In fact, the finding that microbial adhesion and biofilm formation may be promoted by environmental conditions present in the food industry indicates that food producers should be aware of the importance of controlling biofilm formation by *Pseudomonas* spp., *L. monocytogenes* and *S. aureus*, which are important bacteria causing respectively food spoilage and food poisoning after their consumption. Moreover, such results could provide valuable insights into the attachment mechanisms and, perhaps, could lead to better methods of biofilm control in food plants.

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Chapter 4. EFFECT OF TEMPERATURE ON BIOFILM FORMATION AND SANITIZERS SENSITIVITY OF *PSEUDOMONAS* SPP.

4.1 INTRODUCTION

Biofilms represent a significant problem for the food industry, because if not properly controlled, they compromise the sanitation process causing hygienic issues and health risks. The main strategy in controlling biofilm formation is to prevent microbial adhesion. This can be carried out, for example, through an effective cleaning system which is essential for the control of biofilm development on surfaces in food processing environments. Microorganisms growing in a biofilm surround themselves with EPS and form a complex multicellular structure. EPS and three-dimensional structures are thought to play an important role in biofilm resistance to sanitizers (Costerton et al., 1995). As a matter of fact, mature biofilms are generally difficult to inactivate and remove, since the structural characteristics of biofilm, constituted of cells and EPS, play protecting roles against various chemical and physical stresses (Kumar and Anand, 1998). Therefore, clarifying the structural characteristics of biofilm is extremely important for obtaining more effective inactivation and removal treatments. Understanding the mechanical and architectural properties of the matrix is closely related with the use of suitable methods that permit accurate analysis of the matrix structure and composition. Ideally, an *in situ*, non destructive approach should be used. In the recent years, confocal laser scanning microscopy (CLSM) has been developed as a three-dimensional optical sectioning technology for the visualization of viable biofilm systems. CLSM is currently one of the most frequently used tools to study biofilm structure, because it allows to a direct *in situ* and non-destructive investigation of biofilm cell structures using specific fluorescent markers. Moreover, dehydration and fixation are not needed in the observation process of CLSM (Caldwell et al., 1992). CLSM has provided some new information on the structural complexity of biofilms and has confirmed their heterogeneity (Stoodley, 1999). However, few studies concerning CLSM observation of developing biofilms and before/after inactivation treatments have been reported.

Members of the genus *Pseudomonas* have frequently been reported to produce exopolymers, and they have the ability to attach rapidly to the surfaces of the food industry, where they are frequently found (Leriche et al., 2004). Among the *Pseudomonas* genus, *P. aeruginosa* is known for its ability to form biofilms on abiotic surfaces, but little is known about the biofilm-forming capacity of *Pseudomonas* spp. isolated from food environments. It has to be highlighted that the *Pseudomonas* spp. relevant for the food industry are psychrophilic, so their presence in food plant areas where the temperature is below the room temperature could be a concern. The aim of this work was to evaluate the biofilm forming ability of three strains belonging to the *Pseudomonas* genus at temperatures of 4 ° C and 15 ° C, in order to simulate relevant temperatures of a food chain. Moreover, the sensitivity to two sanitizers of the biofilms formed at the temperatures tested was evaluated. Furthermore, CLSM was used to follow the biofilm formation of a *Pseudomonas* sp. strain on stainless steel under dynamic conditions, as well as its sensitivity to a sanitizer product.

4.2 MATERIALS AND METHODS

4.2.1 Biofilm formation by *Pseudomonas* spp.

P. fluorescens Ps_019, *P. fragi* Ps_053 and *P. putida* Ps_071 strains were used in this study (see List of Strains). The biofilms were grown both on polystyrene microtiter plates and on stainless AISI 304.

The biofilm production assay in 96-well polystyrene flat bottom microtiter plates was performed in Luria Bertani broth (LB) in three biological replicates as described in paragraph 2.2.1. The microtiter plates were incubated at 4 °C and 15 °C for seven days. Every 48 hours the microtiter plates were subjected to a refreshing of the exhausted broth with fresh LB. At the end of the incubation, the microtiter plates were rinsed and stained as described in paragraph 2.2.1, and OD_{570} values were calculated.

Biofilms were also grown on stainless steel AISI 304 coupons in the CDC Biofilm Reactor (CBR) in LB as described in paragraph 2.2.2. The incubation of the CBR was performed for seven days at 4 °C and 15 °C. Each experiment was repeated twice. After incubation, the coupons were removed and rinsed, as described in paragraph 2.2.2. Subsequently, the evaluation of viable counts was carried out using the Drop Plate method (paragraph 2.2.3).

4.2.2 Biofilm susceptibility towards disinfectants

The strains in the sessile state (biofilms grown in microtiter plates and on stainless steel) were subjected to a susceptibility test against a peracid-based product (PA; peracetic acid 7%, hydrogen peroxide 26%, acetic acid 6%) and a chloramine-T based product (CL-T; active chlorine minimum 24%). The products were tested respectively at concentrations of 1% and 0.3%, which are the recommended industrial use conditions. In order to better understand the tolerance of the strains against sanitizers, they were also tested in the planktonic state.

The test was carried out in 96-well polystyrene flat bottom microtiter plates to set up the biofilm formation by the strains, as described in paragraph 2.2.1 using LB broth as the culture medium. The microtiter plates were incubated at 4 °C and 15 °C for seven days and were subjected to a refreshing of the exhausted broth with fresh LB every 48 hours. For each strain four wells were treated with each disinfectant and four wells were used as the controls. The treatment with sanitizer products was carried out as follows: the medium was gently removed and each well was washed twice with 200 µL sterile saline solution (0.9% NaCl). Each well was treated with 250 µL of 1% PA or 0.3% CL-T for 5 min at 20 °C; the sanitizer solutions were then removed, replaced with 250 µL of neutralizer solution (0.5% sodium thiosulfate) and left in contact for 5 min at 20 °C. In the control wells the sanitizer solutions were replaced with 250 µL of sterile tap water at the same temperature and for the same contact time, and subsequently removed and replaced by an equal volume of neutralizing solution through the same procedures described above. After the neutralizer removal, biofilm cells were resuspended in 250 µL of Maximum Recovery Diluent (MRD), then scraped with a pipette tip. Afterwards, the resuspended biofilm was transferred in 500 µL Eppendorf tubes

and subjected to sonication as described in paragraph 2.2.2. The bacterial counts were assessed by plating serial 10-fold dilutions of the biofilm cell suspension on Gelatin Sugar Free Agar (GSFA, Oxoid, Milan, Italy) plates, using the spread plate method (see paragraph 2.2.3). The plates were incubated at 30 °C for 24-48 hours. Each experiment was conducted twice.

Regarding the biofilms formed on stainless steel AISI 304 coupons, the CDC Biofilm Reactor was used, as described in the paragraph 4.2.1, using LB broth as culture medium. For each trial, two coupons were used as the control and two were treated with each sanitizer product. After incubation, each coupon was rinsed twice with a sterile saline solution to remove the non-adherent cells and subsequently placed in contact with 15 mL of sanitizing solution at the appropriate concentration for 5 min at 20 °C. After the treatment, the coupons were treated with 15 mL of neutralizing solution for 5 min at 20 °C. Then, the biofilm cells were detached from coupons by using sterile cell-scrapers; detached cells were resuspended in 1 mL of MRD and sonicated as described in paragraph 2.2.2. The bacterial counts were carried out by plating serial ten-fold dilutions of the biofilm cell suspension on GSFA plates, using the spread plate method. The plates were incubated at 30 °C for 24-48 hours. The coupons used as controls, after washing twice with a sterile saline, were placed in contact with a sterile saline solution for 5 min and subsequently subjected to detachment of the biofilm and microbiological count, as previously described. Each trial was repeated twice.

As regards planktonic cells susceptibility test, 10 mL of LB were inoculated with 50 µL of an overnight culture of each strain and incubated at 4 °C and 15 °C for seven days. Subsequently, 1 mL of each culture was washed twice with a sterile saline and resuspended in 1 mL volume of sterile saline. Afterwards, the suspension was placed in contact for 5 min at 20 °C with 9 mL of each sanitizer solution. Then 1 mL of the suspension was put in contact for 5 min at 20 °C with 9 mL of neutralizing solution. The bacterial counts were assessed by plating serial ten-fold dilutions of the cell suspension on GSFA using the spread plate method. The plates were incubated at 30 °C for 48 hours. Each experiment was repeated twice.

For each type of treatment, both for sessile and planktonic cells, the efficacy of the disinfecting treatments was evaluated by taking the ratio of the Log CFU/cm^2 (or /mL) before (N_0) and after treatment (N_t), presented as -Log (N_t/N_0) (Sudhaus et al., 2014).

4.2.3 Kinetics of adhesion and biofilm formation on stainless steel AISI 304, and resistance to peracid-based sanitizer

The effect of incubation temperature on adhesion and biofilm formation by *P. fluorescens* Ps_019 was evaluated during a five-day incubation on stainless steel in CBR. During incubation viable counts were evaluated and microscopic observation using CLSM was performed. Moreover, the sensitivity of biofilm to peracid-based sanitizer was evaluated during incubation.

The biofilms were grown at 4 °C and at 15 °C on stainless steel in the CDC Biofilm Reactor, as described in the paragraph 4.2.1, and subjected to microbiological and CLSM observation at times of 2, 4,

8, 24, 48 h and 5 days. At each sampling time, three coupons (two for microbiological analysis and one for CLSM observation) were rinsed twice with 10 mL of a sterile saline solution, in order to eliminate the non-adherent cells. For microbiological analysis, two coupons were treated as described in paragraphs 2.2.2. The bacterial counts were carried out by plating serial ten-fold dilutions of the biofilm cells suspension on GSFA plates, incubated at 30 °C for 48 hours.

At the times of 48 h and 5 days, three coupons were rinsed with 10 mL of a sterile saline solution and treated with 15 mL of 1% PA solution for 5 min at 20 °C. After treatment, the coupons were neutralized with 15 mL of neutralizing solution for 5 min at 20 °C. Then, the biofilms were detached from two coupons and subjected to microbiological analysis as previously described. The third coupon was used for the CLSM observation.

4.2.3.1 CLSM Microscopy

After washing in the sterile saline solution, each coupon was stained with Live/Dead BacLight kit (Molecular Probes, Italy) with a concentration of 6 µM for Syto-9 and of 30 µM for propidium iodide, and Concanavalin A at a concentration of 200 µg/mL (Molecular Probe, Italy). The Live/Dead BacLight kit monitors the viability of bacterial populations as a function of the membrane integrity of the cell. Cells with a compromised membrane that are considered to be dead or dying will stain red (propidium iodide), whereas cells with an intact membrane will stain green (Syto-9). After 15 min, each coupon was rinsed with 7 mL of sterile phosphate buffer saline (pH 7.4) and observed. CLSM analysis was performed with an LSM 510 META laser scanning microscope attached to an Axiovert 200 microscope (Zeiss, Jena, Germany) using 100x oil NA 1.4 objective. The excitation wavelengths were 488, 543 and 633 nm for green, red and far red emission respectively. The emitted fluorescence was filtered by a primary dichroic filter (488, 543, 633 nm), splitted with NTF 635 vis and recorded using BP 505-530 for green emission, BP 585-615 for red emission while for far red emission the meta detector in the channel mode setting wavelength was used. Reconstructions of imaged samples were obtained by x-y, y-z projections created by LSM Zeiss Image Examiner (ver. 3.0).

4.3 RESULTS AND DISCUSSION

Three *Pseudomonas* species strains were tested in this study, firstly because the microorganisms belonging to the genus *Pseudomonas* include psychrophilic bacteria known for their ability to contaminate food products. The second reason comes from the evidence that these microorganisms are able to form biofilms even at low temperatures, which makes them efficient colonizers of chilled zones in the food industry. Moreover, in the literature the studies about biofilm formation by bacteria belonging to the genus *Pseudomonas* regard almost exclusively *P. aeruginosa* strains of medical origin. Therefore, it was particularly interesting to study in depth the biofilm ability of foodborne isolates belonging to this microbial group.

As shown in Figure 4.1 strain *P. fluorescens* Ps_019 evidenced a greater attitude in developing biofilm at 4 ° C as compared to 15 ° C in polystyrene plates, while *P. putida* Ps_071 showed an opposite

behavior, although with different values of optical density (p<0.05). Regarding *P. fragi* Ps_053, the effect of temperature was not significant for the biofilm formation. It is known that many environmental parameters, and in particular the temperature, are able to influence biofilm formation, although an important component of variability is related to the strain (Lianou et al., 2012). The ability to form biofilms at low temperatures is a physiological characteristic particularly hazardous in the food production area, since it might indicate the ability of a microbial species to cause cross-contamination in food processing plant areas at low temperatures (e.g. chilled areas).

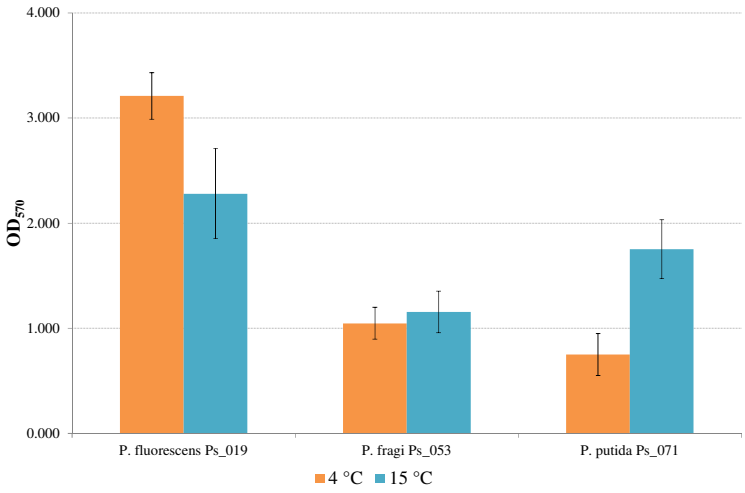

Figure 4.1 Biofilm biomass (mean OD_{570} ± SD; n=3) formed on microtiter plates by *Pseudomonas* spp. at 4 °C and 15 °C

In Figure 4.2 the viable counts of biofilms formed are reported, both on polystyrene and on stainless steel, by the three strains tested at the two temperatures. For all the isolates the viable counts were higher on plastic surfaces compared to stainless steel ones (p<0.05), with mean values always over than 10^7 CFU/cm^2, index of a high ability to form biofilm.

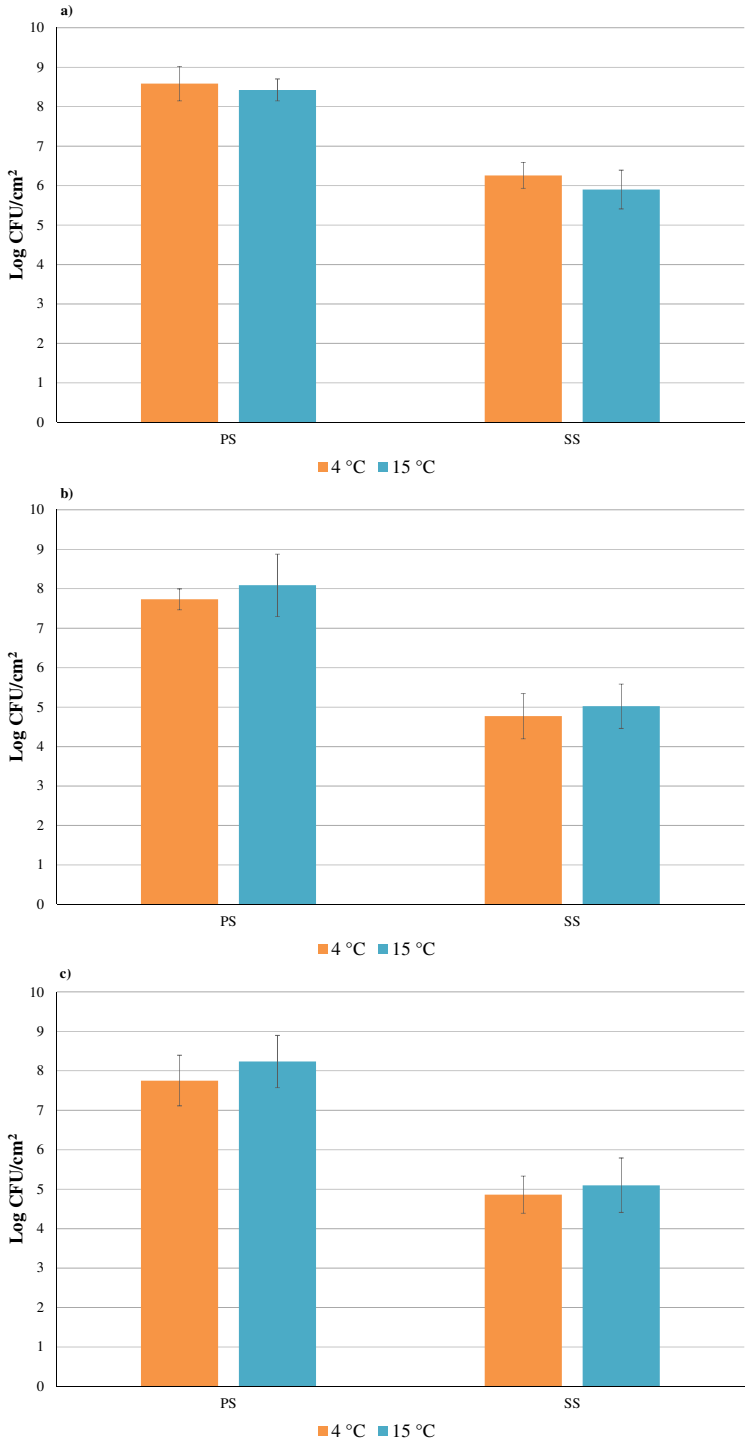

Figure 4.2 Biofilm cell viable counts (mean Log CFU/cm^2 ± SD; n=2) of *P. fluorescens* Ps_019 (a), *P. fragi* Ps_053 (b) and *P. putida* Ps_071 (c) on polystyrene (PS) and stainless steel (SS) at 4 °C and 15 °C

Mean Log CFU/cm^2 on stainless steel were always greater than 4. It has to be highlighted that polystyrene in microtiter plates is specially treated to promote cell adhesion and it is considered the reference material for the screening of the bacterial ability to form biofilm. Stainless steel surface is, instead, a very common material used in food processing plant for its lack of toxicity and its resistance to high temperatures and to physical, chemical and microbiological corrosions. The high ability to form biofilms on stainless steel makes these strains particularly hazardous for the food industry. To our knowledge, there is

a lack of information on the biofilm-forming ability of foodborne *Pseudomonas* spp. on surfaces present in food plants, so these results could help in completing the picture on the characterization of species very widespread in the food industry.

