Universidade Vigo

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Biofilm formation by *Listeria monocytogenes*. Resistance to industrial biocides and cross-response caused by adaptation to benzalkonium chloride.

Paula Saá Ibusquiza

Tesis Doctoral

DOUTOR EUROPEUS

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Memoria presentada en la Universidad de Vigo
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Dña. Rosana Álvarez Rodríguez, Profesora Titular de Química Orgánica de la Universidade de Vigo

INFORMA: que el trabajo titulado *"Biofilm formation by Listeria monocytogenes. Resistance to industrial biocides and cross-response caused by adaptation to benzalkonium chloride"*, que constituye la Memoria que presenta Dña. Paula Saá Ibusquiza para optar al Grado de Doctor por la Universidade de Vigo, ha sido realizado bajo la dirección de Dra. Marta López Cabo y Dr. Juan José Rodríguez Herrera en el Instituto de Investigaciones Marinas (C.S.I.C.).

Vigo, Marzo 2011.

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Dra. Marta López Cabo y Dr. Juan José Rodríguez Herrera, Científicos Titulares del Instituto de Investigaciones Marinas (C.S.I.C.):

CERTIFICAN: que la memoria adjunta, titulada *"Biofilm formation by Listeria monocytogenes. Resistance to industrial biocides and cross-response caused by adaptation to benzalkonium chloride",* que constituye el trabajo que presenta Dña. Paula Saá Ibusquiza para optar al Grado de Doctor por la Universidad de de Vigo, ha sido realizada bajo nuestra inmediata dirección en el Instituto de Investigaciones Marinas (C.S.I.C.).

Vigo, Marzo 2011.

Fdo. *Dra. Marta López Cabo*

Fdo. *Dr.* Juan José Rodríguez Herrera

“Sorprendernos por algo es el primer paso de la mente hacia el descubrimiento"

Louis Pasteur (1822-1895)

Microbiólogo y químico orgánico francés.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications:

1. Effects of mussel processing soils on the adherence of *Listeria monocytogenes* to polypropylene and stainless steel.

   Authors: P. Saá, M. L. Cabo M. and J. Herrera

   Reference: J. Food Prot 72(9), 1885-90 (2009).

2. An efficient method to obtain benzalkonium chloride-adapted cells of Listeria monocytogenes CECT 5873.

   Authors: P. Saá Ibusquiza, J. R. Herrera, D. Vázquez-Sánchez and M. L. Cabo

   Reference: in preparation.

3. Resistance to benzalkonium chloride, peracetic acid and nisin during formation of mature biofilms by *Listeria monocytogenes*

   Authors: P. Saá Ibusquiza, J. R. Herrera and M. L. Cabo

   Reference: Accepted in FoodMicrobiol. doi:10.1016/j.fm.2010.09.014

4. Adherence kinetics, resistance to benzalkonium chloride and microscopic analysis of mixed biofilms formed by *Listeria monocytogenes* and *Pseudomonas putida*.

   Authors: P. Saá Ibusquiza, J. R. Herrera, D. Vázquez-Sánchez and M. L. Cabo

   Reference: submitted.

5. Comparison between the resistance of benzalkonium chloride-adapted and non-adapted biofilms of *Listeria monocytogenes* to modified atmosphere packaging (MAP) and nisin once transferred to mussels

   Authors: P. Saá Ibusquiza, J. R. Herrera and M. L. Cabo

   Reference: accepted in J. Food Prot.

1. Introduction

1.1. *LISTERIA MONOCYTOGENES*

1.1.1. History, biological characteristics and classification

L. monocytogenes was discovered by EGD Murray in 1924 following an epidemiy that affected rabbits and guinea pigs in animal care houses in Cambridge (Murray et al., 1926). It was firstly described as a pathogen in 1929 and initially considered as an animal pathogen with rare human cases, *L. monocytogenes* emerged as a "new" human pathogen in the 1980s, with several food-associated listeriosis outbreaks. The case fatality rate is about 20-30%. In the US, listeriosis accounts for approximately 28% of the deaths and the highest hospitalization rate (91%) caused by known food-borne infections.

Prior to the 1980s the origins of human infections caused by *Listeria monocytogenes* were uncertain. However, at the end of the 1970s and the start of the 1980s, the number of reports on *Listeria* isolations began to increase and in 1983, the first human listeriosis outbreak directly linked to the consumption of *Listeria* contaminated foodstuffs was reported (Schlech et al., 1983). From 1983 onwards, a series of epidemia outbreaks in humans in North America and Europe clearly established listeriosis as a severe food-borne infection (Farber and Peterkin, 1991; Fleming et al., 1985; James et al., 1985; Schlech et al., 1983), and thereby *L. monocytogenes* as a food-borne pathogen.

The genus *Listeria* consists of a group of Gram-positive bacteria of low G+C content closely related to *Bacillus* and *Staphylococcus* (Sallen et al., 1996). It includes six species: *L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri* and *L. grayi* (Sallen et al., 1996). Two of them, *L. monocytogenes* and *L. ivanovii*, are pathogenic. While *L. ivanovii* is mainly pathogenic for animals, *L. monocytogenes* can infect humans, and domestic and wild animals (Seeliger and Jones, 1986). A third species, *L. seeliegeri*, is considered avirulent, although it has been isolated from at least one case of human listeriosis (Rocourt et al., 1986).

Listeria monocytogenes is a Gram positive facultative anaerobic bacterium (Seeliger and Jones, 1986) that can resist acid pHs, low Aw, low O_2 concentrations and low temperatures (Kathariou, 2002; Ross et al., 2000). Morfologically, *L. monocytogenes* are non-spore forming rods of 0.4 μm in diameter and 0.5 – 2 μm in length. They are motile by means of peritrichous flagella when cultured below 25 °C (Seeliger and Jones, 1986). *L.*

monocytogenes can grow over the temperature range of 0.5-45 °C with an optimum between 30 °C and 37 °C. However, its growth at temperatures below 4 °C is generally very slow and the lag phase can be very long. As the temperature increases above 4 °C, the growth rate of *L. monocytogenes* increases and the lag phase time decreases considerably; consequently, storage at slightly abusive temperatures (e.g. 7 to 10 °C) of refrigerated ready-to-eat (RTE) greatly increases the risk that *L. monocytogenes*, if present, will reach numbers that could cause human disease (ILSI Research Foundation/Risk Science Institute Expert Panel on *L. monocytogenes* in Foods, 2005; International Commission on Microbiological Specifications for Foods, 1996). Freezing at −18 ºC, and even repeated freezing, has little effect on the survival of *L. monocytogenes*; these conditions are more likely to injure than to inactivate this organism.

L. monocytogenes is very well equipped to survive typical hurdles applied in food preservation. It grows across a broad pHs range (4.3-9.8), but depends on the acid type and temperature, *L. monocytogenes* can grow at pHs as low as 4.0 (Lado and Yousef, 2007, Martin and Fisher, 1999). *L. monocytogenes* grow in complex medium containing up to 10% (w/v) NaCl, but some strains can tolerate up to 20 % (w/v) NaCl, so it can resist very low water activities (aw 0.91) (Lado and Yousef, 2007, Seeliger and Jones, 1986). Moreover, it can grow in aerobic modified atmospheres also with competitive microorganisms (Wimpfheimer et al., 1990).

L. monocytogenes has been classified under different criteria:

1. According to the presence of O (somatic) and H (flagelar) antigens *L. monocytogenes* is classified in 13 serovars (Seeliger and Höhne, 1979; Seeliger and Jones, 1986): 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 7. Although human listeriosis may be caused by all 13 serovars of *L. monocytogenes,* 1/2a, 1/2b, 1/2c and 4b serovar cause at least 95% of the cases (Doumith et al., 2004; Farber and Peterkin, 1991; Swaminathan and Gerner-Smidt, 2007). Among the outbreaks of invasive listeriosis, serovar 4b strains have caused the majority of the outbreaks worldwide from 1980-2005, whereas strains of serovar 1/2 have caused the majority of the non-invasive, gastrointestinal listeriosis outbreaks worldwide from 1993-2001 (Swaminathan and Gerner-Smidt, 2007). However, among food isolates, serotype 1/2 is the most frequently found (Farber and Peterkin, 1991; Jacquet et al., 2002).

2. According to the genotypic analyses: whereas enzyme electrophoresis (Piffaretti et al., 1989), pulsed-field gel electrophoresis (PFGE) (Brosch et al., 1994) and ribotyping (Graves et al., 1994) can divide *L. monocytogenes* into two major subgroups, virulence gene analysis have grouped *L. monocytogenes* into three groups: lineage I, II and III (Rasmussen et al., 1995; Wiedmann et al., 1997). At the same time, each lineage includes several serotypes: lineage I, comprising serotypes 1/2b, 3b, 3c and 4b; lineage II, comprising serotypes 1/2a, 1/2c and 3a, and lineage III comprising serotypes 4a and 4c (Nadon et al., 2001). Invasive listeriosis is primarily caused by lineage I strains, whereas lineage II strains are most frequently isolated from food. In comparison, serotypes belonging to lineages II and III are less significant, being rarely associated with foodborne listeriosis.

Typing studies involving *L. monocytogenes* isolates from clinical, food and food processing sources led to the consensus of considering the division of *L. monocytogenes* in those three lineages (Harvey and Gilmour, 1992).