The conventional approach to microbial biofilms in the food field is the use of chemical disinfection, even if it is generally accepted that biofilm organisms are more resistant to biocides than their planktonic counterparts. In this step of the study *Pseudomonas* strains were tested against two oxidizing agents, i.e. one peracid-based product (PA) and one chlorine-releasing agent (CL-T), frequently used in food sanitation programs. Usually the suspension test (performed on planktonic cells) is used to assess the bactericidal activity of a biocide against a specific microorganism (Holah et al., 2002). This test consists in exposing planktonic cells to the product to be tested under specific conditions of time, temperature and concentration, evaluating the survival rate to the treatment. There are some concerns about this protocol, as a good test must be able to predict the efficacy of the used disinfectants. However, in practice, cells are found much more frequently in the sessile form than in suspension, which makes them more resistant to the sanitation treatments with respect to free cells. Thus, the use of the suspension test could overestimate the effectiveness of a sanitation protocol, resulting in an increased risk of food cross-contamination. In the light of these considerations, in this study the effectiveness of two oxidizing agents were tested against both planktonic and sessile cells grown at the two tested temperatures.

In Figure 4.3 the efficacy of the disinfecting treatments was evaluated shown by graphing the ratio of the Log CFU/cm^2 (or /mL) before (N_0) and after treatment (N_t), and is presented as -Log (N_t/N_0).

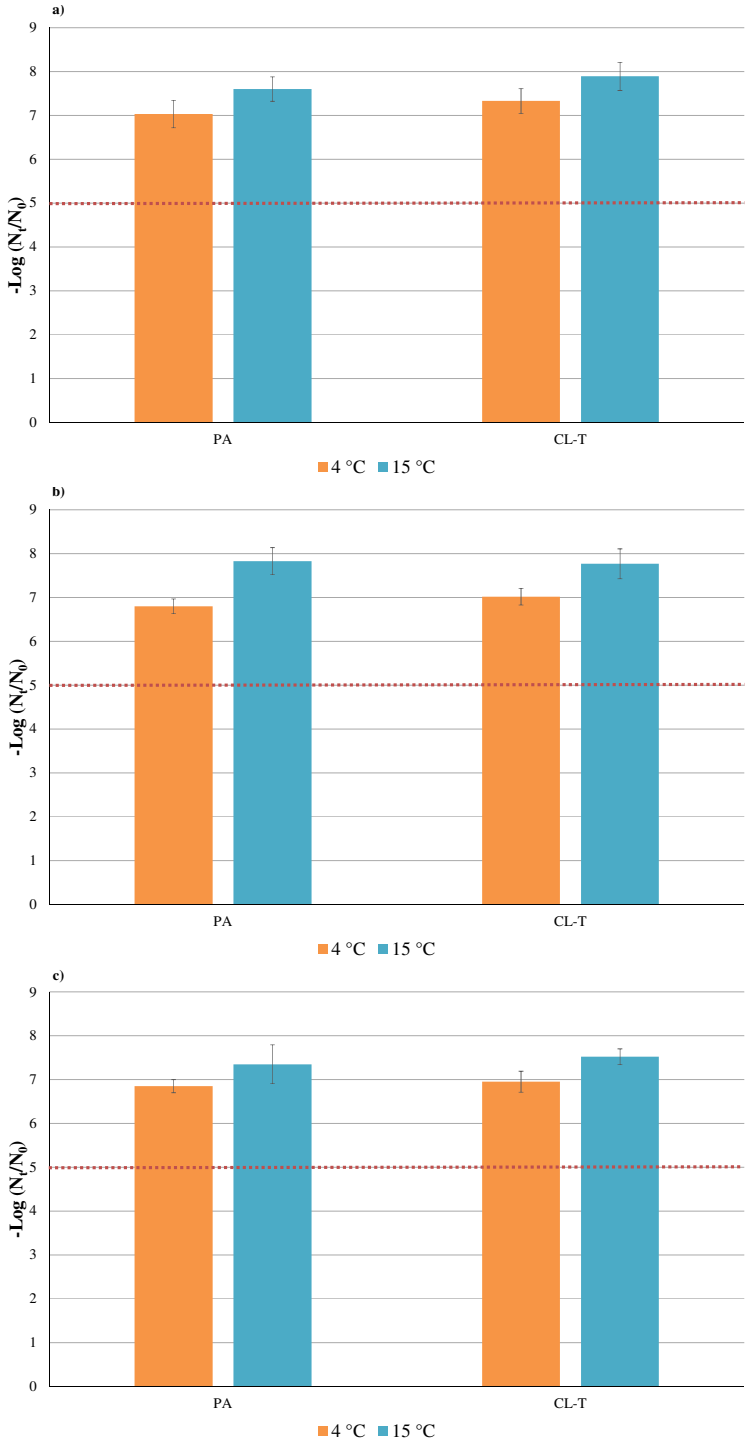

Figure 4.3 Efficacy (mean -Log (N_t/N_0) ± SD; n=2) of PA and CL-T towards planktonic cells of *P. fluorescens* Ps_019 (a), *P. fragi* Ps_053 (b) and *P. putida* Ps_071 (c) grown at 4 °C and 15 °C; dotted red line refers to the minimal efficacy required to a sanitizing agent (Payne et al., 1999)

The results clearly indicate that, regardless of the incubation temperature, for all the tested strains the reduction of viable counts abundantly exceeds 5 Log CFU/cm^2, which is considered acceptable to define a product as a disinfectant according to the European standards (Payne et al., 1999). No significant differences ($p>0.05$) were observed between PA and CL-T efficacies for each strain. However, when considering the microbial cells in the sessile state, the inactivation was considerably lower. As regards biofilms formed on polystyrene (Figure 4.4), PA caused a reduction of more than 5 Log CFU/cm^2 in almost all the tested

cases, except for *P. putida* Ps_071, whose biofilm, formed at 15 °C, was inactivated by less than 2 Log CFU/cm^2. According to Mosteller and Bishop (1993), a product with a disinfectant action against bacterial biofilm must be able to reduce the cellular populations of 3 logarithmic units. *P. putida* Ps_071 formed different amounts of biofilm mass on polystyrene at the two tested temperatures, as the OD_{570} values were higher at 15 °C. Instead, the viable counts were not statistically different. It is therefore conceivable that in response to different thermal stimuli this species produces different amounts of EPS, which can protect the cells from the bactericidal agent. This hypothesis cannot be applied to *P. fluorescens* Ps_019, which showed higher OD_{570} at 4 °C and similar reductions following PA contact.

Regarding CL-T, the effect of inactivation was lower than that obtained with PA. Mean efficacies were statistically different ($p<0.05$) for each strain, except for *P. putida* Ps_071 grown at 15 °C. In most cases the reduction of the biofilm cell population was lower than 3 Log CFU/cm^2, which makes this commercial product ineffective for the treatment of *Pseudomonas* biofilms. The lower effect of inactivation of CL-T compared to PA could be due to the effect of EPS matrix, which can inactivate the chlorine-based biocide, as observed by Toté et al. (2010). Regarding the biofilm treated with chloramine-T, the sensitivity of the biofilm was quite different at the two tested temperatures: in particular, the biofilms formed at 15 ° C were more resistant, which could indicate a higher production of EPS in these conditions.

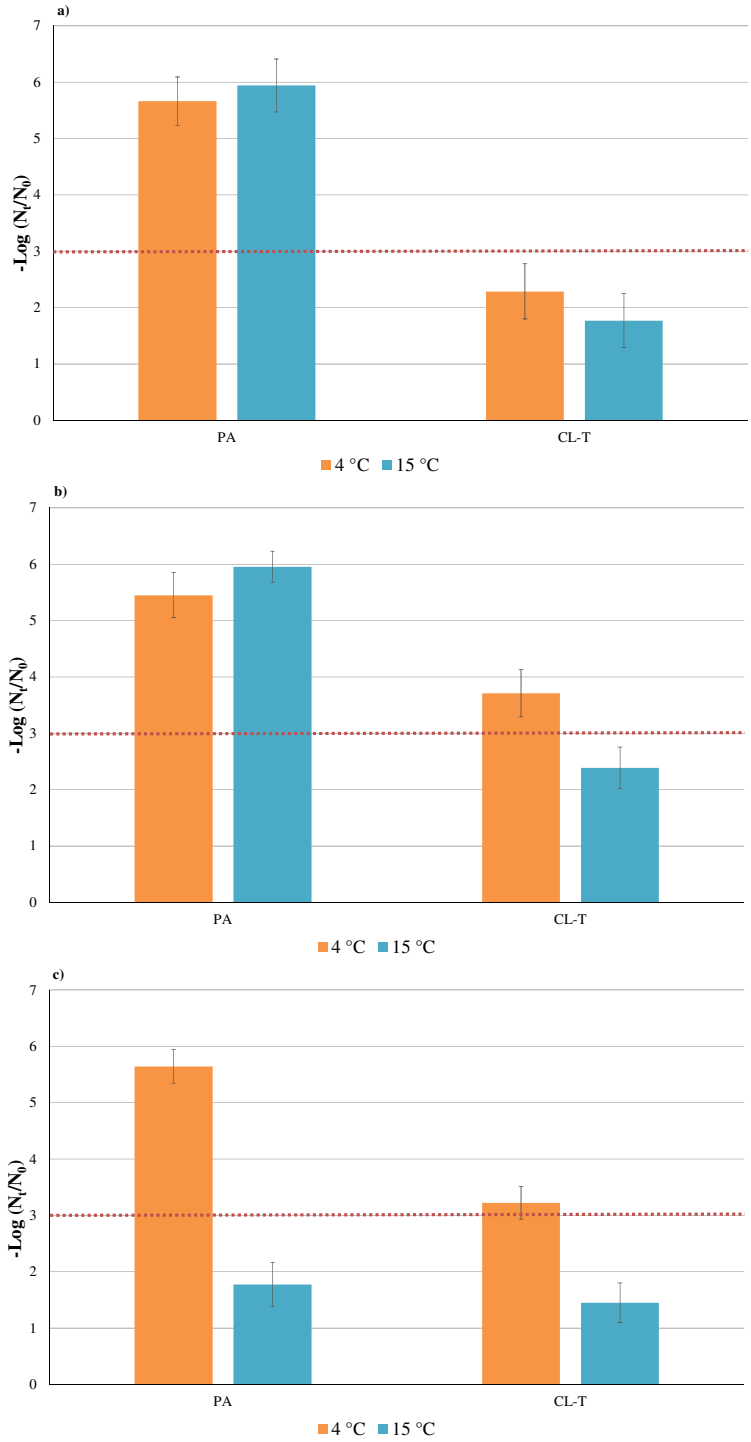

Figure 4.4 Efficacy (mean -Log (N_t/N_0) ± SD; n=2) of PA and CL-T towards biofilms of *P. fluorescens* Ps_019 (a), *P. fragi* Ps_053 (b) and *P. putida* Ps_071 (c) grown on polystyrene at 4 °C and 15 °C; dotted red line refers to the minimal efficacy required to a sanitizing agent towards biofilm cells (Mosteller and Bishop, 1993)

The results obtained on biofilms formed on stainless steel clearly indicate that the effect of the peracid-based product fulfils the goal of biofilm microbial inactivation, regardless of the growth temperature (Figure 4.5). CL-T instead failed at inactivating the biofilm formed by *P. fragi* Ps_071 at 4 °C. Contrary to polystyrene, there was no clear influence of temperature on the sensitivity of biofilms. It should be emphasized that the nature of the material can deeply influence its interactions with the cell biomass and the EPS matrix, which results in significant differences in the biofilm sensitivity.

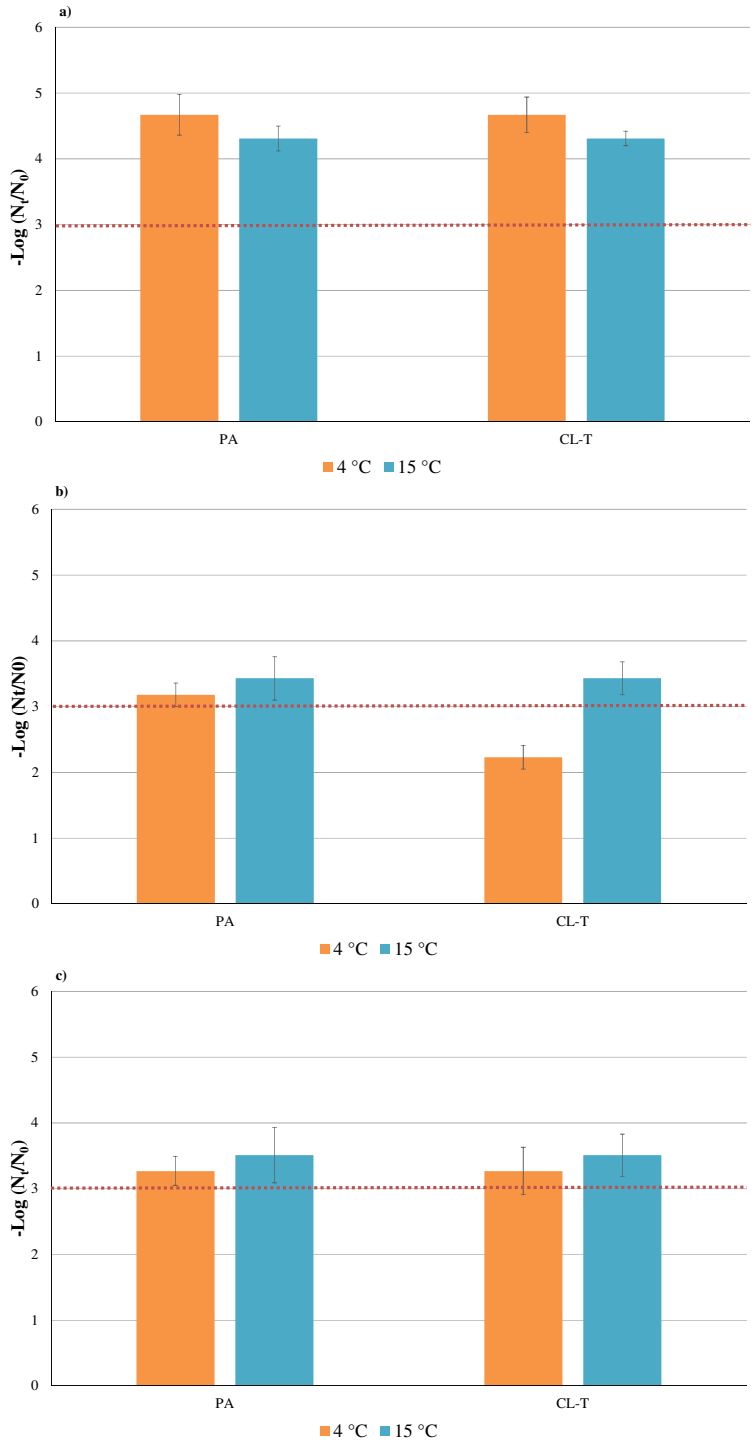

Figure 4.5 Efficacy (mean -Log (N_t/N_0) ± SD; n=2) of PA and CL-T towards biofilms of *P. fluorescens* Ps_019 (a), *P. fragi* Ps_053 (b) and *P. putida* Ps_071 (c) grown on stainless steel at 4 °C and 15 °C; dotted red line refers to the minimal efficacy required to a sanitizing agent towards biofilm cells (Mosteller and Bishop, 1993)

In order to better elucidate the effect of temperature on biofilm formation by *Pseudomonas* spp., *P. fluorescens* Ps_019 was chosen for a study on the kinetics of biofilm formation during a five-day growth on stainless steel in CBR. During the incubation, the cells were enumerated by a viable count evaluation,

and contextually the different components of the biofilm (cells and EPS matrix) were differentially stained and observed using CLSM. Moreover, PA-treated biofilms were characterized in the same way.

Figure 4.6 reports the data related to the formation of biofilm by *P. fluorescens* Ps_019 at 4 °C and 15 °C. At 4 °C the adherence was similar in the first 24 h of incubation, whilst it was significantly higher ($p<0.05$) after 2 d and 5 d. At 15 °C Log CFU/cm^2 means were not affected by the incubation temperature within the first 8 h of growth, while these values were significantly higher at 24 h and 2 d/5 d ($p<0.05$). The adherence of *P. fluorescens* Ps_019 was higher ($p<0.05$) at 15 °C than at 4 °C at each sampling time, except after 2 h. *P. fluorescens* Ps_019 attached on stainless steel in the first eight hours as single cells at 4 °C, while at 15 °C they attached as loosely packed microcolonies. A greater difference in biofilm formation at 4 °C and 15 °C was observed after 24 h of incubation ($p<0.05$).

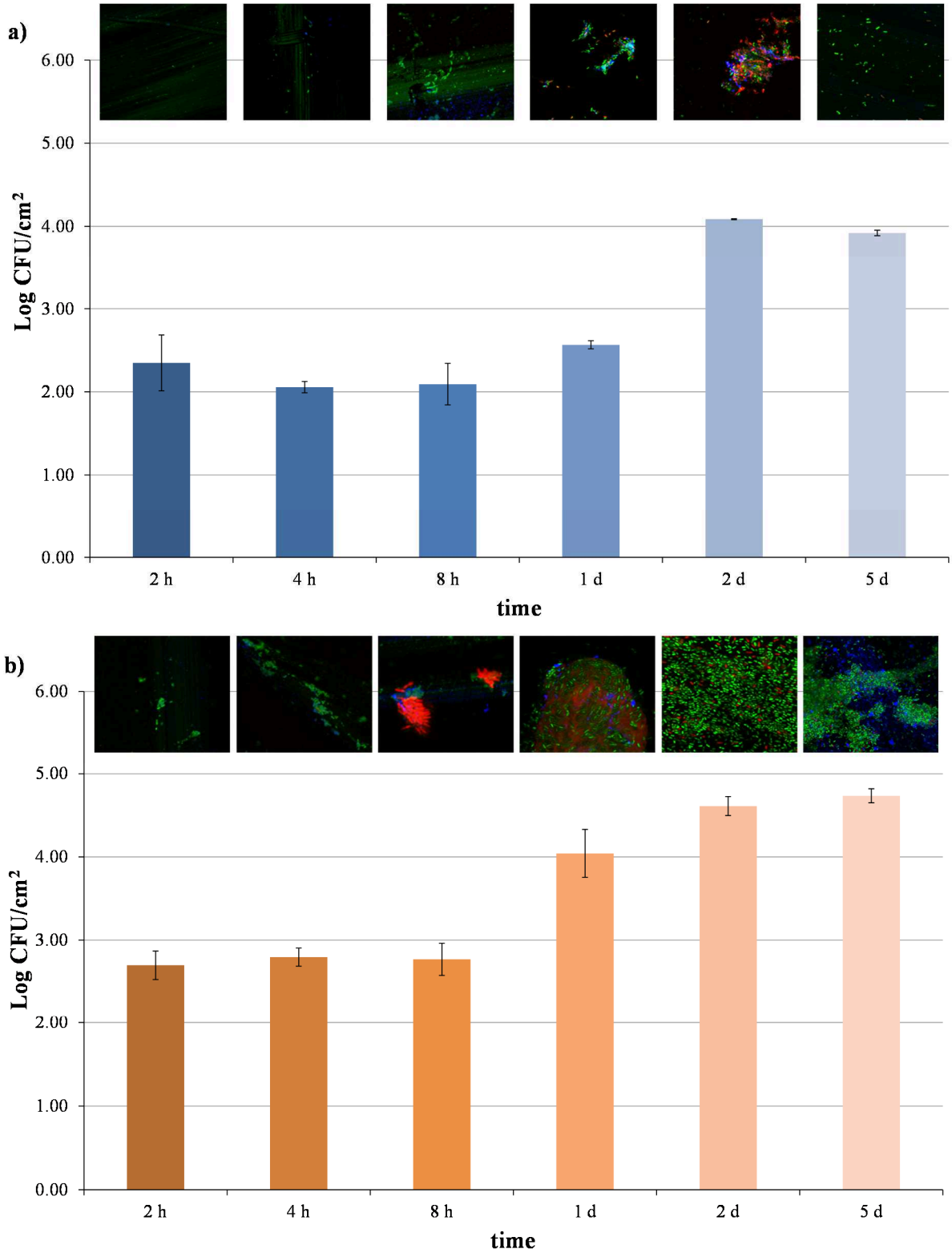

Figure 4.6 Biofilm cell viable counts (mean Log CFU/cm^2 ± SD; n=4) and CLSM images of *P. fluorescens* Ps_019 grown in dynamic conditions on stainless steel at 4 °C (a) and 15 °C (b). Biofilm samples were stained with Syto-9 (green fluorescence, indicating live cells), propidium iodide (red fluorescence, indicating dead cells) and Con-A (blue fluorescence, indicating extracellular matrix)

After 24 h, *P. fluorescens* Ps_019 formed small irregularly shaped microcolonies both at 4 °C and 15 °C, even if the colony sizes were more dense at 15 °C than 4 °C. Moreover, after 24 h at 15 °C, *P. fluorescens* Ps_019 formed ball-shaped microcolonies.

Despite the observed differences in biofilm cell counts at 4 °C and 15 °C, the strong ability of *P. fluorescens* Ps_019 to adhere to and form biofilm on stainless steel at both temperatures should be emphasized. As a matter of fact, already in the first eight hours of growth the number of adherent cells to stainless steel was always higher than 2 Log CFU/cm^2 both at 4 °C than at 15 °C. These amounts of biofilm cells should not be underestimated as they referred to very low temperatures, which are widely used in food plants. Consequently, since the safety of food supply is dependent upon proper cold-chain operations and since the initial attachment is the key to successful biofilm development, it is worrisome that *P. fluorescens* Ps_019 may have the ability to establish biofilms on food processing equipment and/or chilled processed foods. After 5 days of incubations the mean counts of adhered cells were 3.92 Log CFU/cm^2 and 4.74 Log CFU/cm^2 at 4 °C and at 15 °C, respectively. The 2- and the 5-days-old biofilms of *P. fluorescens* Ps_019 were clearly different at the two incubation temperatures. In fact, while at 15 °C it was possible to observe compact and tightly packed microcolonies and the presence of high amounts of blue-stained EPS, at 4 °C small irregularly shaped and loosely packed microcolonies were formed, as well as lower amounts of EPS. From these data it is possible to highlight that the incubation temperature strongly influenced the biofilm formation and structure of *P. fluorescens* Ps_019 during the 5 days of incubation in this study. The obtained results suggest that structural changes in biofilm formation occur in response to changing environments. It is conceivable that different amounts of sessile cells and EPS produced in different temperature conditions are diversely affected by the treatments implemented in the control of biofilms.

After the sanitizer treatment with PA-based products, *P. fluorescens* Ps_019 sessile cells were lower than the detection limit of the microbiological sampling method (Table 4.1). In CLSM images, biofilm cells stained mostly red, indicating cell death, both at 4 °C than 15 °C (Figure 4.7 and Figure 4.8). However, after the PA treatment on 2- and 5-day biofilms grown at both temperatures a significant number of cells was observed still alive, and their size was quite diminished. It has been shown that, after a chlorine-treatment at 100 ppm for 5 min, a significant amount of *P. fluorescens* sessile cells were only damaged, and not killed. Moreover, after the sanitizer treatment, the cell length appeared modified, which may indicate cell injury (Lindsay and von Holy, 1999). It has to be stressed that injured cells may recover within few hours, and consequently re-grow and re-colonize surfaces. CLSM allowed also to evidence the presence of blue-stained EPS, both in untreated cells and in PA-treated cells, though in smaller amount in the latter case. In pure biofilms EPS contributes to the biofilm structure during maturation, and significant amounts were visible in 5-days old biofilms. After PA-treatments, EPS was still detected, probably due to the fact that peracetic acid is not known to remove EPS from surfaces, as does chlorine instead (Alasri et al., 1992). The presence of EPS after a sanitation protocol is not desirable, as it has been hypothesized that it can contribute in enhancing the attachment of Gram-positive bacteria, such as *L. monocytogenes*, to stainless steel surfaces (Sasahara and Zottola, 1993).

Table 4.1 Efficacy of PA on biofilms (mean Log CFU/cm^2 ± SD; n=4) formed by *P. fluorescens* Ps_019; *ND, not detectable, <1 CFU/cm^2

Temperature	Biofilm age	Untreated	PA-treated
4 °C	2-day	4.09 ± 0.01	ND*
4 °C	5-day	3.92 ± 0.03	ND
15 °C	2-day	4.61 ± 0.11	ND
15 °C	5-day	4.74 ± 0.08	ND

Significant reductions in biofilm cells were observed after treatment with PA. The Log CFU/cm^2 reductions obtained in the treatment of biofilms of these strains with peracetic acid were sufficient enough to qualify the peracid-based product used in this study as an efficient sanitizer for biofilm control. In fact, peracid-based disinfectants have been usually used in the food industry especially in the sanitation step of the CIP system (Orth, 1998), as this substance works quickly and is effective against bacteria thanks to its high oxidation capacity of cellular molecules by releasing free oxygen and hydroxyl radicals, which decompose in oxygen, water and acid acetic. Moreover, it does not produce toxic or carcinogenic compounds as it does not react with proteins, it has low environmental impact and it has been reported to be more active against biofilm (Loukili et al., 2006).

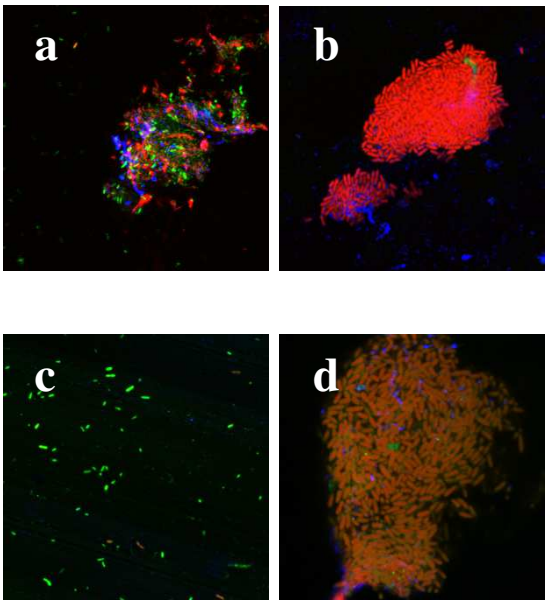

Figure 4.7 CLSM images of *P. fluorescens* biofilm formed at 4 °C; (a) 2-days-old biofilm before and (b) after sanitizer treatment, (c) 5-days-old biofilm before and (d) after sanitizer treatment

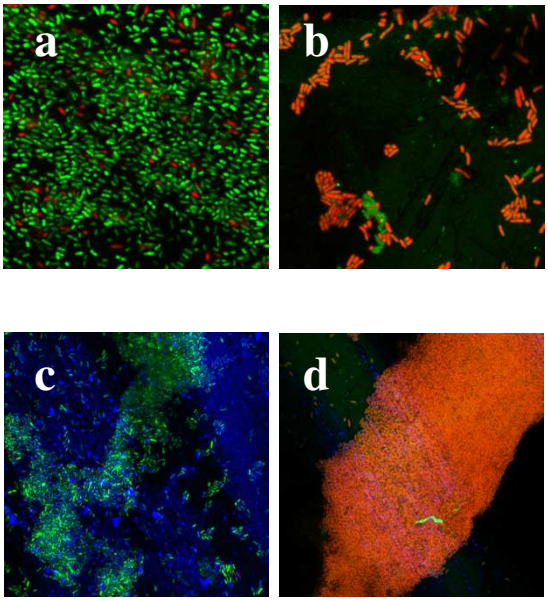

Figure 4.8 CLSM images of *P. fluorescens* biofilm formed at 15 °C; (a) 2-days-old biofilm before and (b) after sanitizer treatment, (c) 5-days-old biofilm before and (d) after sanitizer treatment

4.4 CONCLUSIONS

In this study the effect of temperature on the formation of biofilms by *Pseudomonas* spp. and the sensitivity of the biofilms to disinfection treatments was evaluated. From the obtained results the strains showed a high ability to form biofilm at low temperatures both on polystyrene and on stainless steel, which makes these strains particularly dangerous in the food industry, as they can represent a possible source of food cross-contamination.

The study of biofilms formed on polystyrene and stainless steel have shown that temperature significantly affects the kinetics of adhesion, but also the cell density and the amount of EPS produced, and consequently the resistance to biocides. The use of the CLSM technique for microscopic observation allowed the study of biofilms under undisturbed conditions, and thus is well suited to a possible online use. The presence of alive, but probably damaged cells, observed using CLSM after a treatment with a peracid-based biocide may represent a possible reserve of contamination and consequent alteration of food products.

It is expected that the findings of this study give useful information about the knowledge of sessile organisms response to environmental stimuli, which could support the research for innovative strategies to prevent, inactivate and remove the biofilm.

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Chapter 5. EFFECTIVENESS OF CHEMICAL SANITIZERS AND PULSED LIGHT FOR THE INACTIVATION OF *LISTERIA MONOCYTOGENES* AND *PSEUDOMONAS FLUORESCENS* BIOFILMS

5.1 INTRODUCTION

Attached bacteria and biofilm formed on surfaces can represent a hygiene risk in food processing due to possible cross-contaminations. The most common approach to fight biofilms is to prevent them or, after their development, treat them with chemicals. It is well known that attached cells are more resistant to biocide than planktonic cells, as the polysaccharide matrix provides a protective barrier limiting the penetration of disinfectants. For instance, active chlorine concentrations as high as 1000 ppm are necessary for a substantial reduction in bacterial numbers in multispecies biofilms compared to 10 ppm for planktonic cells (Norwood and Gilmour, 2000). Therefore, biofilm elimination from food processing facilities represents a big challenge.

The selection of detergents and disinfectants for the food industry depends on the efficacy, safety and rinsability of the agent, as well as on its corrosiveness or its effects on the sensory values of the manufactured products. The key to effective cleaning and disinfection of food plants is understanding the type and nature of the soiling agent (sugar, fat, protein, mineral salts etc.) and the microbial growth to be removed (Gibson et al., 1999). Oxidising substances like chlorine or peroxyacetic acid are frequently used. Chlorine is commonly applied as a sanitizer due to its oxidizing and disinfecting power. Peracetic acid is the most widely used among peracid sanitizers and it is often more effective than chlorine, since it maintains activity in the presence of an organic load. Hence, peracetic acid is effective against biofilm bacteria and is advantageous to use if biofilm contains food residues (Chmielewski and Frank, 2003). Other substances, known as surfactants (e.g. acid anionics and quaternary ammonium compounds) are relatively unaffected by an organic load or hard water and are fast acting on yeast but slower acting on bacteria, as reported by Frank and Koffi (1990).

When a microbial population is put into contact with high concentrations of a biocide, susceptible cells will be inactivated. However, some cells may possess a degree of natural resistance and physiological plasticity or they may acquire it later, so they can survive and grow even after a sanification protocol. Indeed, bacterial resistance to all classes of biocides has been reported in the literature, and anecdotal reports within the biocide industry are common (Chapman, 2003). Thus, the increased biofilm resistance to chemical treatments enhances the need to develop new control strategies. Moreover, conventional chemical and mechanical cleaning and disinfection methods tend to be too harsh and time-consuming, and chemical residues remaining on the surface might be a risk upon coming in contact with foods (Wirtanen and Salo, 2003). The use of disinfectants, which are often provided to the user in a concentrated form, is also a potential risk to the safety of operators involved in the sanitation program if this is not properly instructed. Also, it is important not to underestimate the possible consequences related to the emission of residual materials, such as environmental pollution and the selection of microbial species, resistant/tolerant to antimicrobial substances.

One alternative technology that can be applied for surface disinfection is the radiation with ultraviolet (UV) light. The antimicrobial activity of short-wave ultraviolet (UV) light in the "UV-C" band (200 to 280 nm) is well known to reduce microbial contamination in hospitals (Andersen et al., 2006), in the pharmaceutical/medical industry (Rastogi et al., 2007), in the water treatment plants (Høibe et al., 2008) and on the food products and contact surfaces (Woodling and Moraru, 2005; Sommers et al., 2009). UV-C light does not contain or produce toxic compounds, it does not have legal restrictions or require extensive safety equipment, and these characteristics make it an interesting disinfection principle for food processing. A method that is receiving considerable attention is pulsed light (PL) radiation, a non-thermal technique for decontaminating food, packaging, water and air. PL is an approach which kills microorganisms by using ultra-short duration pulses of an intense broadband emission spectrum that is rich in UV-C germicidal light. PL is produced using techniques that multiply power manifold by storing electricity in a capacitor over a relatively long time (fractions of a sec) and releasing it in a short time (millionths or thousandths of a sec) using sophisticated pulsed compression techniques. The emitted flash has a high peak power and usually consists of a wavelength from 200 to 1100 nm broad spectrum light enriched with shorter germicidal wavelengths (Gómez-López et al., 2007). By killing the surface spoilage microflora, PL treatment was found to inactivate microorganisms naturally present on vegetables, fruits, food powders and seeds (Gómez-López et al., 2005). Currently, only little information is available on the PL effect on microbial biofilms formed on materials used in food plants.

The objective of this study was to evaluate the performances of different approaches aimed to the inactivation of *L. monocytogenes* and *P. fluorescens* biofilms. These microorganisms are known for their ability to effectively colonize food processing surfaces and for representing a hygienic risk for food products. Thus, biofilms formed in dynamic flow conditions on two types of material were treated with both commercial chemicals and PL, in order to compare the performances of both approaches and to acquire useful information for the development of non-conventional strategies.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains and culture conditions

The microorganisms used in this study were *L. monocytogenes* Lm_284 and *P. fluorescens* Ps_019 (see List of Strains). Both strains were classified as strong biofilm-producers according to Stepanović et al. (2000).

5.2.2 Biofilm formation on stainless steel and PTFE

Biofilms were grown in the CDC Biofilm Reactor (CBR) on stainless steel AISI 304 and polytetrafluoroethylene (PTFE) coupons. In these trials, Luria Bertani broth (LB) was used as culture medium in the CBR, which was inoculated as described in paragraph 2.2.2. The biofilm growth was carried out for 48

hours under dynamic conditions (in batch with rotation of the magnet to 125 rpm) at 20 °C. Each experiment was repeated three times for each strain.

5.2.2.1 Biofilm treatment with disinfectants

After the biofilm growth, stainless steel and PTFE coupons were removed from each rod of the CBR and subjected to rinsing, as described in paragraph 2.2.2. Afterwards, coupons were immersed for 5 min in 5 mL of each sanitizing solution at the concentrations recommended by the manufacturer. The commercial products used in this test included formulations based on chlorine, iodophors, quaternary ammonium salts, glycolic acid, alcohols, organic acids, peroxides and amines (Table 5.1).

Table 5.1 Sanitizing products used for biofilm treatments

Abbreviation	Active compounds
CL-1	sodium hypochlorite
CL-2	chloramine T
CL-3	potassium hydroxide, sodium hypochlorite
PER	peracetic acid, acetic acid, hydrogen peroxide
ALC	alcohols, hydrogen peroxide, chlorhexidine digluconate
QAC	quaternary ammonium salt, EDTA, monoethanolamine
QAC-G	glutaraldehyde, quaternary ammonium salt
GLY	glycolic acid
IOD	iodine
ANF	surfactants, alkyl amine, alcohol

After the treatment, the coupons were neutralized with 5 mL of sodium thiosulfate at 0.5% for 5 min, then the cells were detached from the surface and treated as described in paragraph 2.2.2. Microbial viable counts were evaluated using the spread plate method on Brain Heart Infusion (BHI, Oxoid, Milan, Italy) agar plates. The plates were incubated at 30 °C for *P. fluorescens* and at 37 °C for *L. monocytogenes* for 48 hours.