1.1.2. Natural niches

Although *L. monocytogenes* is ubiquitous, its prevalence in the outdoor environment is not high (Porsby et al., 2008). *Listeria* spp. are isolated from a diversity of environmental sources, including soil, water, effluents, a large variety of foods, and the feces of humans and animals (Barbuddhe et al., 2009). It is thought to be widespread, being a saprophytic organism adapted to the plant-soil environment (Weis and Seeliger, 1975). The bacteria is widely present in plants, soils, sediments and surface water samples, and has also been found in sewage, human and animal faeces (MacGowan et al., 1994; Weis and Seeliger, 1975). Generally, the proportion of positive samples is low in the outdoor environment, between 0 and 6%, but studies indicate that the prevalence increases with the degree of human activity (Hansen et al., 2006; MacGowan et al., 1994; El Marrackchi et al., 2005). Animals are susceptible to listeriosis but can carry *L. monocytogenes* asymptomatically. Fecal carriage of *L. monocytogenes* in livestock animals such as cattle beef, dairy, poultry and horses has been found with varying frequency around 0-13%, and in wildlife up to 40% (Lyautey et al., 2007). Prevalence of *L. monocytogenes* has also been reported in wild life animals like deer, moose and birds (Hellström et al., 2008; Lyautey et al., 2007). Similarly, humans can be

asymptomatic carriers of *L. monocytogenes*, but generally with prevalence below 1% (MacGowan et al., 1994; Sauders et al., 2005).

1.1.3. The disease: clinic, invasiveness and virulence

The invasive disease is fatal in 30% of cases although it can be treated with amoxicillin antibiotics if early caught (Williams and Nadel, 2001). Clinical manifestations range from febrile gastroenteritis to more severe invasive forms, including sepsis, meningitis, rhombencephalitis, perinatal infections, and abortions (Allerberger et al., 2010). In pregnant women, listeriosis may lead to spontaneous abortion, stillbirth or fetal death.

Recent outbreaks demonstrated that *L. monocytogenes* can cause gastroenteritis in otherwise healthy individuals and more severe invasive disease in immunocompromised patients. Common symptoms include fever, watery diarrhea, nausea, headache, and pains in joints and muscles. As it is shown in **Figure 1.1.1**, the intestinal tract is the major portal of entry for *L. monocytogenes*, whereby strains penetrate the mucosal tissue either directly, via invasion of enterocytes, or indirectly, via active penetration of the Peyer's patches. Additionally, some evidences have shown that listeriolysin (a protein directly involved in the infectiveness of *L. monocytogenes*, as described below) can act as an extracelular virulent factor that caused gastroenteritis in the host (Richter et al., 2009).

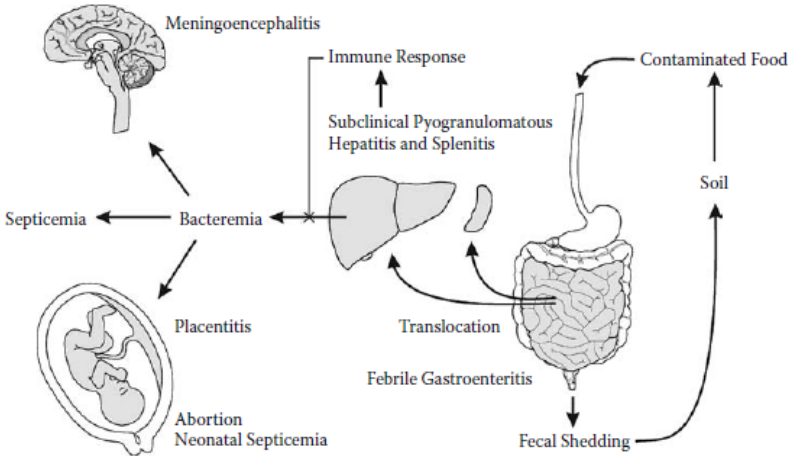

Figure 1.1.1: Schematic representation of the physiopathology of *L. monocytogenes* infection (taken from Liu 2008)

Although all the strains belonging to this species are assumed to be pathogenic, epidemiological evidence indicates that strain-to-strain differences in virulence. Thus, only 4 of the 13 *Listeria* serotypes—namely, 1/2a, 1/2b, 1/2c, and 4b—account for 95–98% of all cases of human and animal listeriosis worldwide. Of these, serovar 1/2c is found in a minority of clinical isolates (2–4%) but predominates among food isolates. Similarly, serovar 4b, belonging to one of the two major genetic lineages of *L. monocytogenes*, predominates among clinical isolates (>50% of listeriosis cases), whereas it is much less frequently found among food isolates than serogroup ½ (i.e., serovars 1/2a, 1/2b, and 1/2c) strains. Moreover, a restricted number of 4b strains, representing distinct genotypes, are responsible for most food-borne outbreaks of human listeriosis world-wide, which suggests that certain clones of *L. monocytogenes* may be more pathogenic for humans (Liu, 2008).

Virulence heterogeneity among *L. monocytogenes* isolates—often associated to natural attenuating mutations in key virulence *loci*—is also supported by experimental evidence. However, the most critical factor is the underlying condition and immunological status of the host as this determines the susceptibility to a given strain of *L. monocytogenes*. The vast majority of listeriosis patients have a physiological or pathological condition that impairs the capacity to mount an effective cellular immune response. Three major population groups at risk for invasive listeriosis are the neonates, elderly (>60 years) and pregnant woman. In neonates and elderly, that risk is due to the inmature or declining immune system. In pregnant woman is associated with depression of cell-mediated immunity to prevent rejection of the fetoplacental allograft. In nonpregnant adults, almost all cases of listeriosis have been found in individuals with chronic, debilitating illnesses or subjected to immunosuppressive therapy. Specific risk groups in the intermediate-age band include cancer and organ transplant patients, HIV-infected and AIDS patients, and individuals with chronic liver disease (alcoholism and cirrhosis), diabetes and lupus (Liu, 2008).

In immunocompetent individuals with no predisposing condition, ingestion of low to moderate doses of *L. monocytogenes* ($\leq 10^5$ CFUs) has no effect other than boosting antilisterial protective immunity, whereas ingestion of large doses of the bacteria ($\geq 10^6$ CFUs, sometimes doses as high as 10^{11}) may cause acute febrile gastroenteritis within 24 h of consumption of the contaminated food due to massive invasion of the intestinal mucosa. Depending on the pathogenicity of the strain, some healthy nonpregnant adults exposed to a large *L. monocytogenes* inoculum may develop invasive listeriosis. In immunocompromised

individuals, however, invasive disease is facilitated by the inefficient mobilization of the host defenses and the blood-borne dissemination of *L. monocytogenes* from the primary infectious foci in the liver and spleen (silent phase of infection). In those cases, bacteremia may lead to meningoencephalitis if bacteria traverse the brain microcapillaries, to abortion or perinatal septicemia if they traverse the placental barrier, or to septicemic disease in cases of severe immunosuppression (**Figure 1.1.2**).

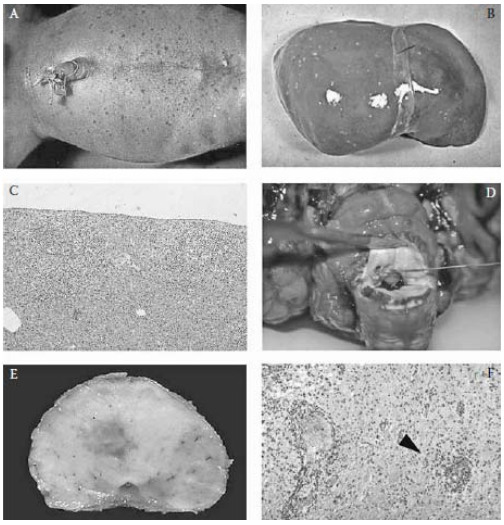

Figure 1.1.2: Clinical and pathological features of *Listeria monocytogenes* infection. (A) Fetomaternal listeriosis. Stillborn fetus with septicemic invasion ("granulomatosis infantiseptica"). (B) Liver from the stillborn fetus in (A) showing typical disseminated pyogranulomatous necrotic foci. (C) Histopathological image of the liver from an experimentally infected sheep with milliary listerial pyogranulomatous hepatitis (hematoxilin/eosin-stained). (D) Meningoencephalitis due to *L. monocytogenes* in a cow. (E) Section of the brainstem of a sheep with listerial rhombencephalitis showing inflammatory lesions in the nerve tissue. (F) Parenchymal inflammatory infiltration of the brainstem in (E) showing typical perivascular cuffing (arrow) indicative of blood-borne invasion of the brain tissue by *L. monocytogenes*. Clinical and pathological manifestations of listeriosis are essentially similar in humans and animals. (taken from Liu 2008 Pag. 99)

1.1.4. Virulence mechanism

Macrophages and epithelial cells are widely used to study the interaction of *L. monocytogenes* with mammalian host cells. However, it was shown that also neutrophils,

dendritic cells, hepatocytes, fibroblasts, endothelial cells, or glial cells may become infected with, and serve as host cells for *L. monocytogenes in vitro* and *in vivo*.

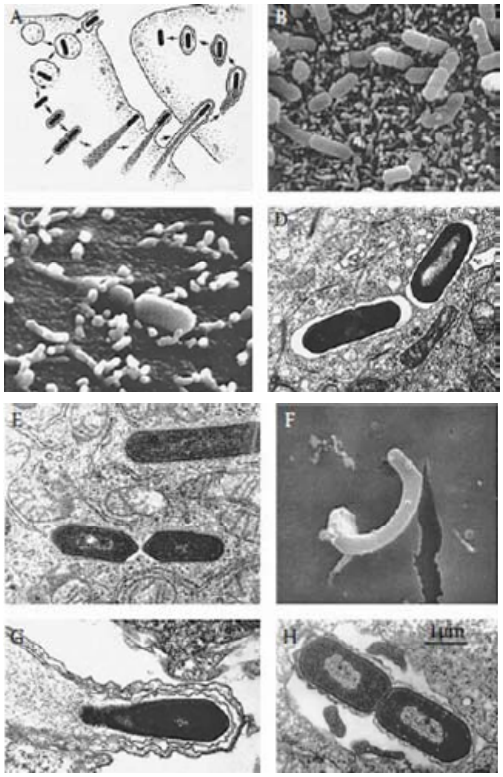

Figure 1.1.3: Stages of listerial intracellular parasitism. (**A**) Scheme of the intracellular life cycle of pathogenic *Listeria* spp. (**B–H**) Scanning and transmission electron micrographs of cell monolayers infected with *L. monocytogenes.* (**B**) Numerous bacteria adhering to the microvilli of a Caco-2 cell (30 min after infection). (**C**) Two bacteria in the process of invasion (Caco-2 cell, 30 min postinfection). (**D**) Two intracellular bacteria soon after phagocytosis, still surrounded by the membranes of the phagocytic vacuole (Caco-2 cell, 1 h postinfection). (**E**) Intracellular *Listeria* cells free in the host cell cytoplasm after escape from the phagosome (Caco-2 cell, 2 h postinfection). (**F**) Pseudopod-like membrane protrusion induced by moving *Listeria* cells, with the bacterium being evident at the tip (brain microvascular endothelial cell, 4 h postinfection. (**G**) Section of a pseudopod-like structure in which a thin cytoplasmic extension of an infected cell is protruding into a neighboring noninfected cell, with the protrusion being covered by two membrane layers (Caco-2 cell, 4 h postinfection). (**H**) Bacteria in a double membrane vacuole formed during cell-to-cell spread (Caco-2 cell, 4 h postinfection). (Taken from Vazquez-Boland et al., 2001.

Macrophages actively engulf *L. monocytogenes* spontaneously, but internalization of the bacterium by normally nonphagocytic cells is triggered by *L. monocytogenes*-specific factors. Aside from the internalization step, the intracellular life cycle (**Figure 1.1.3 A**) of the bacterium in phagocytes or normally nonphagocytic mammalian cells is, however, essentially identical. *L. monocytogenes* induces its own internalization without an extensive remodeling of the host cell surface. Entry occurs via zipper-like phagocytosis, characterized by the emission of small pseudopods that firmly entrap the bacteria and the intimate contact of the bacterial surface with the host cell plasma membrane. Upon uptake, the pathogen appears in a membrane-bound vacuole (**Figure 1.1.3 D**), which is subsequently lysed by the combined action of the pore-forming hemolysin, listeriolysin (LLO), and two phospholipases (see below). The bacteria that are released into the cytoplasm begin to replicate while making use of specific transporters to gain carbohydrates from the host cell, whereas those remaining in the phagosome are killed and digested.

Concomitant with the onset of intracellular replication, *L. monocytogenes* induces the expression of the surface protein ActA which, through the activation of the cellular rp2/3 complex, induces the nucleation of host actin filaments. The formation of a polar tail and the permanent polymerization of F-actin at the interface between the bacteria and the actin tails produce a propulsive force, which moves the bacteria through the cytoplasm. Those bacteria that in their random movement reach the plasma membrane push outwards inducing the formation of pseudopod-like structures with the bacterium at the tip. These invading pseudopods or “listeriopods” are taken up by the neighboring cells, in which the bacteria become entrapped within a double membrane. This vacuole is again lysed by LLO and the phospholipases, a broad-specificity phospholipase, releasing the bacteria into the cytoplasm of the newly infected host cell where they initiate a new cycle of replication and actin-based motility. This direct cell-to-cell invasion mechanism allows the bacteria to spread through host tissues without leaving the host cytosolic compartment, protected from the humoral effectors of the immune system and phagocytosis (Liu, 2008)

L. monocytogenes has become not only an important paradigm for immunological investigation but also an important model system for analysis of the molecular mechanisms of intracellular parasitism (Vázquez-Boland, 2001)

1.2. BIOFILM FORMATION BY *L. MONOCYTOGENES*

1.2.1. What is a biofilm? History and definition

Although bacteria grow preferentially in the biofilm mode in industrial and natural systems (Blaschek et al., 2007), studies on bacteria in laboratories are still generally carried out in planctonic cells. The vast majority of microorganisms tested end up by forming biofilms, on practically any surface and any environmental conditions, although their adhesion and growth rates, extracellular polymeric substances (EPS) yields and final structure is highly variable (Watnick and Kolter, 2000).

During the last 25 years, definitions of biofilms have been proposed by several authors (Marshall, 1976; Costerton et al., 1978; Costerton et al., 1987; Characklis and Marshall, 1990; Costerton et al., 1995; Costerton and Lappin-Scott, 1995; Davies and Geesey, 1995; Prigent-Combaret and Lejeune, 1999). Finally, the following definition is widely accepted: "Biofilm is a microbially derived sessile community characterized by cells that are irreversively attached to a substratum or interface or to each other, embedded in a matrix of extracellular polymeric substances that they have produced, and with an altered phenotype with respect to growth rate and gene transcription (Donlan and Costerton, 2002)".

1.2.2. Some important consequences of biofilm formation

1.2.2.1. Transference of gases and nutrients

Diffusion limitations create gradients of nutrients and oxygen from the top to the bottom of biofilms that are associated with decreased bacterial metabolic activity and increased doubling times of the bacterial cells. Anaerobic conditions may be present in the centre of the biofilm. Likewise, growth, protein synthesis and metabolic activity are stratified in biofilms, i.e. a high level of activity at the surface and a low level and slow or no growth in the centre. In fact, this is one of the explanations for the reduced susceptibility of biofilms to antibiotics (Høiby et al., 2010). Such gradients will increase in extent as biofilms thicken and will become particularly marked in aged biofilms (Gilbert et al., 2002). A major contributor towards the failure of biofilms to rapidly succumb to antimicrobial treatments must, therefore, be associated with its physiological heterogeneity (Allison et al., 2000; Gilbert et al., 2002).

1.2.2.2. Cell disposition in the biofilm

Bacterial cells have the ability to aggregate into particular three-dimensional assemblages, differentiate, divide labor within these assemblages, and then disperse as part of their life cycle (Davey and O´Toole, 2000). The structures that form in biofilms contain channels in which nutrients can circulate. The complexity of biofilm structure and metabolism has led to the analogy of biofilms to tissues of higher organisms. The complex biofilm architecture provides an opportunity for metabolic cooperation and could imply the generation of niches where antimicrobial-resistant phenotypes are formed within the spatially well-organized system (Davies, 2003; Klapper et al., 2007).

So, beside the individual cell changes, intercellular interactions and communication are required for biofilm development and persistence. Dissecting these interactions provides one of the future challenges in biofilm research. Particularly challenging is the attempt to understand the complexity of the interactions within the biofilm community. Communication between species may include extracellular compounds whose sole role is to influence gene expression, metabolic cooperativity and competition (possibly encompassing global changes in gene expression and metabolism), physical contact, and the production of antimicrobial exoproducts. One or all of these interactions may be occurring simultaneously (Davey et al, 2000).

1.2.2.3. Resistance to external stimulus: implications in medicine and in food industry.

Biofilms constitute a protected mode of growth that allows survival in a hostile environment (Costerton et al., 1999) under changing environmental conditions and hostile chemical or physical agents: predators, host cells, innate defense compounds, antibiotics and, in the food industry, antimicrobial additives and cleaning and disinfection agents. Biofilm-embedded cells can stand up to 100-1000 times higher biocide concentrations than planktonic cells. As a consequence, bacterial cells persist in biofilm form, causing problems mainly in the medical ambit and in the food industry.

Concerning the medical ambit, the US National Institutes of Health estimates that upwards of 75% of microbial infections that occur in the human body are underpinned by formation and persistence of biofilms. Some examples are recopilated in **Table 1.2.1.** Biofilms increase the tolerance to antibiotics and disinfectant chemicals in association with medical devices as

well as resisting phagocytosis and other components of the body's defence system (Campanac et al., 2002, Costerton et al., 1999, Høiby et al., 2010). Biofilms are the most likely environmental nidus of resistance development and selection that might relate to active efflux of the treatment agent or to others less well-understood mechanisms (Gilbert et al., 2002).

Table 1.2.1: Some human infections involving biofilms (taken from Costerton et al., 1999).