5.2.2.2 Biofilm treatment with pulsed light

After removal from the CBR, the coupons were rinsed twice with a sterile saline, placed in Petri dishes covered with a transparent plastic film to UV-C light (polycoupled Combiflex PA/PE 090, 20/70, Savonitti, Codroipo, Italy) and processed using a pulsed light mobile decontamination unit (Claranor, Rouaine, France) equipped with 4 xenon lamps with a maximum emission in the range 200-1000 nm. The samples were treated with increasing fluence (incident light energy per unit of surface area, KJ/m^2) as a function of the distance of the sample from the light source and the number of pulses. After PL treatment, the biofilm cells were detached from coupons by using sterile cell-scrapers; detached cells were resuspended in 1 mL of MRD and sonicated as described in paragraph 2.2.2. The bacterial counts were carried

out by plating serial ten-fold dilutions of the biofilm cell suspension on BHI plates, using the spread plate method. The plates were incubated at 30 or 37 °C for 24-48 hours. Each trial was repeated twice.

5.2.3 Statistical analysis

The data were statistically analysed using the analysis of variance and the means separated according to Tukey's HSD test with a significant level (*p* value) of 0.05 using Statistica 8.0 (StatSoft, Tulsa, Oklahoma, USA).

5.3 RESULTS AND DISCUSSION

In this work the sensitivity of *L. monocytogenes* and *P. fluorescens* biofilms formed on stainless steel and PTFE surfaces to sanitizers and PL was studied. These microbial species were chosen as they are potentially pathogenic and food spoilage bacteria, and they are known for their biofilm forming ability in the food industry. Considering the safety issues related to the presence of such microbial species in the food plants, it is of interest to identify the most efficient strategy in killing biofilm cells.

In Table 5.2 the data related to the formation of biofilm by *L. monocytogenes* and *P. fluorescens* on stainless steel and PTFE in dynamic conditions are reported.

Table 5.2 Mean biofilm viable counts (mean Log CFU/cm^2 ± SD; n=3) formed by *L. monocytogenes* Lm_284 and *P. fluorescens* Ps_019 in CBR

Strain	Stainless steel	PTFE	statistical significance[§]
L. monocytogenes Lm_284	4.57 ± 0.07	5.24 ± 0.09	*
P. fluorescens Ps_019	4.87 ± 0.11	5.22 ± 0.06	*

[§] statistical significance for data within rows (*, p<0.05)

Both strains are able to adhere and form a biofilm on surfaces tested, which are materials commonly used in the food industry. The mean viable counts varied from 4.57 to 5.24 Log CFU/cm^2 for *L. monocytogenes*, with the highest adhesion on PTFE ($p<0.05$). Regarding *P. fluorescens* strain Ps_019, the adhesion varied between 4.87 and 5.22 Log CFU/cm^2 ($p<0.05$). Cunliffe et al. (1999) observed a weaker adhesion of *L. monocytogenes* on hydrophilic with respect to hydrophobic surfaces, whilst Chavant et al. (2002) reported a faster biofilm formation on stainless steel compared to PTFE. Moreover, Smoot and Pierson (1998) observed a higher attachment on polymers and rubber compared to stainless steel. Microbial adhesion is influenced not only by the contact surface, as several factors are involved in the adhesion process. Biological mechanisms, such as the presence of adhesive molecules on cell surfaces could be major factors in the process of microbial adhesion and further biofilm development (Sauer and Camper, 2001). Moreover, microorganisms can adapt their attachment strategies to the nature of the surface. Data obtained from several studies on the effect of surface materials on adhesion and biofilm formation are contradictory, probably owing to experimental culturing conditions and/or strain differences. Although it is difficult to compare the

different studies, it is apparent that the surface material affects adhesion and biofilm formation. This consideration is particularly relevant when planning the use of different materials in food plants, as the knowledge of microbial adhesion affinity of the different materials could help in avoiding the use of the worst material in the areas of a food plant where the biofilm formation is more probable.

To evaluate the antimicrobial activity of commercial sanitizers against biofilms, the efficacy of the disinfecting treatments was evaluated by calculating the ratio of the Log CFU/cm^2 before (N_0) and after the treatment (N_t). The results are reported in Table 5.3 and Table 5.4 presented as -Log (N_t/N_0).

Table 5.3 Reduction (mean -Log (N_t/N_0) ± SD; n=3) of *L. monocytogenes* Lm_284 biofilms treated by commercial sanitizers; [#]ND, not detectable, < 1 CFU/cm^2

Sanitizers	stainless steel	PTFE	statistical significance[§]
CL-1	4.21 ± 0.08	4.10 ± 0.05	
CL-2	3.75 ± 0.03	3.99 ± 0.02	*
CL-3	ND[#]	ND	
PER	4.21 ± 0.11	5.07 ± 0.09	*
ALC	ND	ND	
QAC	ND	ND	
QAC-G	ND	ND	
GLY	ND	ND	
IOD	ND	ND	
ANF	ND	4.73 ± 0.13	*

[§] statistical significance for data within rows (*, p<0.05)

Table 5.4 Reduction (mean -Log (N_t/N_0) ± SD; n=3) of *P. fluorescens* Ps_019 biofilms treated by commercial sanitizers; [#]ND, not detectable,< 1 CFU/cm^2

Sanitizers	stainless steel	PTFE	statistical significance[§]
CL-1	2.59 ± 0.11	1.16 ± 0.19	*
CL-2	ND[#]	ND	
CL-3	ND	4.99 ± 0.02	*
PER	ND	ND	
ALC	2.09 ± 0.15	3.56 ± 0.11	
QAC	3.33 ± 0.16	4.99 ± 0.04	*
QAC-G	ND	4.11 ± 0.02	*
GLY	ND	4.99 ± 0.01	*
IOD	ND	3.89 ± 0.11	*
ANF	3.73 ± 0.06	1.76 ± 0.23	*

[§] statistical significance for data within rows (*, p<0.05)

All disinfectants had a significant effect on the viability of biofilm microbial cells, regardless of species tested. Regarding *L. monocytogenes*, an almost total microbial inactivation of the formed biofilm in all the experimental conditions was observed for QACs, iodophors, glycolic acid and alcohols and CL-3, while two chlorine-based products and the peracid-based product were not sufficient to ensure a complete inactivation of the biofilm cells. This finding is rather surprising as, according to many authors, chlorine-based compounds and products containing oxidants like peracetic acid are the most used for the treatment of microbial biofilms. As recommended by Mosteller and Bishop (1993), disinfectant products used against biofilms must be able to ensure a microbial inactivation of at least 3 Log in the cell population. Therefore,

observing the data reported in Table 5.3, all the commercial products were found sufficiently effective against *L. monocytogenes*, even though the survival of a number of cells, although limited, can generate the formation of a new biofilm.

Regarding *P. fluorescens*, a lower sensitivity of biofilms to disinfectants compared to those of *L. monocytogenes* was observed. The treatments with some disinfectants did not always guarantee 3 Log-reductions of the biofilm viable cells. For example, the chlorine-based product CL-1 was rather ineffective. The higher resistance of *P. fluorescens* biofilms could be related to the high amount of EPS secreted during biofilm formation, which can protect the biofilm from the penetration of antimicrobials (Allison et al., 1998) or it is linked to the reaction of chlorine species with organic matter in the surface layers of biofilm, which is faster than their diffusion into the biofilm interior (Chen and Stewart, 1996). *P. fluorescens* biofilms were quite sensitive to the other chlorine-based products (CL-2 and CL-3), probably due to the chemical characteristics of the specific commercial formulations. Even glycolic acid and peracetic acid were quite effective in inactivating *P. fluorescens* biofilm.

In order to investigate the potential of PL treatments in inactivating microbial biofilms, in a preliminary step the effect of the distance between the light source and the sample was evaluated by treating *L. monocytogenes* biofilms with 1 pulse at three different distances, conventionally defined near, mid and far, corresponding to fluence values of 18, 12 and 8 KJ/m^2. In the case of the farther distance, the samples were also treated with 2 pulses (16 KJ/m^2). As can be observed in Figure 5.1, the treatment with PL was effective against *L. monocytogenes* biofilm cell viability, with higher inactivation at a decreased distance of the lamp from the coupon. This effect was expected since decreasing distance strongly increases the energy supplied to the sample, and consequently increases the antimicrobial effect.

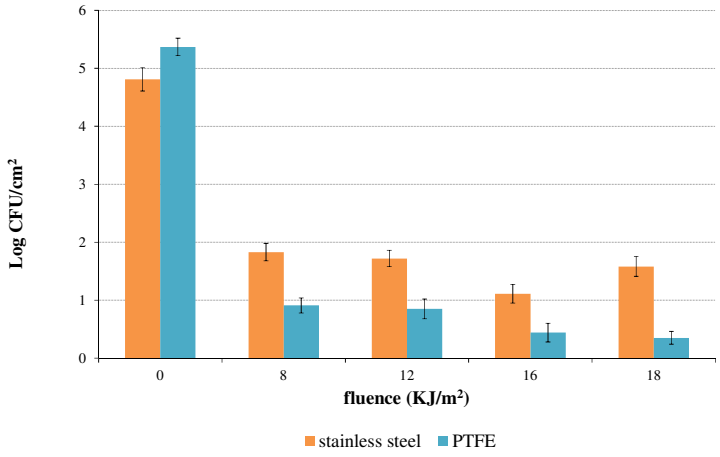

Figure 5.1 PL inactivation (mean Log CFU/cm^2 ± SD; n=2) of *L. monocytogenes* Lm_284 biofilms as affected by the distance between lamps and sample

The PL treatments caused higher inactivations on biofilms formed on PTFE with respect to stainless steel ($p<0.05$). This difference can be linked to the interactions between the cell surface and the material

on which the biofilm is formed, or to possible different amounts of EPS secreted by the cells on the different materials. Woodling and Moraru (2007) evidenced that the rougher surfaces can protect biofilm cells from the effect of pulsed light holding and "hiding" them inside the micro-cracks. The most effective treatment, with reductions of viable counts higher than 3 Log CFU/cm^2 on stainless steel and PTFE, was obtained for the nearest distance and 1 pulse (p<0.05). However, after the treatment, still alive cells were present. Thus, for the following tests the treatments were performed against *L. monocytogenes* and *P. fluorescens* biofilms at the nearest distance with increased numbers of pulses, corresponding to fluence values of 18, 36 and 54 KJ/m^2 (for 1, 2, and 3 pulses respectively).

Both for *L. monocytogenes* and *P. fluorescens* biofilms, an increased number of pulses caused a higher inactivation of the cells (Figure 5.2 and Figure 5.3). In particular, for *L. monocytogenes* a fluence of 54 KJ/m^2 caused a significantly higher inactivation than 18 and 36 KJ/m^2 (p<0.05), while for *P. fluorescens* a fluence of 36 KJ/m^2 were already sufficient to allow a significantly higher inactivation than 18 KJ/m^2 (p<0.05)

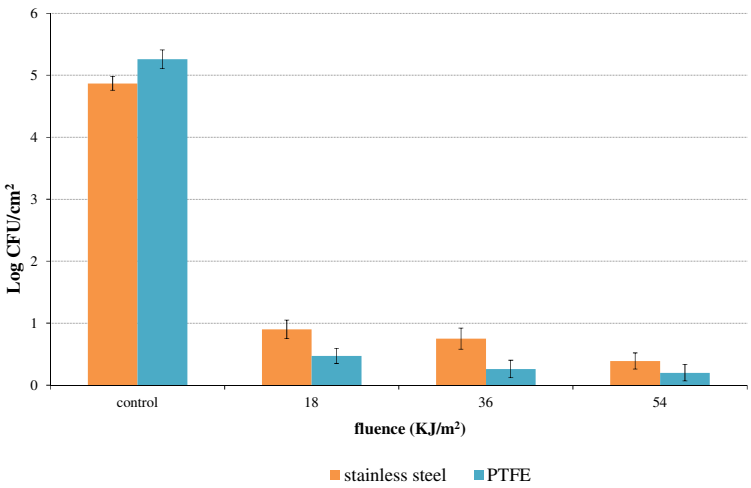

Figure 5.2 PL inactivation (mean Log CFU/cm^2 ± SD; n=2) of *L. monocytogenes* Lm_284 biofilms as affected by the number of pulses at the nearest distance

Also for fluence values higher than 18 KJ/m^2 the inactivation of *L. monocytogenes* was higher on biofilms formed on PTFE than stainless steel for treatments with fluence of 18 and 36 KJ/m^2 (p<0.05). The most antimicrobial effect was observed just after the first pulse. In fact, in the case of PTFE the difference between the initial count and the viable count after the treatment was 4.79 Log CFU/cm^2 (p<0.05). However, increasing the number of pulses up to 3, only a minimal improvement in the efficacy of the treatment was observed. For stainless steel, the highest inactivation was observed for fluence values of 54 KJ/m^2, even if for 18 KJ/m^2 the reduction was already 3.97 Log CFU/cm^2.

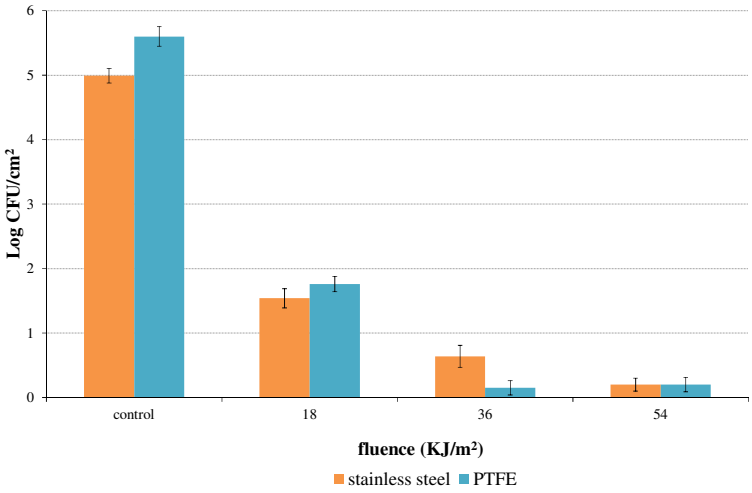

Figure 5.3 PL inactivation (mean Log CFU/cm^2 ± SD; n=2) of *P. fluorescens* Ps_019 biofilms as affected by the number of pulses at the nearest distance

P. fluorescens Ps_019 biofilms were more resistant than *L. monocytogenes* Lm_284 to PL treatmens for the lowest fluence values (p<0.05). This result is in contrast with the observation of Anderson et al. (2000), who showed that the Gram-positive bacteria are more resistant than Gram-negative to this type of treatment. Farrell et al. (2010) observed that *P. aeruginosa* showed some resistance to PL related to the production of coloured pigments (pyocyanin and pyoverdin), as these pigments were able to absorb the wavelengths corresponding to the region of the germicidal UV light.

From the data it is possible to observe that PL treatments were able to produce a strong antimicrobial effect on *L. monocytogenes* and *P. fluorescens* biofilms formed on different materials. As observed by Woodling and Moraru (2005) the PL treatment might induce a sub-lethal damage on microbial biofilms, which the cells are able to overcome after a revitalization step in a nutrient rich medium. In the conditions used in this study, it was not possible to evidence a sub-lethal damage of the biofilm cells as the microbiological counting technique always allowed to obtain a countable number of colonies on agar plates. In any case, the use of PL is promising for a hurdle approach, in which any surviving damaged cells are then inactivated by other treatments. For example, it is conceivable to obtain an almost total biofilm cell inactivation effect using one pulse followed by a treatment with a chemical disinfectant used at a low concentration.

The use of PL at low fluence values (18 KJ/m^2) has not been able to cause a complete inactivation of the biofilms, so it does not seem feasible to use this technology to sanitize the surfaces of the production areas at the end of the day. However, in some processing sites a lower rate of inactivation is still sufficient to control the risk of cross contamination. For example, the use of the PL treatment on conveyor belts during the production cycle would be conceivable, so as to minimize the risk of formation of biofilm, which is well documented in many industries (Somers and Wong, 2004).

5.4 CONCLUSIONS

In this study the effects of chemical and physical treatments on *L. monocytogenes* and *P. fluorescens* biofilms formed in dynamic culture conditions and on two types of material were evaluated. From the results it was possible to observe how the environmental conditions in which biofilms were formed influenced their sensitivity to the individual treatment. In fact, the surface material on which biofilms were formed might affect their sensitivity to treatment, probably due to a different production of EPS substances by the cells.

A comparison between chemical and physical treatments shows that some chemical products used for disinfection in the food industries are not effective in inactivating the biofilm cells, and unexpectedly the products usually used for inactivating biofilms are not the most effective. The treatments made with PL showed that this strategy proved to be very promising, since also at the lowest applied fluence a strong inactivation of viable counts on stainless steel and PTFE was observed. Although survivor cells are present, the PL treatments can be useful for the surface decontamination of equipment, for example conveyor belts, during the production cycle. Moreover, thanks to the ability of this technology to inactivate or damage the cells at a sub-lethal level, a treatment with PL might precede treatment with biocides applied at lower concentrations and for a shorter time than the routine uses, allowing a significant reduction of the risk for operators and environmental damage. It is possible that the use of these techniques in a unique strategy for the control of biofilm is able to guarantee the solution to problems of health and hygiene in the production of foodstuffs.