Infection disease	**Common biofilm bacterial species**
Dental caries	Acidogenic Gram-positive cocci (e.g., *Streptococcus*)
Periodontitis	Gram-negative anaerobic oral bacteria
Otitis media	Nontypable strains of *Haemophilus inßuenzae*
Musculoskeletal infections	Gram-positive cocci (e.g., staphylococci)
Necrotizing fasciitis	Group A streptococci
Biliary tract infection	Enteric bacteria (e.g., *Escherichia coli*)
Osteomyelitis	Various bacterial and fungal species often mixed
Bacterial prostatitis	*E. coli* and other Gram-negative bacteria
Native valve endocarditis	Viridans group streptococci
Cystic fibrosis pneumonia	*P. aeruginosa* and *Burkholderia cepacia*
Meloidosis	*Pseudomonas pseudomallei*
Nosocomial infections	
ICU pneumonia	Gram-negative rods
Sutures	*Staphylococcus epidermidis* and *S. aureus*
Exit sites	*S. epidermidis* and *S. aureus*
Arteriovenous shunts	*S. epidermidis* and *S. aureus*
Schleral buckles	Gram-positive cocci
Contact lens	*P. aeruginosa* and Gram-positive cocci
Urinary catheter cystitis	*E. coli* and other Gram-negative rods
Peritoneal dialysis (CAPD) peritonitis	A variety of bacteria and fungi
IUDs	*Actinomyces israelii* and many others
Endotracheal tubes	A variety of bacteria and fungi
Hickman catheters	*S. epidermidis* and *C. albicans*
Central venous catheters	*S. epidermidis* and others
Mechanical heart valves	*S. aureus* and *S. epidermidis*
Vascular grafts	Gram-positive cocci
Biliary stent blockage	A variety of enteric bacteria and fungi
Orthopedic devices	*S. aureus* and *S. epidermidis*
Penile prostheses	*S. aureus* and *S. epidermidis*

Conventional resistance mechanisms such as chromosomal beta-lactamase, upregulated efflux pumps and mutations in antibiotic target molecules in bacteria also contribute to the survival of biofilms (Høiby et al., 2010). Proposed explanations for the observed resistance of biofilm communities include diffusional resistance of the extracellular matrix, augmented by chemical/enzymatic modification of the agent (reaction-diffusion limitation), physiological changes due to slow growth rate and starvation responses and the induction of attachment-specific drug resistant physiologies (Allison et al., 2000; Gilbert and Allison, 1999). Whilst there is evidence to support each of these explanations no single mechanism can account for

the general observation of resistance. Rather, these mechanisms act in concert within the biofilm and amplify the effect of small variations in phenotype susceptibility.

In the food industry, it is generally admitted that *L. monocytogenes* can be present in food processing environments and some strains are persistently present (**see section 1.2.6**). These resident strains are expected to form biofilms on food processing equipment, on conveyor belts, in pipes, on floors, and in drains. Since biofilms are generally more difficult to eradicate than planktonic cells during disinfection treatments (Mah and O'Toole, 2001; Robbins et al., 2005), the capability of *L. monocytogenes* to form biofilms poses a major concern for the food industry. However, to our knowledge, none of the reported outbreaks have been related with contamination from biofilms.

1.2.3. Biofilm development in *L. monocytogenes*

In general, as suggested by Donlan and Costerton (2002), several factors should be considered in the development of a biofilm: i) the medium (composition, temperature, presence of antimicrobial agents), ii) the inoculum (identity of organism, number of cells), iii) hydrodynamics (flow rate, presence of shear, batch versus open system, retention time), and iv) the type of substratum (roughness, chemistry, conditioning films). These factors can influence the different steps of biofilm formation: initial attachment, maduration and dettachment (Campanac et al., 2002).

1.2.3.1. Initial attachment

1.2.3.1.1. Effect of external conditions: nutrients, type ofsurface, pH.

The development of a biofilm is initiated by planctonic (freely moving) bacteria that reversibly attach to a surface, which may be covered by a layer of, for example, proteins (a pellicle). At this stage, the bacteria are still susceptible to antibiotics (Høiby et al., 2010). All the factors previously enumerated are determinant in this initial phase of biofilm formation. Here we describe the most relevant according with previous works:

Nutritional conditions: Contradictory results concerning the influence of nutritional conditions on the initial adherence of *L. monocytogenes* were found. Whereas several authors observed that attachment of *L. monocytogenes* ATCC 19111 to stainless steel surface was

greater when cultivated in a rich medium (Hood and Zottola, 1997; Mai and Corner, 2007; Stepanovic et al., 2004; Takhistov and George, 2004), the studies of Jaradat and Bhunia (2002), observed that nutrient-rich media and high glucose concentration suppressed *Listeria* adhesion protein (LAP) expression. Folsom et al. (2006) demonstrated that the formation of biofilms at different nutrient levels in tryptic soy broth (TSB) or in a 1:10 dilution of TSB (DTSB) for 24 h at 32 ºC on stainless steel by *Listeria monocytogenes* was genotype dependent. They found that serotype 4b strains produced more biofilm in TSB than did serotype 1/2a strains, whereas serotype 1/2a strains produced more biofilm in DTSB than did serotype 4b strains. These results indicate that strains of serotype 1/2a and serotype 4b differ in the regulation of their biofilm phenotype. By the way, the poor biofilm accumulation of serotype 4b isolates when grown in DTSB could be a factor in the predominance of serogroup 1/2 strains in food processing plants, where nutrients may be limited. Finally, the contribution of nutrients to biofilm formation may differ from their roles in attachment, reinforcing the distinction between attachment and biofilm growth.

Type of surface: Most of the published results reached the consensus that material nature determines the level of adherence in bacteria (Blackman and Frank, 1996; Di Bonaventura et al., 2008; Krysinski and Brown 1992; Meylheuc et al., 2001; Rodríguez et al. 2008; Saá et al., 2009; Sinde and Carballo, 2000; Somers and Wong, 2004; Smoot and Pierson, 1998; Teixeira et al., 2008). In fact, we could only found one previous work (Gamble and Muriana, 2007) in which strongly adherent strains of *L. monocytogenes* adhered equally well to four different abiotic surfaces (glass, plastic, rubber, and stainless steel).

pH: Several authors have demonstrated that pH has a great influence on the adhesion capacity of *L. monocytogenes,* although with some discrepancies between them. Whereas Herald and Zottola (1988) concluded saying that a decrease in the adhesion capacity of *L. monocytogenes* at acid pH in comparison with alkaline pH, Poimenidou et al., 2009 pointed out that low pH stimulates initial adhesion of *L. monocytogenes* to stainless steel due to the protonation of the negative groups on the cell surface. In the same line, Smoot and Pierson (1998) showed that after short contact times (less than 30 min) the levels of attached cells were lower under alkaline conditions. However, the maximum levels of adhered cells to Buna-N rubber were not affected by adjustments of pH between 4 and 9. Stopforth et al., 2002 observed no significant differences in attachment and biofilm formation between acid-adapted and nonadapted *L. monocytogenes.*

Concerning the effect of temperature on initial attachment, it has been stated in several works that attachment of *L. monocytogenes* increase with the temperature (Mai and Corner, 2007; Poimenidou et al., 2009).

1.2.3.1.2. Effect of the presence of conditioning film in the initial bacterial attachment

During the first stage of biofilm formation, molecules present in a bulk flow, both organic and inorganic, are carried toward the surface either by diffusion or turbulent flow. This accumulation of molecules at the solid–liquid interface on surfaces found in many food industries is commonly called conditioning film and it leads to a higher concentration of nutrients at the surface than in the liquid phase. The adsorption of organic molecules such as proteins to surfaces could play an important role in bacterial attachment, as this conditioning of the surface may alter its physical and chemical properties. Factors involved can include surface free energy, hydrophobicity and electrostatic charges. Conflicting opinions exist on the importance of a conditioning film in initial bacterial attachment (Palmer et al., 2007). Some authors demonstrated that the presence of nutrients on the surface reduced attachment of *L. monocytogenes* on different surfaces (Parker et al. 2001; Helke et al., 1993). One reason for this reduced attachment may be that proteins in the bulk fluid phase may act as competitors for binding sites on the surface of stainless steel, reducing the ability of bacteria to attach. But others reported that conditioning films increased the attachment of *Listeria monocytogenes* and another species (Palmer et al., 2007; Jeong and Frank, 1994; Verran and Whitehead, 2001; Speers and Gilmour 1985).

1.2.3.1.3 Effects of flagellar motility

Flagellar motility facilitates localization and potentially cellular adhesion which may be vital for colonization during the infectious cycle. *L. monocytogenes* is capable of motility and produces between four and six peritrichous flagella. It is flagellated and motile at temperature of 30 ºC and below; however, it is non-motile at human body temperature or higher (Peel et al., 1988). Then, during biofilm formation temperature affects the motility and thus the adherence capacity to surfaces. Guerieri et al., (2008), Lemon et al. (2007), Tresse et al., (2006), and Vatanyoopaisarn et al. (2000) demonstrated that flagellar motility is critical for *L. monocytogenes* biofilm formation in the first stages. Nonetheless, Bonaventura Di et al. (2008) did not find any correlation between swimming and biofilm production by *Listeria monocytogenes* in different food contact surfaces.

1.2.3.2. Maturation of attached bacteria into a differentiated biofilm

Maturation starts with the irreversible binding to the surface within the next few hours and subsequent multiplication of bacteria cells, which form microcolonies and begin to produce a polymer matrix around them (Høiby et. al., 2010). Takhistov and George (2004) observed that following the initial attachment of *Listeria monocytogenes* new cells attach to the extracellular polymeric substances (EPS). The number and size increased with time, eventually forming intercolony bridges. They describe this appearance as a "bacterial web" similar to the net-like and honey comb patterns that were reported by Marsh et al., 2003. As in attachment, several factors have an important effect on this phase of biofilm formation:

1.2.3.2.1. Effects of the external medium in the maduration of the biofilm

Whereas initial attachment of the cells has been extensively studied, biofilms maturation has received less attention. Results from most studies indicate that *Listeria monocytogenes* prefers forming a biofilm under relatively high nutrient conditions, unlike many other species (Blackman and Frank 1996; Helke and Wong 1994; Takhistov and George 2004; Stepanovic et al., 2004).