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Chapter 6. SUSCEPTIBILITY OF MICROBIAL BIOFILMS TO ENZYMATIC TREATMENTS

6.1 INTRODUCTION

There are several strategies for biofilm removal that may be applied to food processing environments. The most widely used approach is the application of biocides and disinfectants, like hypochlorite, peroxyacetic acid and quaternary ammonium compounds. However, the increased biofilm resistance to conventional chemical treatments enhances the need to resort to alternative control strategies. An attractive alternative to conventional chemical methods of sanitation processes is represented by the use of enzyme-based detergents, also known as "green chemicals". As a matter of fact, enzymes can be used for degradation of biofilm, although a mixture of enzyme activities may be necessary for a sufficient degradation of bacterial biofilm, due to the heterogeneity of the extracellular polysaccharides in the biofilm (Sutherland, 1995). Promoting detachment is one of the least investigated possible strategies to remove biofilms. The use of substances to induce biofilm removal by directly destroying the physical integrity of the biofilm matrix represents an attractive alternative for food industrial applications, where complete biofilm removal is essential. This approach has also the advantage of reducing reliance on inherently toxic antimicrobial agents, whose continued use is fundamentally at odds with the trend towards increasingly restrictive environmental regulations (Chen and Stewart, 2000). Augustin et al. (2004) demonstrated the efficacy of enzymatic cleaning products against biofilms formed by microorganisms commonly found in dairy products, concluding that these products may be useful for inactivating biofilms produced in milk and other lipid food residues that may remain after poor cleaning in many industrial types of equipment. Oulahal-Lagsir et al. (2003) found interesting results when synergistically applying ultrasonic waves and proteolytic and glycolytic enzymes against stainless steel biofilm cells of *Escherichia coli* developed with milk. Proteinase disinfectants showed a good effect against *P. aeruginosa* biofilms, even if the performances of the disinfectants was reduced in presence of organic residues such as milk (Augustin and Ali-Vehmas, 2004). Amylases are another type of enzyme widely used in the formulation of enzyme detergents, mainly for food residue removal of starch-based foods. An α-amylase showed to be very efficient in removing *P. fluorescens* biofilms from stainless steel (Lequette et al., 2010). At any rate, in each formulation there must be either both protease and enzymes that break down carbohydrates, due to the heterogeneity of the matrix (Meyer, 2003). Currently the use of enzymes as an alternative to chemical disinfectants is still limited due to the expensive process to produce commercial formulations of enzymes compared with the low costs of the chemicals, as technology and production of enzyme-based detergents are mostly patent-protected.

The aim of this study was to analyze the effectiveness of commercially available enzymes in removing biofilms formed by *L. monocytogenes*, *S. aureus* and *P. fluorescens*. Three enzymatic products were tested against *L. monocytogenes, S. aureus* and *P. fluorescens* biofilms preformed on microtiter plates under different concentration and temperature conditions. Then, the most performing enzymatic product was tested for removing *L. monocytogenes*, *S. aureus* and *P. fluorescens* biofilms preformed under dynamic flow condition on stainless steel AISI 304 and PTFE coupons.

6.2 MATERIALS AND METHODS

6.2.1 Biofilm formation on polystyrene microtiter plates and quantification by using the crystal violet assay

L. monocytogenes Lm_284 (moderately adherent according to Stepanović et al., 2000; see paragraph 2.2.1), *S. aureus* St_059 (strongly adherent) and *P .fluorescens* Ps_019 (strongly adherent) were used in this study (see List of Strains). The biofilm production assay was performed in three biological replicates in 96-well polystyrene flat bottom microtiter plates, as described in paragraph 2.2.1. Briefly, eight wells of each microplate were filled with 200 µL of Tryptone Soya Broth (TSB, Oxoid, Milan, Italy) and inoculated with 10 µL of each overnight culture (~ 10^8 CFU/mL). The microtiter plates were incubated for seven days at 37 °C for *L. monocytogenes* and *S. aureus* and 30 °C for *P. fluorescens.* Every 48 h the microtiter plates were subjected to a refreshing, i.e. the replacement of 150 µL of exhausted broth with an equal volume of fresh broth. For each experiment, eight wells were used as negative controls and filled with 200 µL of TSB not inoculated. At the end of the incubation, the microtiter plates were rinsed and stained as described in paragraph 2.2.1.

6.2.2 Enzymatic products

The enzymatic products used in this study were: PecP (a mixture of pectinase, polygalacturonase and pectinmetylesterase isolated from *Aspergillus niger*; T_{opt} 40 °C, T_{range} 20-70 °C; pH_{opt} 4.2, pH_{range} 2.4-5.2; industrial use: apple and pear juice clarification by ultrafiltration), CelA (a mixture of cellulase and hemicellulase isolated from *Aspergillus niger*; T_{opt} 45 °C, T_{range} 20-60 °C; pH_{opt} 3.85, pH_{range} 3.2-4.7; industrial use: viscosity reduction and hydrolysis of substrates containing cellulose and pectins), and CelT (mixture of cellulases isolated from *Trichoderma reesei*; T_{opt} 50 °C, T_{range} 20-70 °C; pH_{opt} 4.8, pH_{range} 4.5-6.5; industrial use: modification and digestion of carbohydrates, such as cellulose, hemicellulose and ß-glucans).

6.2.3 Enzymatic treatment of biofilms developed in microtiter plates

After incubation, the biofilm growth medium was gently removed and each well was washed three times with sterile saline (paragraph 2.2.1). Then for each treatment eight wells were filled with 200 µL of enzyme solution dissolved in acetate buffer at pH 4. The treatment were performed at 25 and 37 °C at different contact times (15, 30 and 50 min) and concentrations of the enzymatic product (1% and 2%). In order to highlight a possible non-enzymatic action in removing biofilm, biofilms were also treated with 200 µL of acetate buffer and 200 µL of sterile water. At the end of the treatments, each well was washed with 200 µL of sterile saline and stained with crystal violet according to the procedure previously described (paragraph 2.2.1). Each trial was performed in triplicate.

6.2.4 Formation of biofilms developed on stainless steel and PTFE coupons

Biofilms were grown in triplicate in Luria Bertani broth (LB, Oxoid, Milan, Italy) in CDC Biofilm Reactor (CBR) on stainless steel AISI 304 and PTFE coupons (paragraph 2.2.2). Biofilm growth was performed for 48 h under dynamic conditions in batch with rotation of the magnet to 125 rpm at 20 °C. Each experiment was repeated three times for each strain.

6.2.5 Enzymatic treatment of biofilms developed on stainless steel and PTFE coupons

After incubation the coupons were removed from each rods of the CBR and subjected to rinsing, as described in paragraph 2.2.2. Subsequently, the coupons were immersed in 5 mL of a 1% enzymatic product for 15, 30 and 50 min at 18 °C. Afterward, the coupons were rinsed with 5 mL of a sterile saline solution and subjected to microbiological analysis, as described in paragraph 2.2.2. For each treatment, two coupons for each material were used as a control and treated at the above conditions with 5 mL of sterile saline solution. Viable counts were estimated on BHI agar plates using the drop plate method (paragraph 2.2.3). Plates were incubated for 48 h at 37 °C for *L. monocytogenes* and *S. aureus* and at 30 °C for *P. fluorescens*.

6.2.6 Statistical analysis

The data were statistically analysed using the analysis of variance and the means separated according to Tukey's HSD test with a significant level (*p* value) of 0.05 using Statistica 8.0 software (StatSoft, Tulsa, Oklahoma, USA).

6.3 RESULTS AND DISCUSSION

The presence of bacterial biofilms in food processing lines is of great concern for the food industry. Chemical products are commonly used in cleaning procedures for removing biofilms. However, in some cases, these procedures are not always sufficient for removing cells and EPS of biofilms. Moreover, it is well known that microbial biofilms can acquire a resistance to physical and chemical treatments applied during sanitizing operations (Chmielewski and Frank, 2003). For these reasons, the enzymatic approach to the removal of biofilms, mostly based on the destabilization of the EPS matrix, can be a possible choice when traditional sanitizing protocols do not give satisfactory results in terms of biofilm eradication. In addition, in industrial applications, this approach would also have the advantage of reducing reliance on inherently toxic antimicrobial agents, whose continued use is in conflict with the trend towards increasingly restrictive environmental regulations (Chen and Stewart, 2000).

Because biofilm EPS is typically composed of diverse substances, mostly polysaccharides (Flemming and Wingender, 2001), three different commercial enzymatic products were used in this study. In a

preliminary study, the enzymatic mixtures were used at different working conditions of temperature, concentration and contact times, to remove *L. monocytogenes, S. aureus* and *P. fluorescens* biofilms grown on microtiter plates. In fact, it is well known that the ideal sanitization protocol for the food industry is the one that requires the concentrations of the active substance to be as low as possible, shorter times and the working conditions (eg. operating temperatures) closer to room temperature.

Before screening enzyme products for biofilm removal, the influence of sterile deionized water and acetate buffer at pH 4, which is the buffer in which the enzyme preparations were dissolved, was first tested in removing biofilms The values of OD_{570} of biofilms treated with water and acetate buffer were similar to control wells (p>0.05). In fact, p values were 0.775454, 0.809875 and 0.797758 for Lm_284, St_059 and Ps_019, respectively. Therefore neither water nor acidic buffer were efficient in removing biofilms (Figure 6.1), and each difference of OD_{570} evidenced in the following stages of the study was attributed to the action of the enzymatic products.

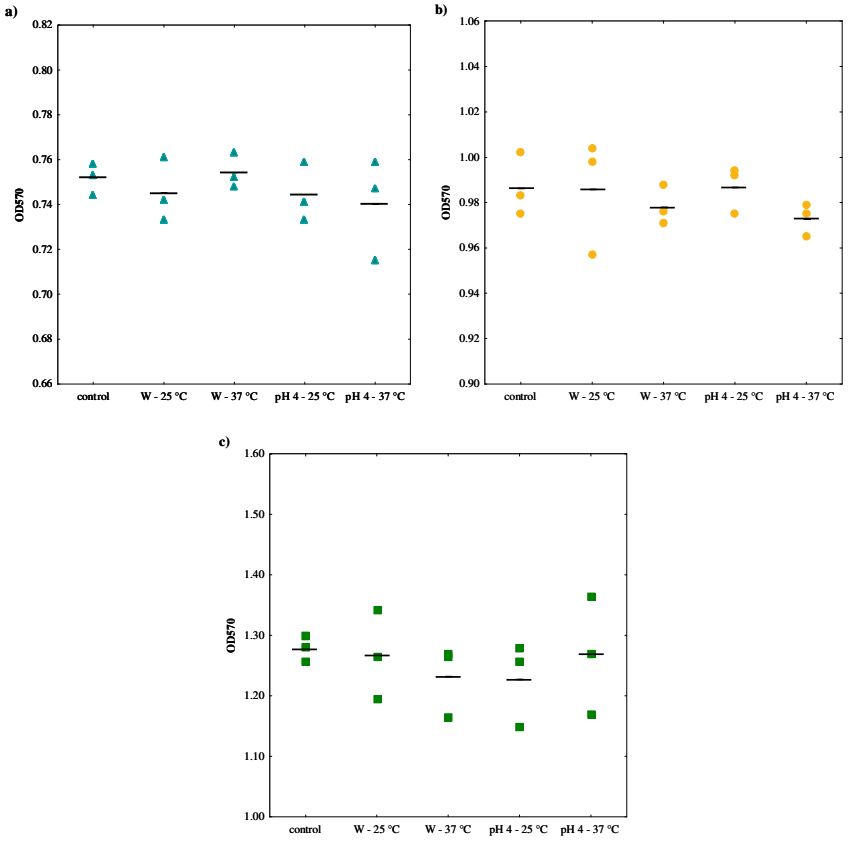

Figure 6.1 Biofilm biomass (mean OD_{570} ± SD; n=3) of (a) *L. monocytogenes* Lm_284, (b) *S. aureus* St_059 and (c) *P. fluorescens* Ps_019 on polystyrene treated with water and acetate buffer; coloured markers are for raw data, line for mean data

In Table 6.1 the results obtained for *L. monocytogenes* treated at 25 °C and 37 °C with enzymatic products are reported. The performance of enzyme mixtures in removing biofilms was evaluated through the calculation of the Percentage Reduction Index (PRI), which estimates the percentage of absorbance

values (OD_{570}) in treated wells in comparison to the OD_{570} value of the control (Pitts et al., 2003). In particular, the removal was considered total if PRI>70%, and only partial if 30%<PRI<70%. All treatments applied at 25 °C were effective in the removal of preformed biofilm (p<0.05), and in several conditions the removal was total (PRI>70%). In particular, the treatment with PecP and CelA allowed higher biofilm removal (p<0.05), regardless of the concentration used. The product CelT was effective in all cases at 25 °C, even though the amount of microbial biomass removed was lower compared to PecP and CelA (p<0.05).

Table 6.1 Biofilm biomass (mean OD_{570} ± SD; n=3) for *L. monocytogenes* Lm_284 after enzymatic treatments; mean values with a different letter within the same temperature treatment indicate statistically different values (p<0.05). The means in bold refer to a total removal of biofilm (PRI> 70%)

L. monocytogenes Lm_284				
temperature	enzymatic product	treatment	concentration	OD_{570nm}
25 °C	PecP	control		0.744^{a} ± 0.026
		15 min	1%	**0.061^{e}** ± 0.050
		30 min		**0.068^{e}** ± 0.080
		50 min		**0.105^{d}** ± 0.012
		15 min	2%	**0.069^{e}** ± 0.012
		30 min		**0.090^{de}** ± 0.011
		50 min		**0.061^{e}** ± 0.016
	CelA	15 min	1%	**0.152^{c}** ± 0.014
		30 min		**0.159^{c}** ± 0.017
		50 min		**0.180^{c}** ± 0.019
		15 min	2%	**0.152^{c}** ± 0.015
		30 min		**0.156^{c}** ± 0.013
		50 min		**0.142^{c}** ± 0.018
	CelT	15 min	1%	**0.141^{c}** ± 0.017
		30 min		**0.103^{d}** ± 0.011
		50 min		**0.125^{dc}** ± 0.012
		15 min	2%	0.239^{b} ± 0.018
		30 min		0.270^{b} ± 0.023
		50 min		0.260^{b} ± 0.027
37 °C	PecP	control		0.728^{a} ± 0.027
		15 min	1%	**0.093^{c}** ± 0.009
		30 min		**0.071^{cd}** ± 0.008
		50 min		**0.052^{d}** ± 0.008
		15 min	2%	**0.102^{c}** ± 0.013
		30 min		**0.085^{c}** ± 0.013
		50 min		**0.039^{d}** ± 0.007
	CelA	15 min	1%	0.254^{b} ± 0.016
		30 min		0.248^{b} ± 0.014
		50 min		**0.194^{b}** ± 0.009
		15 min	2%	**0.193^{b}** ± 0.012
		30 min		0.237^{b} ± 0.013
		50 min		**0.125^{c}** ± 0.018
	CelT	15 min	1%	0.282^{b} ± 0.017
		30 min		0.252^{b} ± 0.011
		50 min		0.232^{b} ± 0.018
		15 min	2%	0.230^{b} ± 0.008
		30 min		0.226^{b} ± 0.023
		50 min		**0.101^{c}** ± 0.027

Enzymatic product CelT, at 25 ° C and at a concentration of 2%, unexpectedly allowed to remove biofilm less than the concentration of 1% (p<0.05), regardless of the time of contact; this phenomenon was not observed in the samples treated at 37 °C. One possible explanation for this observation could be a temperature-dependent conformational change of one or more enzymes in the CelT mixture, which could reduce the enzyme activity in the presence of high concentrations of substrate (Petsko and Ringe, 2004). As regards the test carried out at 37 ° C, the performances of the three enzymatic mixtures were different. While PecP was able to almost completely remove biofilm of *L. monocytogenes* regardless of concentration and contact time, the effectiveness of CelA and CelT was lower (p<0.05). The increase of the contact time up to 50 min in some cases allowed an almost total elimination of the biofilm. The activity of the most effective enzyme mixture (PecP) was found to be similar regardless of the temperature tested (p>0.05),

although the technical file indicates an optimal temperature of 40 °C. This apparent independence of effectiveness on the temperature can be explained by the fact that the commercial specifications refer to the action of clarification of fruit juices, while in the case of biofilms the EPS composition could make the enzymatic activity of the mixture less sensitive to temperature.

Also *S. aureus* biofilms were susceptible to removal by enzymatic products. Even for this microorganism, the more effective mixture was PecP, which at concentration of 2%, irrespective of the contact time, allowed an almost total removal of biofilm (Table 6.2). Similar results were obtained for the mixture CelT at 1% for the longer contact times and at 2% for all of contact times.

Table 6.2 Biofilm biomass (mean OD_{570} ± SD; n=3) for *S. aureus* St_059 after enzymatic treatments; mean values with a different letter within the same temperature treatment indicate statistically different values (p<0.05). The means in bold refer to a total removal of biofilm (PRI> 70%)

S. aureus St_059				
temperature	enzymatic product	treatment	concentration	OD_{570nm}
25 °C	PecP	control		0.954^{a} ± 0.031
		15 min	1%	0.378^{b} ± 0.015
		30 min		0.306^{c} ± 0.008
		50 min		0.348^{b} ± 0.012
		15 min	2%	**0.160^{e}** ± 0.012
		30 min		**0.172^{e}** ± 0.011
		50 min		**0.156^{e}** ± 0.016
	CelA	15 min	1%	0.366^{b} ± 0.012
		30 min		0.414^{b} ± 0.027
		50 min		0.308^{c} ± 0.009
		15 min	2%	0.294^{c} ± 0.015
		30 min		0.337^{b} ± 0.013
		50 min		**0.277^{c}** ± 0.018
	CelT	15 min	1%	0.325^{c} ± 0.011
		30 min		0.447^{b} ± 0.021
		50 min		**0.234^{d}** ± 0.012
		15 min	2%	**0.253^{d}** ± 0.018
		30 min		**0.212^{d}** ± 0.018
		50 min		**0.223^{d}** ± 0.013
37 °C	PecP	control		0.978^{a} ± 0.018
		15 min	1%	**0.243^{f}** ± 0.015
		30 min		**0.079^{g}** ± 0.008
		50 min		**0.094^{g}** ± 0.012
		15 min	2%	**0.242^{f}** ± 0.012
		30 min		**0.105^{g}** ± 0.011
		50 min		**0.130^{g}** ± 0.016
	CelA	15 min	1%	0.447^{d} ± 0.012
		30 min		0.405^{d} ± 0.027
		50 min		0.350^{de} ± 0.029
		15 min	2%	0.557^{c} ± 0.015
		30 min		0.314^{e} ± 0.013
		50 min		0.303^{e} ± 0.018
	CelT	15 min	1%	0.639^{b} ± 0.011
		30 min		0.414^{d} ± 0.021
		50 min		0.464^{d} ± 0.012
		15 min	2%	0.526^{c} ± 0.018
		30 min		**0.267^{ef}** ± 0.018
		50 min		**0.270^{ef}** ± 0.013

The test carried out on *S. aureus* biofilms treated with enzyme mixtures at a temperature of 37 ° C showed a greater sensitivity of biofilms compared to 25 °C (p<0.05). Indeed, PecP at 1% concentration

allowed an almost total removal of biofilm, as well as CelT at concentration of 2% for a long time. This result could be attributed to the temperature dependence of the enzymatic activity, which could promote, for some mixtures, a better biofilm removal.

Regarding *P. fluorescens*, preformed biofilms were susceptible to the enzymatic removal , even if the performances of the three enzymatic preparations were different. Also for this microorganism the more effective mixture was PecP, which at both concentration and temperature, irrespective of the contact time, allowed a total removal of biofilm (Table 6.3). The products CelA and CelT were effective (p<0.05) only at the higher concentration and at 25 °C, while at 37 °C they were effective (p<0.05) only when the contact time increased up to 50 min.

Table 6.3 Biofilm biomass (mean OD_{570} ± SD; n=3) for *P. fluorescens* Ps_019 after enzymatic treatments; mean values with a different letter within the same temperature treatment indicate statistically different values (p<0.05). The means in bold refer to a total removal of biofilm (PRI> 70%)

P. fluorescens Ps_019				
temperature	enzymatic product	treatment	concentration	OD_{570nm}
25 °C	PecP	control		1.280^{a} ± 0.013
		15 min	1%	**0.214**^{h} ± 0.005
		30 min		**0.202**^{h} ± 0.008
		50 min		**0.142**^{i} ± 0.012
		15 min	2%	**0.123**^{il} ± 0.012
		30 min		**0.090**^{l} ± 0.011
		50 min		**0. 081**^{l} ± 0.016
	CelA	15 min	1%	0.561^{b} ± 0.014
		30 min		0.521^{bc} ± 0.017
		50 min		0.510^{cd} ± 0.019
		15 min	2%	**0.382**^{f} ± 0.015
		30 min		**0.380**^{f} ± 0.013
		50 min		**0.350**^{fg} ± 0.018
	CelT	15 min	1%	0.485^{cde} ± 0.017
		30 min		0.472^{de} ± 0.011
		50 min		0.463^{e} ± 0.012
		15 min	2%	**0.321**^{g} ± 0.018
		30 min		**0.318**^{g} ± 0.017
		50 min		**0.312**^{g} ± 0.015
37 °C	PecP	control		1.320^{a} ± 0.016
		15 min	1%	**0.200**^{g} ± 0.013
		30 min		**0.198**^{gh} ± 0.013
		50 min		**0.157**^{h} ± 0.007
		15 min	2%	**0.185**^{gh} ± 0.013
		30 min		**0.174**^{gh} ± 0.013
		50 min		**0.168**^{h} ± 0.007
	CelA	15 min	1%	0.692^{b} ± 0.016
		30 min		0.651^{b} ± 0.014
		50 min		0.634^{bc} ± 0.009
		15 min	2%	0.581^{c} ± 0.012
		30 min		0.463^{e} ± 0.013
		50 min		**0.380**^{f} ± 0.018
	CelT	15 min	1%	0.521^{cd} ± 0.017
		30 min		0.518^{cd} ± 0.011
		50 min		0.515^{cd} ± 0.018
		15 min	2%	0.520^{cd} ± 0.008
		30 min		0.503^{d} ± 0.023
		50 min		**0.378**^{f} ± 0.027

According to the results of the preliminary screening, the most performing enzymatic product appeared to be PecP, a mixture of pectinase, polygalacturonase and pectinmetylesterase. Different sensitivities

observed between strains could be due to differences in biofilm composition in terms of EPS and microbial cell quantity. It is well known that the amount and composition of EPS are dependent on cultural conditions as well as on microbial species (O'Toole et al., 2000; Flemming et al., 2007). In addition, *S. aureus* and *P. fluorescens* strains produces higher amounts of biofilm than *L. monocytogenes* ($p < 0.05$), which could justify a different sensitivity to enzymatic treatments.

In agreement with the above results, the enzymatic product PecP was chosen to be tested against biofilms formed in dynamic conditions on stainless steel AISI 304 and PTFE surfaces by *L. monocytogenes*, *S. aureus* and *P. fluorescens*. Biofilms were grown in the CDC Biofilm Reactor (CBR), which allows the formation of biofilms on a high number of surfaces under standardized conditions. In this test biofilms were grown in a nutrient-poor culture medium (LB) and in stirring conditions, to simulate both a stress of nutritional nature and one of a mechanical nature. These stresses may commonly occur on the surfaces in the food industry, for example in the presence of small amounts of organic residues or in flow conditions that occur in closed vessels, in which the liquid product is subjected to stirring. In Table 6.4 the viable counts of microbial biofilms formed on stainless steel and PTFE surfaces are reported.

Table 6.4 Viable counts (Log CFU/cm2) ± SD of biofilms formed by *L. monocytogenes* Lm_284, *S. aureus* St_059 and *P. fluorescens* Ps_019 on stainless steel and PTFE surfaces. Different letters within each row indicate statistically different means ($p<0.05$)

strain	stainless steel	PTFE
L. monocytogenes Lm_284	$4.58^{b} \pm 0.08$	$5.22^{a} \pm 0.08$
S. aureus St_059	$6.73^{b} \pm 0.18$	$7.60^{a} \pm 0.11$
P. fluorescens Ps_019	$5.37^{a} \pm 0.06$	$5.69^{a} \pm 0.28$

The biofilm viable counts of *L. monocytogenes* varied between 4.58 and 5.22 Log CFU/cm^2, with the highest adhesion on PTFE surface compared to stainless steel ($p<0.05$). As showed by Møretro and Langsrud (2004), the maximum adhered cell concentration (Log CFU/cm^2) of *L. monocytogenes* can vary from 3.6 to 8.5, depending on the strain, the culture medium, the time and temperature of incubation, as well as the type of surface. Regarding this parameter, stainless steel and PTFE differ for their hydrophobicity, as stainless steel is hydrophilic, while PTFE is hydrophobic. These characteristics strongly influence the interactions between the outside of the microbial cell and the surface on which the cell adheres. The literature reports rather contradictory data, since the behaviour of the isolates of *L. monocytogenes* on these surfaces can be very variable. Although it is difficult to compare the results of different experiments, the type of material strongly influences microbial adhesion and biofilm formation by *L. monocytogenes*. This consideration is particularly important when planning the use of different materials in food processing plants. The biofilm counts of *S. aureus* varied between 6.73 and 7.60 Log CFU/cm^2, which are comparable to values found by other authors (Rushdy and Othman, 2011). An influence of the surface in biofilm forming ability of St_059 was found ($p<0.05$), as observed for Lm_284, probably correlated to the hydrophobic characteristics of this material (da Silva Meira et al., 2012). These results are in agreement with Cerca et al. (2005), according to whom adhesion of bacteria belonging to *Staphylococcus* genus to hydrophobic

substrata occurred to a greater extent than to hydrophilic surfaces. Regarding *P. fluorescens* Ps_019, the surface colonization ranged between 5.37 and 5.69 Log CFU/cm^2, with values comparable to the few data available in the literature for this microbial species (Sillankorva et al., 2008). For this strain no significant effects on biofilm formation ability by surface materials were found. Likewise, not-*aeruginosa Pseudomonas* species seem to be able to adhere to and colonize various surfaces, probably thanks to their ability to produce a rather dense EPS matrix, which fixes the biofilm to the surface (Simões et al., 2008).

The results of the treatment of biofilms with PecP are shown in Figure 6.2 and in Figure 6.3.

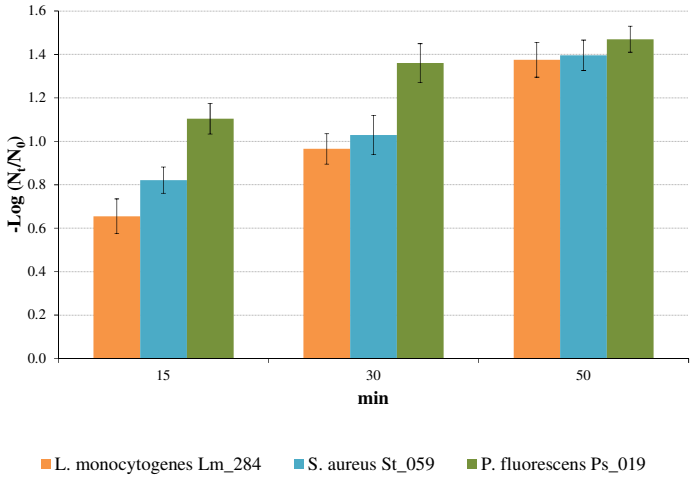

Figure 6.2 Reduction (mean -Log (N_t/N_0) ± SD; n=3) of viable counts in biofilms formed by *L. monocytogenes* Lm_284, *S. aureus* St_059 and *P. fluorescens* Ps_019 on stainless steel after treatment with PecP

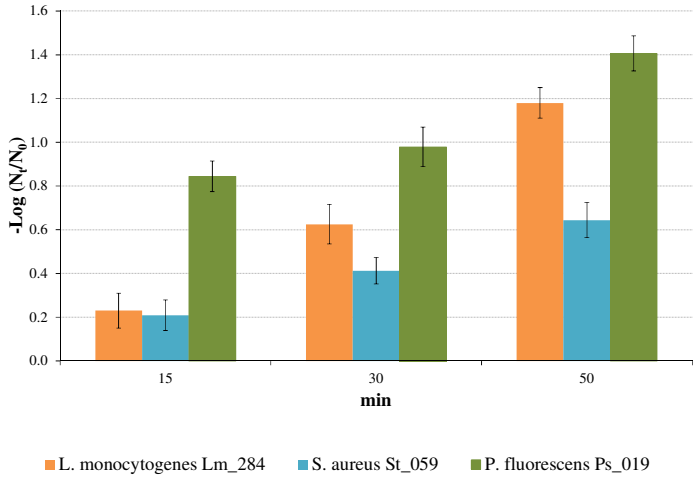

Figure 6.3 Reduction (mean -Log (N_t/N_0) ± SD; n=3) of viable counts in biofilms formed by *L. monocytogenes* Lm_284, *S. aureus* St_059 and *P. fluorescens* Ps_019 on PTFE after treatment with PecP

The efficacy of the enzyme treatment with the product PecP was differently affected by the treatment time, the type of microorganism and the surface on which the biofilms were formed. After 15 min it is possible to observe a certain action in removing biofilms, even if with the longer contact time most

biofilm detachments were obtained. These results are in agreement with the few data present in the literature, according to which the enzyme mixtures, containing enzymes active towards saccharidic components, are able to reduce the number of biofilm cells, depending on the type of microorganism, disaggregating the EPS matrix (Johansen et al., 1997). The best performances of enzymatic preparation PecP were observed on biofilms formed on stainless steel compared to those formed on PTFE, probably due to a different EPS composition of biofilms on different surface materials (Chaignon et al., 2007). Moreover, the greater Log reductions were obtained treating *P. fluorescens* biofilm, both for biofilms preformed on stainless steel and on PTFE. Even this result can be explained through variations in the composition of the extracellular polymers in *P. fluorescens* biofilms compared to those of *L. monocytogenes* and *S. aureus*. This result is in contrast with Johansen et al. (1997), according to which *S. aureus* biofilms were more sensitive to enzymatic removal by enzymes than *P. fluorescens*, which formed the most resistant biofilm. The more efficient activity in removing *P. fluorescens* biofilms was also observed by Lequette et al. (2010), according to which polysaccharidase-degrading enzymes were effective in detaching *P. fluorescens* biofilms. Therefore, the efficacy of enzymes strictly depended on bacterial species EPS matrix. As a matter of fact, the enzyme product used is a mixture of enzymes that degrade the uronic acids present in the matrix of the biofilm (pectinmethylesterase, polygalacturonase and endopectinlyase): this kind of approach could cause a destabilization of the EPS matrix, allowing an easier detachment of the biofilm from surfaces. In all the tested conditions, the biofilm cell removal was less than 2 Log units. However, the data are encouraging, since it may be a good starting point for the development of more effective enzyme formulations and process conditions. The use of products such as bio-enzymatic cleaners is certainly a "green" approach to solve the problem of biofilms in the food industry, and may represent an optimal strategy, especially if a mixture of enzymes capable of degrading the EPS matrix of a heterogeneous group of microorganisms with biofilm forming ability is used. The enzymatic treatment may also be performed in conjunction with a chemical strategy carried out at concentrations lower than those commonly used, allowing for a risk reduction both for the operator and for the environment.

6.4 CONCLUSIONS

The results of this study clearly indicate that the enzymatic treatments were able to partially or totally remove preformed biofilms of *L. monocytogenes, S. aureus* and *P. fluorescens* on polystyrene surfaces, even if less efficiently on biofilms formed on stainless steel and PTFE surfaces. The effects of the concentration, contact time and temperature in removal biofilms were different in function of the tested strain, and this is probably related to the different composition of the polysaccharide fraction of the EPS of the strains. Although the biofilm forming ability in food processing plants is well known in *L. monocytogenes, S. aureus* and *P. fluorescens*, the data relating to the sensitivity of biofilms of these microorganisms enzymatic treatments are very limited, if not completely missing regarding *L. monocytogenes*. The results of this study can represent a first step in the development of non-conventional sanitation strategies for reducing the risk of cross contamination caused by these microorganisms.

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Chapter 7. BIOFILM FORMATION IN THE FOOD FIELD: TWO CASE STUDIES

7.1 STUDY OF MICROBIAL BIOFILM FORMATION IN DRINKING WATER SYSTEM PIPES

Distribution water systems are known to harbour microbial biofilms, even though these environments are oligotrophic and often contain a disinfectant. Biofilm formation in drinking water systems cause several problems, like contamination of drinking water, reducing the quality of potable water, corrosion of pipes and a microbiological safety reduction through increased pathogen survival (Niquette et al., 2000). The examination of a drinking water system reveals the complexity of such a technical system. There are not only many different materials used for the transportation and regulation of the water flow but also several variations in the temperature values, and flow conditions of different locations. Microorganisms encounter a diversity of habitats with distinct physicochemical and nutritional conditions during treatment, storage and distribution of drinking water. Bacteria are affected not only by the environment they live in, but also by the variety of other species present. A range of interactions has been observed among microorganisms in biofilms, including antagonistic, mutualistic, competitive and commensal relationship (Burmølle et al., 2006). Optimizing the management of drinking water systems and controlling microbial growth are difficult due to the complexity of these systems. The study of bacterial ecology and behaviour might help to improve the understanding of biofilms persistence in drinking water systems. As a matter of fact, biofilm community diversity can affect disinfection efficacy and could allow pathogens to survive within biofilms (Elvers et al., 2002). The evaluation of the composition of drinking water bacteria biofilms represent an important tool because it provides information on the predominance of the best-adapted species for that set of conditions. The knowledge of biofilm biodiversities and its species physiology may facilitate the development of drinking water disinfection and biofilm control processes.

The purpose of this study was to evaluate the microbial heterogeneity within the biofilm present in the pipes of drinking water systems, as well as to evaluate the ability of one representative strain to grow in water and on pipes used in drinking water system.

7.1.1 Materials and methods

7.1.1.1 Bacterial isolation and identification

Pipes and tanks of drinking water systems were sampled using sterile swabs, in order to evaluate autochthonous bacterial species of these surfaces: pipes carrying water, filters, water collection pipes, water output nozzles and also the water collected from the pipes. The swabs were subsequently spread on different agar media. From selected colonies, the isolates were subjected to DNA extraction by using InstaGene Matrix kit (BioRad, Italy). The strains were then submitted to partial 16S rRNA gene amplification with primers 16S rRNA F and 16S rRNA R (Carraro et al., 2011). The amplified fragments were sequenced and the sequences obtained were aligned with the closest sequences available in the GenBank database (≥98%

of homology, http://www.ncbi.nml.nih.gov/BLAST). Among all the isolates, the autochthonous isolate *Pseudomonas gessardii* Ps_331 was chosen for the following tests.

7.1.1.2 Biofilm formation in microtiter plates using crystal violet assay

The first step was to evaluate the biofilm forming ability of *Pseudomonas gessardii* Ps_331 using the microtiter plate assay with crystal violet staining, as described in paragraph 2.2.1. Briefly, eight wells of a microtiter plate were filled with 200 µL of Tryptone Soya Broth (TSB, Oxoid, Milan, Italy) and inoculated with 10 µL of an overnight culture of *P. gessardii* Ps_331 (~ 10^8 CFU/mL). The microtiter plates were incubated for 2 days at 30 °C. The test was repeated three times. For each experiment, eight wells were used as control and filled with 200 µL of non- inoculated TSB. At the end of the incubation, the microtiter plates were rinsed and stained as described in paragraph 2.2.1. The strain was classified for its biofilm-forming ability as described by Stepanović et al. (2000).

7.1.1.3 Planktonic bacterial growth in drinking water

The ability to grow in drinking water of *P. gessardii* Ps_331 was tested. Bacterial cells were grown overnight in TSB at 30 °C. Cells were then harvested by centrifugation (5 min at 13.000 rpm), washed 5 times in Maximum Recovery Diluent (MRD, Oxoid, Milan, Italy) and resuspended in a volume of potable water necessary to achieve a cellular density of 10^3-10^4 CFU/mL. This suspension was incubated at 30 °C for 7 days in order to trigger an adaptation stage of the strain in potable water, a nutrient-low medium for bacterial growth. Afterwards, 1 mL of this suspension was resuspended in another certain volume of potable water in order to achieve a cellular density of 10^3-10^4 CFU/mL. This suspension was then incubated for 30 days at three different temperature values (4, 20 and 40 °C), which are temperatures found in the different areas of a drinking water system, and then subjected to microbial sampling at times of 3, 7, 10, 20 and 30 days.

7.1.1.4 Biofilm formation on inner surfaces of different pipes

Subsequently, a growth test in potable water and in pipes and the ability to form biofilm on the inner pipe surface by *P. gessardii* Ps_331 was performed. For this test, three different pipes in polyethylene (labelled pipe A, pipe B and pipe C) with different characteristics and three different incubation temperatures (4, 20 and 40 °C) were used to follow the microbial growth and biofilm formation kinetics. An aliquot of the microbial suspension in potable water, obtained as described above, was used to inoculate each pipe, which was incubated at 4, 20 and 40 °C for 30 days. At times of 3, 7, 10, 20 and 30 days, microbiological samplings were performed. In particular inoculated water within each pipe was subjected to bacterial analysis through ten-fold dilutions in Pseudomonas Agar Base (PSA, Oxoid, Milan, Italy) plates. In order to evaluate the biofilm forming ability of *P. gessardii* 331, each pipe, at each time of sampling, was washed twice with sterile water, immersed in Maximum Recovery Diluent and subjected to sonication in an ultrasonic bath LBS2 (Falc Instruments, Treviglio, Italy) at room temperature at 40 kHz for 4 min in order to

remove adherent cells from the pipe surface (Asséré et al., 2008). The microbial suspension was then subjected to microbiological analysis through ten-fold dilutions in PSA plates. All the plates were incubated at 30 °C for 24-48 h.