The effects of several specific nutrients on *L. monocytogenes* during biofilm formation have also been studied. Kim and Frank (1995) examined the effects of phosphate, amino acids, tryptone, and various carbohydrates in MWB on *L. monocytogenes* biofilm formation. A reduction or increase in phosphate level from that occurring in MWB (37.52 g/l) reduced biofilm development. A reduction in amino acid levels produced a corresponding decrease in biofilm formation during the first 7 days of incubation, but after 12 days the amount of biofilm was the same regardless of amino acid concentration. Among the carbon sources studied, *L. monocytogenes* produced significantly greater biofilm amounts in presence of mannose and trehalose than in the presence of glucose and even less with glucosamine as carbon source. Similary, Somers and Wong (2004) have shown that small amounts of meat and fat residue in the medium reduced biofilm formation initially but, on prolongated incubation, the cell number increased and, on some surfaces, exceded the number present on unsoiled chips.

Finally, some studies have demonstrated that the effect of the type of feeding is also relevant. Rodrigues et al. (2009) studied the influence of feeding conditions (batch and fed-

batch) at different temperatures on the formation of *L. monocytogenes* biofilms. They showed that biofilms formed under fed-batch conditions were metabolically more active than those formed in batch mode.

1.2.3.2.2. Importance of the extracellular polymeric substances (EPS) matrix

Up-regulation of EPS biosynthesis generally occurs within minutes of the irreversible attachment of a cell to a surface and proceeds with the development of a microcolony over a period of several hours (Gilbert et al., 2002). A biofilm is a structured consortium of bacteria embedded in a self-produced polymer matrix consisting of polysaccharide, protein, lipids, minerals and DNA originating from the microbes, and the bacterial consortium can consist of one or more species living in a sociomicrobiological way (Høiby et al., 2010). Most of the components are secreted by the embedded living cells; others may come from dead and decaying cells and some may be trapped from the environment. The matrix is important since it provides structural stability and protection to the biofilm. The development of bacterial biofilms over time has been intensively studied *in vitro* by confocal scanning laser microscopy employing green fluorescent protein (GFP)-tagged bacteria. This technique has been combined with the advances in silico image analysis to produce three-dimensional images (Høiby et al., 2010). The first matrix components to be known were the polysaccharides; few of them have been quantitatively analyzed, due to the difficulties to obtain sufficiently large amounts. In the particular case of *L. monocytogenes*, EPS structure has not been characterized yet. Whereas the role of polysaccharides as part of the extracellular matrix remains elusive it has been demostrated the presence of extracellular DNA (eDNA) in the biofilm matrix that plays an important role for both initial attachment and early biofilm formation in *L. monocytogenes* (Harmsen et al., 2010; Renier et al., 2010).

The presence of a charged, hydrated exopolymer matrix around individual cells and microcolonies profundly affects the access of solutes. The polymers of the extracellular matrix thereby act as an ion exchange resin and actively remove strongly charged molecules (i.e. glycopeptides; Hoyle et al., 1992) from solution (Slack and Nichols, 1981, 1982; Costerton et al., 1987). Curiously, the effectiveness of macrolide antibiotics, which are positively charged and very hydrophobic, is relatively unaffected by the presence of exopolymers (Ichimiya et al., 1994). Poor penetration through anionic matrices might be a phenomenon restricted to more hydrophilic, positively charged agents. Alternatively, the

matrix polymers might react chemically with and directly neutralize reactive molecules. The latter effects would be most pronounced with biocides, such as iodine and iodine–polyvinylpyrollidone complexes (Favero et al., 1983), and chlorine and peroxygens (Huang et al., 1995), which react directly in a consumptive manner with the exopolymer and cellular materials. However, diffusion limitation studies have generally focused on antibiotics rather than biocides and upon medically relevant biofilm populations rather than biofouling situations.

1.2.3.3. Dettachment

Detachment is defined as a stage where focal areas of the biofilm dissolve and the liberated bacterial cells can then spread to another location where new biofilms can be formed. There are several hypotheses to explain detachment:

i. That cells may detach individually from biofilms as a result of cell growth and division within the biofilms, or cell aggregates or clusters may detach or be sloughed from biofilms (Donlan and Costerton, 2002).
ii. That is the result of different detachment regulating systems that have been demonstrated in various bacterial species, such as those based on catabolic repression (Sauer et al., 2004), in the production of specific enzymes (Liu et al., 2007) or other molecules like ramnolipids in *Pseudomonas* (Boles et al., 2005) that could specifically disrupt the external matrix (EPS) of the biofilms. Other authors believed that EPSs can be used as nutrients by cells, making it easier for starving cells to detach from biofilms (Takhistov and George, 2004).
iii. That is the result of bacteriophage activity within the biofilm. The mature biofilm may contain water-filled channels and thereby resemble primitive, multicellular organisms like it has been demonstrated in other microorganisms (Wang et al., 2009).
iv. That is the result of using type IV pili to mount or climb biofilms formed by other bacteria and colonise the top of the biofilm, resembling a hat (Høiby et. al., 2010).

The amount of biofilm in a given system after a certain period of time depends on biofilm accumulation, which has been defined as the balance between bacterial attachment from the planktonic phase, bacterial growth within the biofilm and biofilm detachment from the

surface. When that balance is null, the biofilm is said to have reached a steady-state (Simões et al., 2009).

Harvey et al., 2007 showed some representative images obtained from microscopic observations of biofilms as they developed during 14 days (**Fig. 1.2.1 a–d)**.

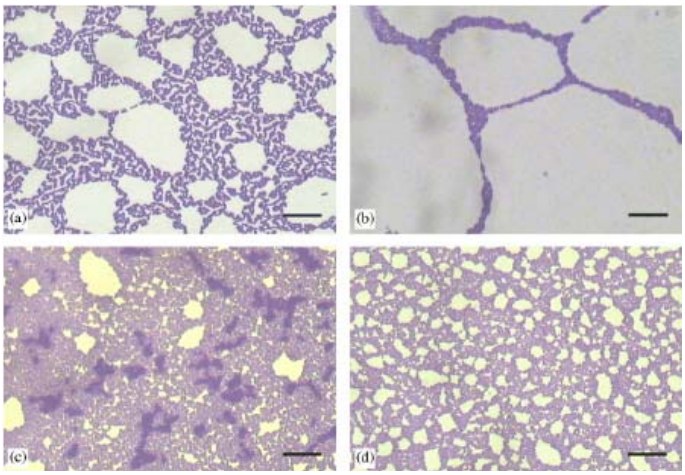

Fig. 1.2.1: Micrographs of *L. monocytogenes* biofilms grown in TSB at 20 ºC on polystyrene Petri dishes. Bacteria stained with crystal violet and observed under a 1000 x oil-immersion objective: (**a**) strain L-12-90 after 1day; (**b**) strain WHO04 after 5 days; (**c**) strain L-12-90 after 5 days; and (d) strain D-11-89 after 14 days. Scale bars, 10.0 mm (taken from Harvey et al., 2007).

The strains developed clearly defined networks of microcolonies completely covering the entire surface of Petri dishes (**Fig. 1.2.1 a**, initial attachment). During the maturation stage, fully developed biofilms were formed in which closely connected microcolonies completely covered the surface of the dish and areas could be seen dispersed throughout the biofilm where layers or stacks of bacterial cells had accumulated. Layers are greater or smaller depending on the strain (**Fig. 1.2.1 c** and **d**). Finally, for a small number of strains, only single cells or small clumps very sparsely distributed over the surface of the Petri dishes were observed throughout the 14-day period (dettachment). Although these steps are most commonly accepted in the physiology of biofilms, small number of the *L. monocytogenes* strains were seen to require an extra stage in biofilm development. These strains, typically after 5–6 days, started to form a network of microcolonies covering the entire surface of the Petri dishes. However compared to the networks formed by the strains of the type shown in

Fig. 1.2.1 a, the unpopulated surface areas between microcolonies in these biofilm networks were very much larger (**Fig. 1.2.1 b**). With continued incubation, the surfaces between these microcolonies gradually became populated until by 11–12 days fully developed biofilms similar to those shown in **Fig. 1.2.1 c** or **d** were produced

1.2.4. Regulation of biofilm formation in *L. monocytogenes*

1.2.4.1. Cell-cell comunication

Two archetypal cell-cell comunication systems have been described in *L.monocytogenes*: the auto-inducer 2 (AI-2) LuxS system found in both Gram-negative and Gram-positive bacteria, and the peptide-mediated QS signalling system Agr characteristic of Gram-positive bacteria (Dunny and Leonard, 1997; Miller and Bassler, 2001; Waters and Bassler, 2005).

Quorum sensing (QS): AI-2 Lux S system

LuxS is responsible for the production of autoinducer-2 (AI-2), which is involved in the *quorum-sensing* response of *Vibrio harveyi* and considered a signal molecule implicated in interspecies communication. Recently, it has also been demonstrated AI-2 plays a role in bacterial biofilm formation (Blehert et al., 2003; Coleet al., 2004; Merritt et al., 2003; Wen et al., 2004). So, Belval et al. (2006) studied the role of autoinducer 2 (AI-2), the gen LuxS, responsible of AI-2 synthesis, and S-adenosylhomocysteine (SAH), S-Ribosyl Homocysteine (SRH), intermediate metabolites in the synthesis of AI-2, in cell attachment during biofilm formation by *Listeria monocytogenes* EGD-e (see **Figure 1.2.2** for metabolic pathway). Their results indicated that *L. monocytogenes* EGD-e produces AI-2 like molecules. However, the authors demonstrated that a mutant luxS-negative (Lux 1) of *L. monocytogenes* EGD-e, can not produce AI-2 and formed denser biofilms than EGD-e strain formed, with 10-17 times more attached cells. To explain these results, they checked the role of the precursors of AI-2, SAR and SHR, that would be accumulated in the mutant Lux1. As a result, they demonstrated that only SRH, but not SAH, plays an important role in biofilm formation. Although the explanation of that mechanism is unclear, for the bacteria it would be beneficial to detect the presence of other bacteria using SRH, especially if we consider this pathway is associated with the detoxification of methyl groups in bacteria.

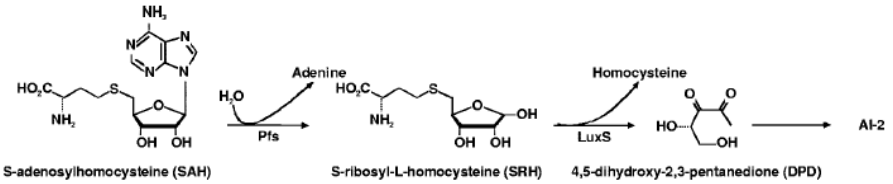

Figure.1.2.2: Partial pathway for conversion of SAH to AI-2.

Agr system

Major adaptive responses are regulated through *agr* autoinduction; these include virulence and biofilm formation (Riedel et al. 2009). Although there is no doubt that the *agr* system is indeed a communication system based on autoinduction, whether it is dedicated to Quorum Sensing (QS) remains an open question. In fact, it has not been demonstrated that the *agr* system is a mechanism to assess cell density in order to coordinate the behaviour of the whole population in *L. monocytogenes* (Garmyn *et al*., 2009). In other words, evidence of a QS system is still awaited in this species (Renier et al., 2010).

Two cell–cell communication systems LuxS and Agr have been shown to take part in the regulation of biofilm formation. But also as mentioned by Renier et al., 2010 several additional molecular determinants have also been identified by functional genetic analyses, such as the (p)ppGpp synthetase RelA (Taylor et al., 2002) and more recently BapL (biofilm-associated protein similar to Bap discovered by Jordan et al., 2008). Further studies on the molecular mechanisms of biofilm formation are needed.

1.2.4.2. General stress sigma factor (sigB)

Schwab et al. (2005) conducted studies that looked at the attachment of wild type *L. monocytogenes* and a sigB mutant to stainless steel. The data suggested that initial attachment of both wild type and mutant to the surface was the same; however, the number of cells of sigB mutant attached was significantly lower than the wild type after 48 or 72 h of incubation. However, van der Veen and Abee (2010a) studies showed that sigB is activated in static and continuous-flow biofilms. These authors also pointed out the implications of HrcA and Dnak genes in biofilm formation (van der Veen and Abee, 2010b).

1.2.5. Mixed biofilms

L. monocytogenes is a relatively poor biofilm former when compared to other bacterial species (Kalmokoff at al., 2001). However, monoculture biofilms are rarely found in natural environment. *Listeria* is mainly found forming mixed biofilms with other species, for example *Pseudomonas. Pseudomonas* spp. are common spoilage organism, particularly at refrigeration temperatures, and are widely distributed in foods (Jay 2003). *Listeria monocytogenes* is capable of integrating itself into EPS and biofilms formed by other bacteria (Hassan et al., 2004; Sasahara and Zottola, 1993). Rickard et al., 2003 defined coaggregations as "a process by which genetically distint bacteria become attached to one another via specific molecules". Moreover there has been increasing reports on the presence of cell-to-cell signalling among different bacteria in recent years. Microorganisms can become part of a microbial ecosystem by acting as primary colonizers, or as later biofilm partners by establishing interactions with other microorganisms (Kolenbrander 2000).

The association between different bacterial species in a biofilm increases the resistance to antimicrobials and the surface colonization by a bacterium can enhance the attachment of others to the same surface (Simões et al., 2007). It is believed that biocide misuse may have an insidious effect, contributing to the evolution and persistence of drug resistance within microbial communities. Whilst such notions are supported by laboratory studies that utilize pure cultures, recent evidence has strongly refuted such linkage within the real environment where complex, multispecies biofilms predominate and where biocidal products are routinely deployed. But the problem is even worst if we consider that this complicates the phenotypic heterogeneity through the establishment of mutualistic and antagonistic partnerships (McBain et al. 2000). As a consequence, the outcome of any antimicrobial treatment of the biofilm community will, therefore, reflect the susceptibility of the most resistant phenotype represented within the consortium. As the biofilm matures, and exopolymer deposition increases, the magnitude of the nutrient and gaseous gradients within them will increase and the net growth rate of the community will become further reduced. This has been shown to cause onset of dormancy in some cells and trigger the expression of stringent response genes (Zambrano and Kolter 1995).

The formation of mixed biofilm could also be a way to control the adherence of *Listeria monocytogenes*. In fact, Jeong and Frank (1994) found that *L. monocytogenes* grew slowly in

the presence of competing biofilms, but increased in numbers in monospecies-biofilm, even at low temperatures and nutrient levels. As a consequence, several studies have been focused in using mixed biofilms with strains that produce antilisterial metabolites to control *Listeria monocytogenes* (**see section 1.3.3.1**). Overall, results from these studies suggest that the specific resident microflora in food processing facilities play an important role in determining the likelihood of *Listeria monocytogenes* establishment and becoming persistent in the environment.

1.2.6. Persistence

Persistence has been noted for "months" by Jacquet et al. (1995); Salvat et al. (1995); Ojeniyi et al. (2000), 2 months at least; Chasseignaux et al. (2001), in poultry meat plant for 1 year and a pork meat plant for 4 months; Rørvik et al. (1995), 8 months; McLauchlin et al. (1990), 11 months; Johansson et al. (1999), 14 months; Pourshaban et al. (2000), 17 months; Lawrence and Gilmour (1995), 1 year; or even years McLauchlin et al. (1990), 2 years; Brett et al. (1998), 3 years; Unnerstad et al. (1996), 40 months; (Nesbakken et al. (1996), Aase et al. (2000) and Fonnesbech Vogel et al. (2001), 4 years; Unnerstad et al. (1996), Miettinen et al. (1999) 7 years; and Kathariou (2003) for 10 years.

The nature of strain persistence may be related to: 1) a number of factors affecting physical adaptation: surface attachment, biofilm formation, attachment strength, reduced growth rate, quiescence, cleaning and disinfection resistance, 2) the whole range of environmental conditions typical in chilled food factory environments: low temperature, wide pH range, fluctuating nutrient supply and moisture levels, frequency of cleaning and disinfection, etc., or to both. However, Jensen et al., 2007 have observed that persistent strains had the same growth rate as the presumed non-persistent strains when grown in LB with 1% glucose with or without the addition of 5% NaCl at both 5 °C and 37 °C. A persistent subtype was as sensitive to the processing steps in cold-smoked salmon production including brine and smoke as compared to two other strains of *L. monocytogenes,* a clinical strain and the EGD strain (Porsby et al., 2008). The persistent strains did not show higher tolerance to acid and heat stresses as compared to other strains of *L. monocytogenes* (Lundén et al., 2008)

Several studies aimed to correlate the persistence of *Listeria monocytogenes* with the resistance to biocides, the level of initial adherence or the capacity to form biofilms were carried out. Obtained results have been , however, contradictory. Some authors demonstrated

that persistent *L. monocytogenes* strains may show enhanced surface adherence and increased disinfection resistance at very low disinfectant concentrations (Gilbert et al., 2002; Lundén et al., 2000, 2003). According to Kasthberg and Gram (2009), persistent strains of *L. monocytogenes* are as susceptible to disinfectants as are presumed nonpersistent strains and attachment does not render the strains more tolerant to disinfectants. Others found no evidence of resistance to in-use concentration of disinfectants in persistant strains (Holah et al., 2002).

Concerning the possible correlation between persistence and initial adherence, Lundén et al. (2000) revealed that persistent *L. monocytogenes* strains showed enhanced adherence at short contact times (less than 72 h), promoting their survival in food processing facilities and possibly having an effect on initiation of persistent plant contamination

One potential cause of confusion is the different opinion about the definition of persistence (Borucki et al., 2003). It is difficult to prove that a persistent strain survives within a factory during continuous production/cleaning cycles and has not merely entered the production area on the day of sampling (Holah et al., 2004). Norwood and Gilmour 1999, denote a subtype as persistent if it has been isolated repeatedly from a product from the same factory. However, it should be taking into account that bacteria are able to switch between a free-living and a surface attached in response to changing environmental conditions (O'Toole, 2004).