7.1.2 Results and discussion

From the microbiological sampling of different pipes and tanks of drinking water distribution systems, and from 16S rRNA gene sequencing of the corresponding microbial isolates, 57 bacterial species were identified. All isolated species (*Aquabacterium* sp., *Bacillus* sp., *Brevundimonas* sp., *Comamonas* sp., *Hydrocarboniphaga* sp., *Pantoea* sp., *Pseudomonas* sp., *Shewanella* sp., *Sphingomans* sp., *Sphingopyxis* sp., and *Staphylococcus* sp.) have previously been detected in drinking water and drinking water systems (Rickard et al., 2004; September et al., 2007; Lee et al., 2010). The obtained results indicated that among microbial populations isolated from drinking water distribution systems, a predominance of Gram negative bacteria was found.

P. gessardii Ps_331, a bacterial species frequently isolated from natural mineral waters (Verhille et al., 1999), was chosen to perform the following biofilm assays. This strain was classified as a strong biofilm producer according to Stepanović et al. (2000).

To determine the ability to grow in an oligotrophic environment like drinking water, the planktonic growth test was performed at three different incubation temperatures (4, 20 and 40 °C).

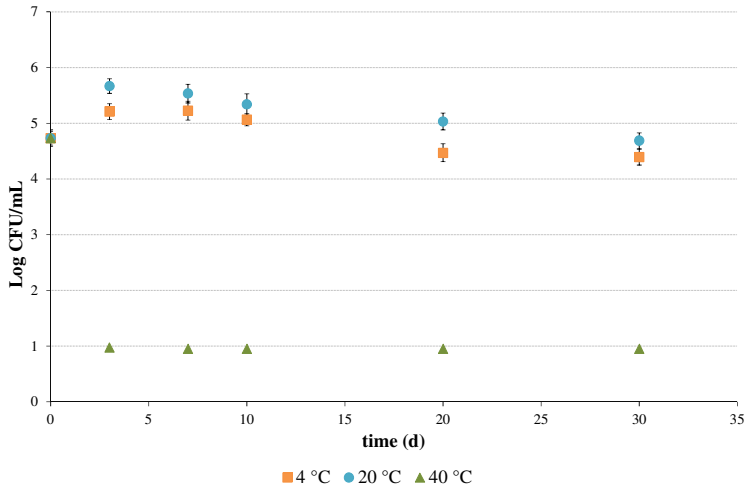

Figure 7.1 Planktonic growth kinetic (mean Log CFU/cm^2 ± SD; n=2) of *P. gessardii* Ps_331 in drinking water.

According to kinetic results (Figure 7.1), *P. gessardii* Ps_331 maintained its vitality both at 4 and 20 °C, while at 40 °C the counts were always lower than the detection limit (<10 CFU/mL). In particular, at 4 and 20 °C *P. gessardii* microbial cells increased of almost 1 Log in the first days and then remained at constant viable level reaching the initial inoculum value.

The biofilm formation of *P. gessardii* Ps_331 was performed testing three polypropylene pipes with different characteristics (pipe A, pipe B and pipe C) at incubation temperatures of 4, 20 and 40 °C. The biofilm growth was performed for 30 days. Results indicated a difference between pipe A, pipe B and pipe C, both regarding biofilm formation on inner pipe surfaces and bacterial growth in drinking water in each pipe. In Figure 7.2 the results obtained from bacterial growth kinetics in drinking water contained in pipe A (a) and biofilm formation kinetics on the inner pipe A surface (b) of *P. gessardii* Ps_331 are reported. Regarding the bacterial growth in drinking water, the results showed that, starting from an inoculum of 5.46 Log/mL, bacterial levels were reduced of 0.5 and 1 Log at 4 and 20 °C respectively, whilst the detection limit of the microbiological method at 40 °C was reached. Bacterial levels remained at these approximate values at the three temperatures over the time period of 30 days. Regarding the biofilm formation on the inner surface of pipe A, biofilms rapidly developed within the pipe. The biofilms levels on the inner pipe A surface averaged between 3 and 4 Log at 4 °C during the time of 30 days. At 20 °C results showed a biofilm development delayed as it was formed after 10 days of incubation, reaching a biofilm level of 5.62 Log/cm^2 and then decreasing to a average level of 2.73 Log/cm^2 during the 20-day period. At 40 °C no biofilm development on inner pipe A surface was evaluated, as for bacterial growth in drinking water within this pipe.

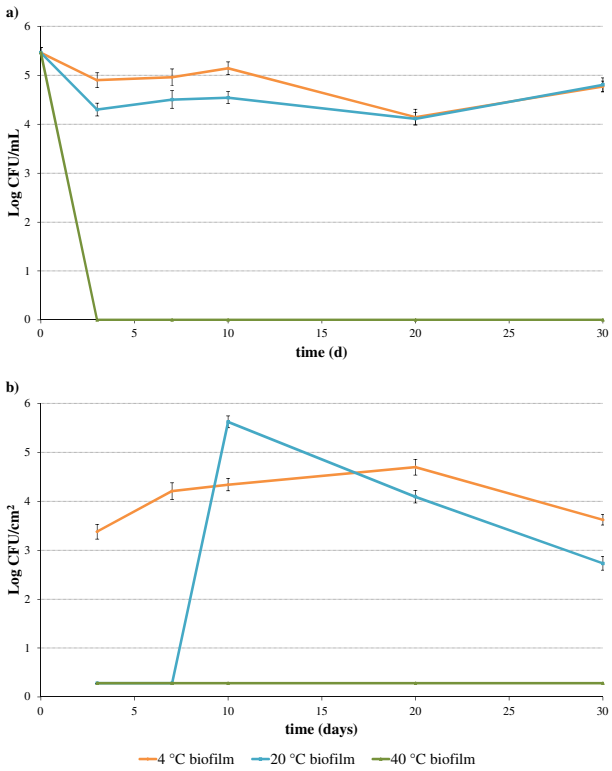

Figure 7.2 Bacterial growth (mean Log CFU/cm^2 ± SD; n=2) a) in drinking water in pipe A and (b) on the inner surface of pipe A.

In Figure 7.3 the results obtained from bacterial growth kinetics in drinking water contained in pipe B (a) and biofilm formation kinetics on the inner pipe B surface (b) of *P. gessardii* Ps_331 are reported. Regarding the bacterial growth in drinking water, the results showed that, starting from an inoculum of 5.46 Log/mL, after 30 days of incubation, the bacterial levels remained at the same value for temperatures of 4 and 20 °C, even if there were increasing and decreasing of about 1 Log in each sampling point at both temperatures. At 40 °C bacterial level decreased to an average value of 3.38 Log/mL in the first days, reaching values below the detection limit over the remaining days. As regards the biofilm formation on inner surface of pipe B, even for this pipe, biofilms rapidly developed within the pipe. In particular, biofilm levels on the inner pipe B surface averaged 3.38 and 3.51 Log/cm^2 at the beginning of the sampling at 4 and 20 °C respectively. During a 30-day period, the biofilm levels at both temperatures increased until 5 Log/cm^2. At 40 °C no biofilm development on the inner pipe B surface was evaluated, as for the bacterial growth in drinking water within this pipe.

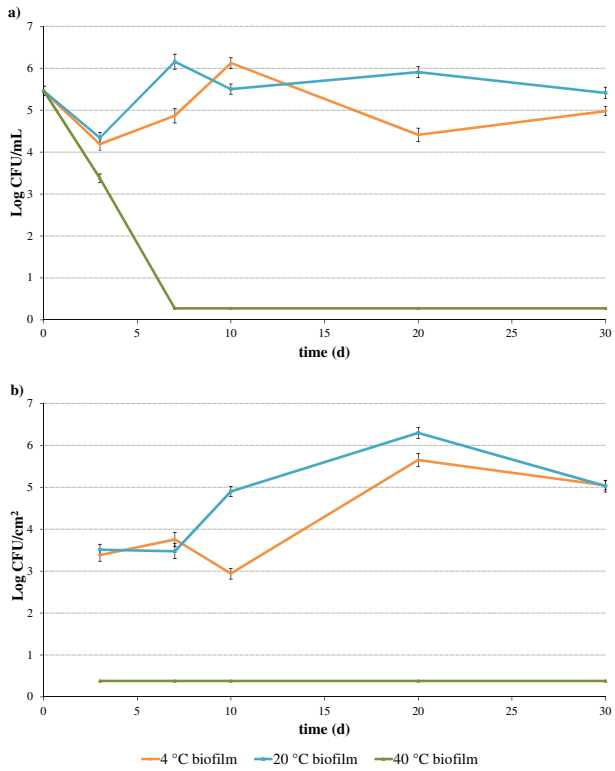

Figure 7.3 Bacterial growth (mean Log CFU/cm^2 ± SD; n=2) (a) in drinking water in pipe B and (b) on the inner surface of pipe B

In Figure 7.4 the results obtained from bacterial growth kinetics in drinking water contained in pipe C of *P. gessardii* Ps_331 are reported. The results showed that, starting from an inoculum of 4.58 Log/mL, after 30 days of incubation, the bacterial levels decreased in the first 10 days reaching values below the detection limit for all temperatures, even if at 40 °C the decreasing was already in the first 3 days. As regards the biofilm formation on the inner surface of pipe C, no biofilm formation was observed there.

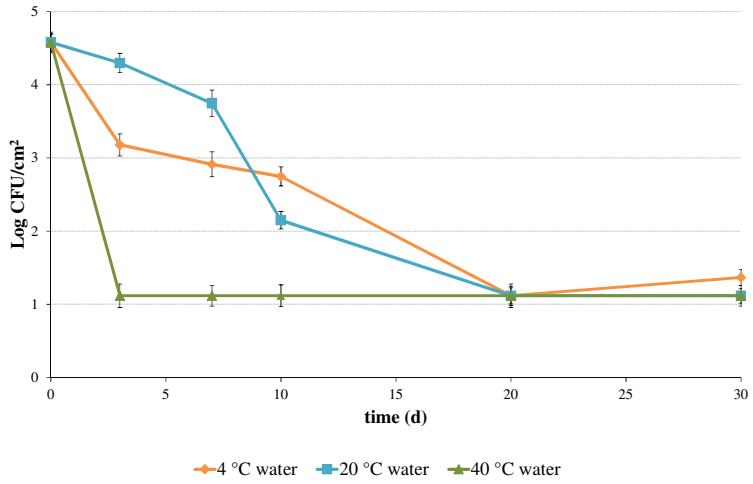

Figure 7.4 Bacterial growth (mean Log CFU/cm^2 ± SD; n=2) in drinking water in pipe C. No biofilm formation on the inner surface was observed

The obtained results showed that the pipe material characteristic considerably influences biofilm formation. Pipe C supported less bacterial growth and none biofilm formation. As already observed by Norton and LeChevallier (2000), pipe materials interact with microorganisms influencing both microbial growth in water and the amount of biofilm development on surface. The results suggest that pipe A and pipe B materials play an important role in stimulating microbial growth in low-nutrient medium, like water, and biofilm development on plastic surfaces. Previous investigations have shown that tubercles materials can concentrate organic nutrients (Liu et al., 2002). Therefore, the higher microbial growth and biofilm formation evaluated in pipe A and pipe B than in pipe C might be due to a combination of nutrient accumulation, and probably a different surface roughness, as well as favouring pipe A and pipe B surfaces as sites for bacterial growth and adhesion.

7.1.3 References

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7.2 BACTERIAL BIOFILM FORMATION IN THE MICROBREWERY ENVIRONMENT

Beer is generally regarded as a safe beverage in terms of food-borne illnesses because it is hard to spoil and has a remarkable microbiological stability. As a matter of fact, pathogens and many other microorganisms are not able to grow in beer due to the presence of ethanol and of the hop bitter compounds, the high content of carbon dioxide, the low pH, the reduced content of oxygen and the presence in traces of nutrient substances such as glucose, maltose and maltotriose. However, a bacterial contamination by spoilage microorganisms is possible because the fermentation process is prone to bacterial growth, due to the nutrient-rich environment of wort and the additional growth factors produced by the brewing yeast (Ingledew, 1979; Sakamoto and Konings, 2003). This is particularly true in the case of beer produced by microbreweries, which usually is not a filtered and pasteurized product, more subjected to microbial contamination than industrial beer (Menz et al., 2010). The sources of microbial beer contaminants originate from the yeast, wort, fermentation, maturation or pressure tanks, as well as from bottling, canning or kegging (Vaughan et al., 2005).

Beer production takes place mainly in closed systems, where cleaning-in-place (CIP) procedures without the need for dismantling are applied. However, long runs between cleanings and short cleaning programs are typical due to the favourable economic aspect. So, these systems are susceptible to bacterial adhesion on surfaces and consequent formation of microbial biofilms which is a time-dependent process (Zottola, 1994). The main causes of biofilm formation and the consequent contamination of beer are commonly due to improper cleaning and disinfection of equipment, mainly of the areas more difficult to clean and disinfect, as bends, edges, dead ends in pipes, seals and joints. It should be highlighted that microbial biofilms are more resistant to antimicrobials compared to planktonic cells and this make their elimination from food processing facilities a big problem (Gilbert et al., 2002).

Beer contaminations by microbial biofilms can occur during production and during bottling, which is considered the major source of the microbial problems in beer production. During production, beer spoilage microrganisms such as lactic acid bacteria, wild yeast and anaerobic bacteria are often present on the equipment, as well as in the air and in the raw materials. These microorganisms can survive in niches like seals, joints and valves, hardly reachable by sanitization, proliferate when residues are present, and contaminate the entire production (Timke et al., 2008). Other critical points are the heat exchangers, for their conformation difficult to disinfect, and the fermentation and maturation tanks, for the presence of sugar not yet fermented and the initial absence of ethanol. Fermentation tanks are usually contaminated by Gram negative and acetic acid bacteria, while maturation tanks by lactic acid bacteria (Timke et al., 2005).

Biofilm production in the brewery environment is a problem when biofilm- producering microrganisms are able to grow in beer and cause off-flavor or turbidity in the final product due to metabolites and sediment production. Despite the fact that much research has focused on the detection of beer-spoiling bacteria (Timke et al., 2008; Matoulková et al., 2012), only little information is available on the composition of brewery biofilms and on the ability of biofilm bacterial isolates to grow in beer, although yeasts are

outnumbered by bacteria in brewery biofilms (Storgårds and Priha, 2009). Thus, the aim of this work was to identify possible niches of bacterial biofilm formation, to verify the potential of isolates to grow in craft beer, and to test the efficacy of a peracetic acid-based sanitizer against preformed biofilms.

7.2.1 Materials and Methods

One craft beer plant located in the North-East of Italy was monitored in this study. The hygienic status of several plant surfaces was controlled for microbiological contamination at two different sampling times at a distance of one month from each other.

7.2.1.1 Sampling of brewery processing plant surfaces

The samples were taken after the application of the sanitation plan, carried out as follows: rinsing with cold water, cleaning with basic detergent (2% for 20 min at 70 °C), rinsing with cold water, disinfection with a 1.5% peroxide-based solution (1.5% peracetic acid/hydrogen peroxide for 15 min at room temperature) and finally a rinse with cold water. Various surfaces of the processing plant, including fermentation tanks, drain valves, pipes, joints, bottling machine, capping machine, heat exchanger and floor drains, were qualitatively sampled through the use of sterile cotton swabs, moistened using a sterile solution (paragraph 2.2.1) immediately before use. The surfaces were sampled, the swabs were then suspended in 5 mL of the sterile saline solution and plated on Tryptone Soya Agar (TSA, Oxoid, Milan, Italy) and de Man Rogosa Sharpe Agar (MRS-A, Oxoid, Milan, Italy). The plates were incubated at 30 °C for 48 h under aerobic (TSA) or anaerobic conditions (MRS-A).

7.2.1.2 Isolation and identification of the strains

Pure cultures of representative colonies from TSA and MRS-A plates were characterized on the basis of their colonial characteristics (colony elevation, size, shape, pigmentation, edge and consistency), cell morphology and Gram-staining using an optical microscope at 1000x magnification, catalase and oxidase activities, and motility, and grouped accordingly. One representative strain of each group was subjected to DNA extraction by using InstaGene Matrix kit (BioRad, Italy). The strains were then submitted to partial 16S rRNA gene amplification with primers 16S rRNA F and 16S rRNA R (Carraro et al., 2011). The amplified fragments were sequenced and the sequences obtained were aligned with the closest sequences available in the GenBank database (≥98% of homology, http://www.ncbi.nml.nih.gov/BLAST).

7.2.1.3 Evaluation of beer-spoilage ability (*forcing test*)

A few colonies of each selected isolate grown on TSA or MRS-A plates were suspended in 5 mL of a sterile saline solution and the turbidity was adjusted to 1.0 McFarland. A top-fermented beer containing ethanol 5.0% v/v and 18 IBU (International Bitterness Units) was degassed in an ultrasound bath, filter-sterilized and inoculated with 200 µL of each culture in two biological replicates in a final volume of 10 mL. The inoculated beers were incubated anaerobically in the dark at 20 °C for up to 6 weeks and examined regularly for visible growth as compared to a sample of uninoculated beer (control).

7.2.1.4 Biofilm formation on polystyrene microtiter plates

The test was carried out in three biological replicates in 96-well polystyrene flat bottom microplates in TSB broth or MRS , as reported in paragraph 2.2.1. The microplates were incubated for 7 days at 30 ° C and subjected every 48 hours to refresh The strains were classified based on their biofilm-forming ability as described by Stepanović et al. (2000).