1.3. INCIDENCE AND CONTROL OF *L. MONOCYTOGENES*

1.3.1. Incidence of *L. monocytogenes* in foods and food processing plants

1.3.1.1. A brief overview of L. monocytogenes outbreaks

Food-borne transmission is considered the most common means of contracting both epidemic and sporadic listeriosis, with 99% of all human cases caused by consumption of contaminated food products. Although the incidence of listeriosis is low relative to other pathogens, such as *Salmonella* and *Campylobacter*, the mortality associated with outbreaks is high, making *L. monocytogenes* one of the most significant pathogens encountered in foods (EFSA 2009, Ivanek et al., 2004). In **Table 1.3.1** the main known listeriosis outbreaks during the last 20 years have been recopilated.

Table 1.3.1: Outbreaks of human food-borne listeriosis

COUNTRY	YEAR	FOOD VEHICLE	Number of cases					Serovar	Reference
			Total	Pregnant	Non-pregnant	With underlying disease	Death		
USA	1976	Raw salad	20	0	20	10	5	4b	McLaughlin et al., 2004
New Zealand	1980	Shell or raw fish	22	22	0	0	7	1/2a	McLaughlin et al., 2004
Canada	1981	Coleslaw	41	34	7	0	18	4b	McLaughlin et al., 2004
USA	1983	Pasteurised whole milk and 2% milk	49	7	42	42	14	4b	McLaughlin et al., 2004
USA	1985	Mexican-style soft cheese made from unpasteurised milk	142	93	49	48	30	4b	McLaughlin et al., 2004
Switzerland	1983-1987	Soft cheese	122	65	57	24	34	4b	McLaughlin et al., 2004
UK	1987–1989	Pate	355[b]	185	129	NK	94	4b and 4 not 4b	McLaughlin et al., 2004
USA	1989	Shrimps	2	NK[a]	NK	NK	NK	4b	McLaughlin et al., 2004
Australia	1990	Pate	9	NK	NK	NK	NK	1/2a	McLaughlin et al., 2004
Australia 1991	1991	Smoked mussels	4	0	4	0	0	1/2a	McLaughlin et al., 2004
New Zealand	1992	Smoked mussels	4	2	2	2	2	1/2a	McLaughlin et al., 2004
France	1993	Pork tongue in aspic	279	NK	NK	NK	NK	4b	McLaughlin et al., 2004
France	1993	Pork rillettes	38	31	7	NK	10	4b	McLaughlin et al., 2004
USA	1994	Commercially pasteurised chocolate milk	445	1	44	1	0	1/2b	McLaughlin et al., 2004
Sweden	1994-1995	Cold-smoked rainbow trout	9	3	6	NK	2	4b	McLaughlin et al., 2004
France	1995	Soft cheese	17	11	9	5	4	4b	McLaughlin et al., 2004
Italy	1997	Sweetcorn salad	1566	0		1566	0	4b	McLaughlin et al., 2004
Canada	1966	Crab meat	2	0	2	0	0	**1/2a**	McLaughlin et al., 2004
USA	1998–1999	Hot dogs and delicatessen meats	50	NK	NK	NK	>8	4b	McLaughlin et al., 2004
Finland	1998-1999	Butter	25	0	25	24	6	3a	McLaughlin et al., 2004
Finland	1999	Cold-smoked trout	5	0	5	NK	NK	1/2a	McLaughlin et al., 2004
England	1999	Cheese and cheese and salad sandwich	2	0	2	2	1	4b	McLaughlin et al., 2004
France	1999–2000	Pork rillettes	10	3	7	6	2	4b	McLaughlin et al., 2004
USA	2000	Turkey meat	29	8	21	NK	7	NK	McLaughlin et al., 2004

France	1999–2000	Pork tongue in jelly	32	9	23	11	10	4b	McLaughlin et al., 2004
USA	2000–2001	Mexican-style soft cheesemade from unpasteurised milk	12	10	2	1	5	NK	McLaughlin et al., 2004
Japan	2001	Cheese	38	NK	NK	NK	NK	1/2b	Makino et al., 2001
UK	2003	Sandwiches (hospital ham salad and tuna salad)	7	5	2	2	2	1/2 (almost 2 cases1/2a)	Dawson et al., 2006
Switzerland	2005	cheese	10	2	8	8	3	1/2a	Bille et al., 2006
Czech Republic	2006	mature cheeses and mixed salads	75	11	64	NK	12	1/2b	Vit et al.,2006
Germany	2006-2007	Cheese (acid curd) made from pasteurized milk	189	11	178	117	26	4b mainly	Koch et al., 2010
Massachusetts	2007	Pasteuriced milk	5	2	3	2	3		CDC 2007
Norway (Oslo)	2007	Camembert cheese	17	3	14	16	3	Serogroup I	Johnsen et al., 2010
Canada	2008	Ready-to-eat meat products	57	NK	NK	NK	22	1/2a	http://www.biomedcentral.com/1471-2164/11/120
Denmark	2009	Beef meat from a meals-on-wheels delivery	8	0	0	8	2	NK	Smith et al., 2009
Austria-Germany	2009	'Quargel' cheese	34	0	34	4	8	1/2a	Fretz et al., 2010

[a] not known

[b]Information was not available to classify 41 patients

Additionally, illness caused due to the consumption of contaminated foods has a wide economic and public health impact worldwide. The Centers for Disease Control and Prevention (CDC) estimate that foodborne diseases are responsible for about 76 million illnesses, which result in 325,000 hospitalizations and 5000 deaths in the United States each year (Mead et al., 1999). Also, the food industry can have major expenses due to product contamination with *L. monocytogenes* as in cases with recall of products, factory closing, extraordinary cleanings, and compensation to infected people. This was seen lately in a large outbreak of *L. monocytogenes* in Canada in 2008 resulting in 57 cases with 22 deaths (Anonymous, 2009a). The outbreak was caused by meat products from a company that itself estimates their costs to be around $43 million for recalls, lost sales and compensations to claimants (Anonymous, 2009b).

1.3.1.2. Incidence of L. monocytogenes in food processing plants

Several studies have confirmed the ability of *L. monocytogenes* to colonize food processing factories (Harvey and Gilmour, 1992). Contamination of the final food products occurs mainly during processing rather than from the raw material (Cox et al., 1989; Hu et al., 2006; Samelis and Metaxopoulos, 1999, Autio et al., 1999, Miettinen et al., 1999; Norton et al., 2001; Rørvik et al., 1995; Vogel et al., 2001, Wulff et al., 2006). Typing methods have been used to point out equipment, floors and drains as important contamination sources in the processing lines (Hoffmann et al., 2003; Miettinen et al., 1999; Norton et al., 2001; Keto-Timonen et al., 2007) and to demonstrate that specific sub-types are able to persist in the food processing environment for up to 10 years (Norton et al., 2001, Wulff et al., 2006). Moreover, from recopilated data during surveys carried out in 2006 and 2007 it can be deduced that the percentage of units in non-compliance with the EU *Listeria* criteria was considerably higher in samples obtained from food processing than in those obtained from retails (**Figure 1.3.1**).

This pathogen represents a problem specifically in dairies, smoked fish houses and RTE meat processing plants where, according to a research carried out by Kornacki and Gulter in 2007, a high prevalence has been detected within processing facilities in North America and Europe. The DNA types found in the final products were also found in many samples from the processing environment (Autio et al., 1999; Norton et al., 2001; Vogel et al., 2001; Wulff et al., 2006). Also, the frequency of positive samples in a fish processing industry was lower for fish before filleting compared to samples of fish after filleting and further in the production process (Rørvik et al., 1995).

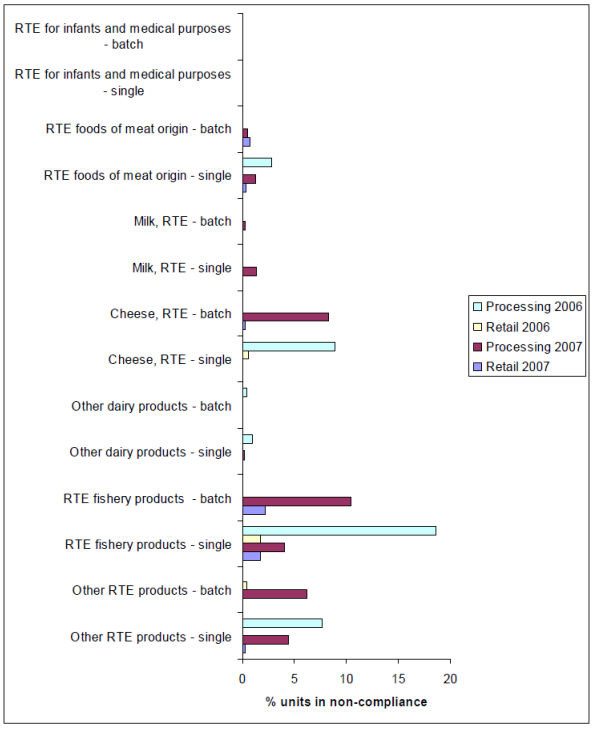

Figure 1.3.1: Proportion of samples in non-compliance with the EU Listeria criteria, 2007. Samples are based on single and batch data from retail, including sample units >=25. Excluding HACPP and own check samples. RTE for infants and medical purposes also include food for special nutritional uses. Taken from EFSA 2009.