7.2.1.5 Biofilm disinfection assay

Stainless steel AISI 304 coupons (50 x 25 x 1 mm) were sonicated in a hot alkali detergent solution for 30 min in an ultrasonic water bath, rinsed in distilled water, sonicated in a 15% phosphoric acid solution at 80 °C for 20 min, rinsed in distilled water for 20 min and sterilized in an autoclave. For each strain, two sterile coupons were placed in a sterile Petri dish containing 15 mL of BHI or MRS broth, inoculated with 150 µL of an overnight culture (~ 10^8 CFU/mL) and incubated at 30 °C for 7 days. At the end of incubation, each coupon was washed three times with a sterile saline solution and then immersed in 15 mL of 1% peracid-based solution for 15 min at 20 °C. After disinfection the coupons were neutralized with 15 mL of 0.5% sodium thiosulphate for 15 min at 20 °C. Biofilm cells were scraped from each coupon, the detached cells were resuspended in 1 mL of a Maximum Recovery Diluent and subjected to two cycles of sonication (59 KHz for 1 min each), interspersed with vortexing for 30 sec. Then, ten-fold dilutions of each suspension were analyzed through the spread plate method. The inactivation efficacy was evaluated by taking the ratio of the Log CFU/cm^2 before (N_0) and after the treatment (N_t), presented as -Log (N_t/N_0). Each trial was performed in triplicate.

7.2.2 Results and discussion

The presence of beer-spoilers yeasts is well documented in surface-associated biofilms of the brewery environment. On the other hand, studies related to the presence of bacterial species in brewery biofilms are quite scarce, although it is rather known that many bacteria may cause an increase in turbidity and unpleasant sensory changes in beer (Sakamoto et al., 2003). For this reason, an investigation of the bacterial community present on process surfaces of a brewing plant was performed, and the impact of the isolates on the hygienic risk was assessed by evaluating their ability to grow in beer and to form biofilms on abiotic surfaces, as well as the tolerance of a preformed biofilm to a peroxide-based treatment.

In total, 253 isolates were obtained from TSA and MRS-A plates. After a colonial characterization, cell morphology, Gram-staining, catalase and oxidase activities, and motility, fifty-eight strains were identified by partial sequencing of the 16S rRNA gene (Table 7.1). The strains were members of different taxa with a focus on *Firmicutes*, *Gammaproteobacteria Actinobacteria* and *Alphaproteobacteria*. Within the 33 strains assigned to the phylum of *Firmicutes* five families were represented, including *Bacillaceae*, *Enterococcaceae*, *Lactobacillaceae*, *Paenibacillaceae* and *Staphylococcaceae*. The well-known beer-spoiling bacteria *Lactobacillus brevis* were detected in three different sampling sites.

Table 7.1. Bacterial species isolated from microbrewery surfaces; *the first two letters indicate the sampling site (Bm, bottling machine; Cb, conveyor belt; Dp, drainage pit; Dv, fermenter drain valve; Pt, pipe thread), the first number indicates the sampling time (_1_, 1st sampling time; _2_, 2nd sampling time)

Strain*	Accession Number	Phylum	Species	Biofilm formation	Forcing test
Bm_1_003	KF360066	*Gammaproteobacteria*	*Enterobacter asburiae*	moderate	-
Bm_1_011	KF477107	*Actinobacteria*	*Microbacterium maritypicum*	strong	-
Bm_1_013	HF936969	*Gammaproteobacteria*	*Stenotrophomonas maltophilia*	strong	-
Bm_1_023	AB854170	*Firmicutes*	*Enterococcus* sp.	weak	-
Bm_1_032	EF061085	*Firmicutes*	*Pediococcus pentosaceus*	moderate	+
Bm_1_033	JX477168	*Firmicutes*	*Pediococcus pentosaceus*	moderate	-
Bm_1_035	KF532949	*Actinobacteria*	*Micrococcus luteus*	strong	-
Bm_1_044	KF279528	*Firmicutes*	*Bacillus* sp.	strong	-
Bm_1_049	FJ429977	*Firmicutes*	*Lactococcus lactis*	weak	-
Bm_2_208	KF010795	*Firmicutes*	*Bacillus nealsonii*	moderate	-
Bm_2_209	KF054958	*Firmicutes*	*Bacillus circulans*	weak	-
Bm_2_213	KF017644	*Alphaproteobacteria*	*Brevundimonas* sp.	strong	-
Bm_2_219	GU994744	*Firmicutes*	*Lactococcus lactis*	weak	-
Bm_2_221	KF060264	*Firmicutes*	*Enterococcus gallinarum*	weak	-
Bm_2_231	JQ800431	*Firmicutes*	*Enterococcus casseliflavus*	weak	-
Bm_2_238	KF158234	*Firmicutes*	*Bacillus cereus*	moderate	-
Bm_2_246	HE610881	*Firmicutes*	*Bacillus thuringiensis*	strong	-
Bm_2_247	FM178868	*Alphaproteobacteria*	*Acetobacter pasteurianus*	moderate	-
Bm_2_248	KC422653	*Firmicutes*	*Bacillus* sp.	moderate	-
Bm_2_253	AM179900	*Gammaproteobacteria*	*Citrobacter* sp.	strong	-
Bm_2_256	FR849936	*Firmicutes*	*Paenibacillus* sp.	moderate	-
Bm_2_260	HF545843	*Gammaproteobacteria*	*Pseudomonas putida*	strong	-
Cb_1_056	KC502974	*Firmicutes*	*Bacillus* sp.	moderate	-
Cb_1_079	FJ982661	*Firmicutes*	*Bacillus cereus*	moderate	-
Cb_1_081	JN863503	*Actinobacteria*	*Leucobacter* sp.	strong	-
Cb_1_094	KF013209	*Gammaproteobacteria*	*Stenotrophomonas* sp.	strong	-
Cb_1_108	FN386776	*Alphaproteobacteria*	*Sphingomonas yabuuchiae*	strong	-
Cb_1_132	AM113741	*Gammaproteobacteria*	*Pseudomonas* sp.	strong	-
Cb_1_139	X95423	*Firmicutes*	*Lactobacillus lindneri*	weak	-
Cb_1_144	JN644610	*Actinobacteria*	*Kocuria carniphila*	strong	-
Cb_1_149	FJ715739	*Actinobacteria*	*Microbacterium oxydans*	strong	-
Cb_1_151	AY271785	*Gammaproteobacteria*	*Pantoea agglomerans*	strong	-
Cb_2_263	AP012167	*Firmicutes*	*Lactobacillus brevis*	weak	-
Cb_2_267	KC920987	*Gammaproteobacteria*	*Pseudomonas fluorescens*	strong	-
Cb_2_270	JN167962	*Alphaproteobacteria*	*Sphingomonas aurantiaca*	strong	-
Cb_2_279	AB680838	*Firmicutes*	*Paenibacillus glucanolyticus*	moderate	-
Dp_1_154	FJ581026	*Gammaproteobacteria*	*Citrobacter freundii*	strong	-
Dp_1_157	GU295951	*Firmicutes*	*Lactobacillus brevis*	weak	+
Dp_1_161	AB512778	*Firmicutes*	*Lactobacillus lindneri*	weak	-
Dp_2_280	JX950894	*Firmicutes*	*Lactobacillus paracasei*	weak	-
Dp_2_283	JX847619	*Firmicutes*	*Enterococcus faecium*	weak	-
Dp_2_288	KF444404	*Firmicutes*	*Bacillus circulans*	moderate	-
Dv_1_162	EU919276	*Firmicutes*	*Staphylococcus saprophyticus*	strong	-
Dv_1_167	AM179861	*Gammaproteobacteria*	*Acinetobacter* sp.	moderate	-
Dv_1_172	JQ389597	*Gammaproteobacteria*	*Acinetobacter johnsonii*	weak	-
Dv_1_176	HM058973	*Firmicutes*	*Lactobacillus brevis*	weak	+
Dv_2_291	AF429480	*Firmicutes*	*Lactobacillus casei*	weak	-
Dv_2_294	FJ609705	*Alphaproteobacteria*	*Brevundimonas intermedia*	strong	-
Pt _1_178	KC113143	*Firmicutes*	*Paenibacillus glucanolyticus*	moderate	-
Pt_1_181	KC113142	*Firmicutes*	*Paenibacillus vortex*	strong	-
Pt_1_187	JX826568	*Firmicutes*	*Lactobacillus* sp.	weak	-
Pt_1_193	AY126244	*Firmicutes*	*Staphylococcus warneri*	strong	-
Pt_1_197	EU741108	*Actinobacteria*	*Leucobacter alluvii*	strong	-
Pt_2_299	AB682347	*Firmicutes*	*Lactobacillus* sp.	moderate	-
Pt_2_304	AM117595	*Firmicutes*	*Lactobacillus* sp.	strong	-
Pt_2_305	JF411252	*Actinobacteria*	*Kocuria atrinae*	strong	-
Pt_2_308	KF358364	*Alphaproteobacteria*	*Sphingomonas* sp.	moderate	-
Pt_2_311	GU584985	*Alphaproteobacteria*	*Sphingomonas aerolata*	strong	-

Bacteria were isolated from several sampled areas, including different sites in the bottling machine, drain valves from tanks, floor drains, and joints. No bacterial contamination was found on capping machine, fermentation tanks, pipes, and heat exchanger. Interestingly, except in the case of the drain valves, the bacterial contamination was present in all the sampling times, underlining that in case of resident biofilms normal sanitation procedures are not sufficient and intensive protocols should be initiated. Bacterial contamination was present mostly on external surfaces of the production plant, and this is a big hygienic issue because it might be distributed throughout by people, splashes or air movements, and consequently reach the final product or clean surfaces (Timke et al., 2005). Indeed, secondary contaminations originating from opened surfaces are responsible for most events of spoilage of non-pasteurized beer (Storgårds and Priha, 2009).

Eleven strains were identified as belonging to the *Gammaproteobacteria* phylum. The presence of members of this group, above all *Pseudomonas* species and *Enterobacteriaceae*, is in accordance with several reports from breweries. The source of this species is usually wort, and they cannot multiply in bottled beer, but they occur frequently in brewery biofilm communities (Timke et al., 2004). Indeed, the strains isolated in this study were classified mostly as moderate or strong biofilm producers. The presence of biofilm formers in the working area of a food processing plant should be regarded as a potential hygienic risk, as it contributes to the overall microbial contamination of the environment, and increases the incidence of cross-contamination. Other microbial groups, belonging to phyla *Actinobacteria* and *Alphaproteobacteria*, were frequently isolated. *Stenotrophomonas maltophilia*, *Bacillus* spp., *Microbacterium* spp., *Staphylococcus warneri* and *Sphingomonas* spp. are often isolated from food environment (Carpentier and Chassaing, 2004) and drinking water distribution systems, in particular regarding *Sphingomonas* sp. (Simões et al., 2010). Even if they are non- beer-spoiling bacteria, they can fulfil important functions like primary surface colonization of, matrix production, acidification and reduction of oxygen presence in the environment for the beer-spoiling bacteria, which are usually facultative or obligate anaerobes and acidophilic or acidotolerant. For example, *Acetobacter pasteurianus*, which was isolated from the bottling machine, is known for reducing the pH in the presence of oxygen, thus providing favorable conditions for the beer-spoilers bacteria. Among *Firmicutes*, *Lactobacillus brevis* and *Pediococcus pentosaceus* were isolated. They represent a potential hazard for the brewing industry as they can be responsible for most of the beer spoilage (Fujii et al., 2005). In this study, two out of five strains belonging to these species were able to multiply in beer.

Within the isolated species, moderate and strong biofilm producers were present mainly within *Actinobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria*, while within *Firmicutes* several weak producers were present. Similar results were obtained for biofilms grown on stainless steel (Figure 7.5). In fact, regardless of the wide range of Log CFU/cm^2 of viable counts on stainless steel, the mean values of the different phyla clearly showed that *Firmicutes* adhered less efficiently on such surfaces, even if some members of this phylum reached more than 6 Log CFU/cm^2.

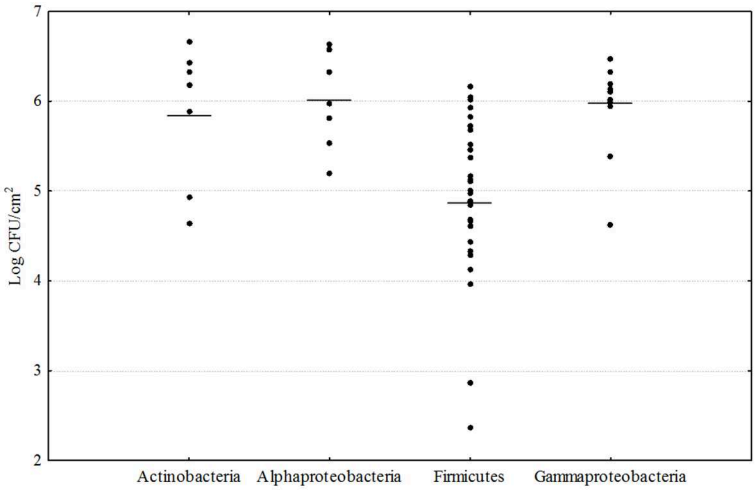

Figure 7.5. Biofilm formation (Log UFC/cm^2) by isolates on AISI 304 stainless steel; horizontal lines represent the mean value for each phylum

As regards the tolerance to a commercial sanitizer widely used in brewing environments, the data showed that *Actinobacteria* was the most tolerant phylum, followed by *Alphaproteobacteria*, *Gammaproteobacteria* and *Firmicutes* (Figure 7.6). According to Møsteller and Bishop (1993), a product with a disinfectant action against bacterial biofilm must be able to reduce the cellular populations by 3 logarithmic units. The results showed that for most of the strains the treatment with a peracid-based product in operating conditions, similar to those of the processing plant, is ineffective in reducing microbial populations on stainless steel surfaces to a safe level, thus establishing a risk for food plant contamination.

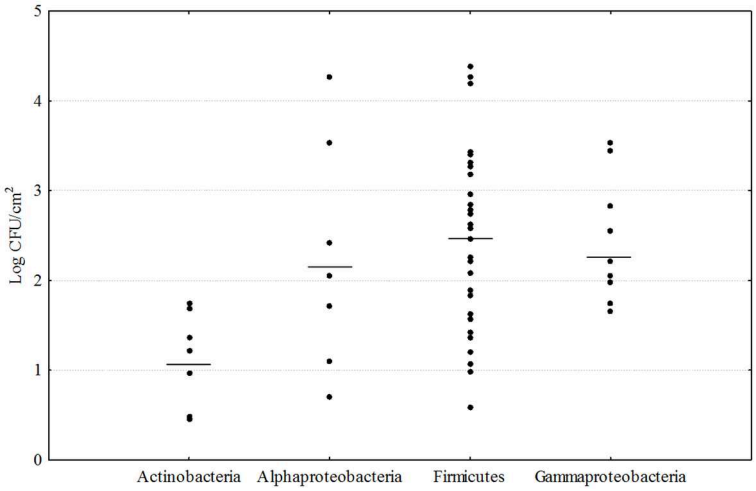

Figure 7.6. Reduction of microbial counts (expressed as -Log (N_t/N_0), where N_t = CFU/cm^2 after treatment, N_0 = initial CFU/cm^2); horizontal lines represent the mean value for each phylum

Actually, peracetic acid-based disinfectants have been usually used in the food industry and in breweries, in particular in the sanitizer step of the CIP system (Orth, 1998) as this substance works quickly and is effective against bacteria (Loukili et al., 2006). Unlike most chemical disinfectants, it does not react with proteins to produce toxic or carcinogenic compounds, has low environmental impact and has been

reported to be more active against biofilm (Holah et al., 1990). The efficiency of peracid acid may be explained by its high capacity of oxidizing cell molecules by releasing free oxygen and hydroxyl radicals decomposing in oxygen, water and acid acetic (Loukili et al., 2006). In this study, a significant fraction of the bacterial population seems to be able to escape disinfection. This is probably due to both microbial aggregation state, which limits diffusion of the oxidant, and the sophisticated antioxidant strategies developed by many microorganisms (Sies, 1993).

This study showed that the sanitation protocol applied in an Italian microbrewery is not always able to ensure a satisfactory hygienic state of the equipment and the plant. Several sampling areas including the bottling machine, conveyor belts, drainage, valves and threads were found to be contaminated by a heterogeneous microflora, and among the microbial species isolated *Lactobacillus brevis* was found, which is a known beer-spoiling bacteria. Most of the strains showed a high ability to form biofilm both on polystyrene and on stainless steel, which makes these strains particularly hazardous in the food industry, and in brewery in particular, as they can represent a possible source of cross-contamination.

Since several strains showed to be insufficiently affected by a widely used sanitizing product, an effective sanitation program should be designed taking into account both the microbial biofilm and its high level of microbial heterogeneity. Therefore, it appears to be essential to resort to the use of sanitizing products efficient for all potentially present microbial species. The sanitizer effectiveness should be tested by in vitro studies that could be invariably repeated under *in situ* conditions in order to control the biofilms presence in the food processing areas.

7.2.3 References

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ACKNOWLEDGEMENTS

I would like to express my special appreciation to Dr. Michela Maifreni and Dr. Marilena Marino, my Ph.D. Supervisors, for their support, help and guidance as advisors in this research project but also as supporters in the everyday life.

I am also very grateful to my colleague Dr. Ingrid Bartolomeoli for her help and support during these past three years of PhD course. I thank her for giving me, especially in times of discouragements, the determination that only she is able to give!

I would also like to acknowledge: Dr. Simone Guarnieri, Dr. Di Bonaventura and his "female staff" Dr. Arianna Pompilio, Dr. Valentina Crocetta and Dr. Serena De Nicola, for giving me the opportunity to study microbial biofilms in a new way and for the time spent in Chieti; Dr. Fabio Spizzo, Dr. Monica Celotto and Dr. Fiorella Trivillin (Electrolux Italia, S.p.A.) for their support, advice and equipment; Dr. Lara Tat for the statistical support; Dr. Dobrila Braunstein for my English lessons and for my PhD thesis corrections; Antonio Ellero for helping me in the "baracco" reconstruction and in every technical critical moments that happen in a lab; the colleagues in the microbiology laboratory for making the lab work less heavy.

I would like to thank all the members of my family for always supporting me.

A special thanks to my husband, Luca, for encouraging me and for loving me during my good and bad times.

Udine, March 2014