1.3.1.3. Incidence of L. monocytogenes in foods

After several *L. monocytogenes* surveys, it is now generally accepted that the highest incidence of *L. monocytogenes* ocurrs in RTE foods. In USA, the prevalence of *L. monocytogenes* in RTE foods was generally determined to be 1.82% in 31,705 tested samples. The highest rate of positive samples were from seafood salads (4.7%) and smoked seafood (4.3%) (Gombas et al., 2003). In the EU, *Listeria* monocytogenes was seldom detected above the legal (Gombas et al. 2003; Wallace et al. 2003) safety limit (contamination level of <10 cfu/g) from RTE foods but findings over this limit were most often found in smoked fish and other ready-to-eat fishery products followed by ready to eat meat products and cheeses (Anonymous, 2009a). In fact, fishery products had the highest proportion of samples

exceeding 100 cfu/g (2.4%) (EFSA, 2009). Incidence data of *L. monocytogenes* in ready-to eat fishery products in different EU countries are showed in **Table 1.3.2.**

Table : 1.3.2: *L. monocytogenes* in ready-to-eat fishery products, 2007

Country	Sampling	Details	N	%POS	N	%POS	%POS
	unit		Units tested presence	*L. monocytogenes* present in 25 mg	Units Tested Enumeration	> detection but =< 100 cfu/g	*L. m.* > 100 cfu/g
Fish							
Belgium	Single	Smoked salmon, at retail	-	-	150	1.3	1.3
Bulgaria	Single	Smoked, at processing	130	1.5	-	-	-
Czech Rep.	Batch	Smoked, at processing	240	13.8	80	12.5	18.8
	Batch	Smoked, at retail	-	-	68	5.9	5.9
Germany	Single	Smoked, at processing	172	9.3	185	1.2	0.6
	Single	Smoked, at retail	447	11.4	622	1.6	1.6
Italy	Single	Smoked,	131	14.5	-	-	-
	Batch	Smoked,	-	-	41	2.4	0
Netherlands	Single	Smoked, at retail	709	22.6	820	4.3	4.6
Poland	Single	Marinated	51	0	-	-	-
	Single	Smoked,	676	29.6	1.098	4.6	0.5
Portugal	Batch	Smoked, at retail	-	-	35	0	0
Romania	Batch	Smoked, at processing	73	0	-	-	-
Slovakia	Batch	Smoked	-	-	90	0	2.2
Total (fish) (10 MSs)			**2.629**	**18.3**	**3.169**	**3.6**	**2.4**
Crustaceans							
Bulgaria	Single	RTE, at processing, cooked	150	0	-	-	-
Germany	Single	RTE, at processing, cooked	210	2.4	241	0.4	0.4
Molluscan shellfish							
Greece	Single	Cooked, at retail	27	3.7	-	-	-
Fishery products, unspecified							
Austria	Single	At retail	166	6.6	166	6.6	0
	Single	-	26	3.8	26	3.8	0
Estonia	Single	RTE, at processing	77	2.6	-	-	-
Germany	Single	At retail	1.008	14.7	779	1.4	1.3
Ireland	Single	At retail	-	-	97	0	0
	Single	At processing	35	0	-	-	-
	Single	Smoked, at retail	32	9.4	52	0	1.9
	Single	Cooked at retail	228	2.2	298	0	0
Slovakia	Batch	-	105	1.9	116	0	0
Spain	Single	RTE	653	5.2	-	-	-
Total (6 MSs)			**2.328**	**2.5**	**1.534**	**1.6**	**0.8**
Norway	Single	Smoked, at processing	70	0	-	-	-

Note: Data are only presented for sample size ≥25
% POS: % positive samples

In contrast with this high incidence, only a few cases of listeriosis were reported from consumption of fish products and they involved a limited number of patients (Brett et al. 1998; Farber et al. 2000). Although *L. monocytogenes* was firstly isolated from imported cooked crabmeat in 1987, the first of several cases of listeriosis positively linked to consumption of fish or seafood was not reported until 1989. Further, some listeriosis outbreaks have been related to the consumption of steamed mussels, shrimp, fish salads and semicooked fish (Ben Embarek, 1994, Brett et al., 1998), cold-smoked rainbow trout (Miettinen et al. 1999) and salmon (Tham et al., 2000).

The frequency with which *L. monocytogenes* can be found during environmental surveys has raised doubts that the food processing industry can effectively eliminate *L. monocytogenes* contamination. In this sense, some authors have hypothesized that three recent anthropogenic practices increase the load within and transmission among reviewed habitats and host populations: extended refrigerated storage of ready-to-eat foods allowing *L. monocytogenes* growth in foods that are contaminated during production or subsequent handling; feeding domestic ruminants with silage often contaminated with *L. monocytogenes*; and dispersal of contaminated products of sewage treatment to agricultural fields and waters (Ivanek et al., 2006). In this frame, prevention relies on progressively better defined tolerance limits in foods and improved risk communication, focused on risk groups. Risk assessment is increasingly used as a scientific process to assess the potencial for adverse health effects to occur and as a basis for management of unacceptable risks (Notermans and Hoornstra, 2000).

1.3.2. Control of *L. monocytogenes* in food processing plants

1.3.2.1. Prerrequisites and Hazard Analysis Critical Control Point (HACCP): importance for food safety

HACCP is an effective and rational means of assuring food safety from harvest to consumption. Preventing problems from occurring is the paramount goal underlying any HACCP system. To meet this goal, seven basic principles are used in developing HACCP plans. These include hazard analysis, Critical Control Point identification, establishing critical limits, monitoring procedures, corrective actions, verification procedures, and record keeping and documentation. Under such systems, if a deviation occurs indicating that control has been

lost, the deviation is detected and appropriate steps are taken to re-establish control in a timely manner to assure that potentially hazardous products do not reach the consumer. But for a HACCP to be succesful some prerequisites programs must be developed and implemented

These programs provide the basic environment and operating conditions that are necessary for the production of safe, wholesome food. Many of the conditions and practices are specified in federal, state and local regulations and guidelines. All prerequisite programs should be documented and regularly audited, and are established and maintained separately from the HACCP plan.

Prerequisites are aimed to prevent contamination of food-products with foodborne pathogens such us *L. monocytogenes* during the manufacturing processing are importat (Kornacki and Gulter, 2007). Thus:

i. The raw material must be of good microbiological quality.
ii. The employees must have a thorough understanding of food hygiene: Holah et al. (2004) showed that although food factory designs, personnel hygiene and cleaning and disinfection regimes are sufficient to control *Listeria* spp. to very low levels, persistent strains can remain within factory high-risk production areas over considerable time periods. Although hygienic conditions of food production in industrially developed countries are continually improving, outbreaks of listeriosis originating from consumption of contaminated food products still occur, ensuring effective control of *L. monocytogenes* has proved to be challenging.
iii. The factory and equipment should be designed so they are easily cleaned to avoid cross-contamination: biofilms may also cause technical problems such as total or partial occlusion of pipes, outlets, valves, drains, filters, filtration membranes and may coat the surfaces of heat exchangers and diverse sensors. Particularly vulnerable are poorly accessible zones, pieces of equipment that are difficult to dismantle, irregular or scratched surfaces and sites where routine cleaning fails to prevent accumulation of organic material. Mechanical aids (high pressure jets, brushing, scrubbing) may be used to supplement cleaning and disinfection chemical treatments, but manual labour is costly and the hygienic results have a low reproducibility. Identification and quantification of biofilm resident

microorganisms and development of specific sanitation practices are increasing concerns among food processors in the last few years (Belessi et al., 2010; Sofos and Geornaras, 2010)

Once these and others prerrequisites are controlled, critical points in the process where contamination can occur should be identified and controlled. Rocourt and Cossart (1997) proposed that *L. monocytogenes* enters processing plants through soil on workers' shoes, clothing, and transport equipment; animals that excrete the bacterium or have contaminated hides or surfaces; raw plant tissue; raw food of animal origin; and, possibly, healthy human carriers. However, the widespread presence of *L. monocytogenes* in natural reservoirs and its ability to grow at harsh conditions make its control in foods difficult (Bonnet and Montville 2005).

1.3.2.2. The problem of cross contamination

Cross-contamination can also occur after listeriocidal treatment (Reij and Aantrekker Den, 2004; Tompkin, 2002) and generally represents post-processing contamination from environmental sources, including in food processing plants, retail operations, and household kitchens (Soumet et al., 2005). Foodborne pathogens can remain viable on common food contact surfaces for days or weeks and cross-contaminate other products (Lappi et al., 2004; Lunden et al., 2000; Nesbakken et al., 1996).

There are numerous studies about the cross-contamination when food is sliced with blades. Keskinen et al. (2008) study the stress and biofilm-forming ability on transfer of surface-dried *Listeria monocytogenes* during slicing of delicatessen meat. They obtained significantly greater transfer for blades used after 6 as opposed to 24 h of incubation. Also, Pappelbaum et al. (2008) monitored the prevalence of *Listeria* spp. in frozen vegetables and environmental samples (swabs) from a large produce processing factory. They conclude that the lack of personnel hygiene was supporting the cross-contamination cycle in the plant. Another possible source of cross-contamination could have been aerosol generation through hosing under high pressure or in the cooling units. In fact protective barriers between the processing lines were limited.

In this frame, it is evident that cleaning and disinfection protocols must be effective: a key point in preventing contamination is the control of hygiene at the food processing plant level, and this is primarily obtained by efficient cleaning and especially disinfection, which is regarded as a critical step in eliminating spoilage and pathogenic bacteria in the food processing environment. The mechanisms by which *L. monocytogenes* survive under these harsh conditions of physical and chemical stresses are not fully understood (Harvey et al., 2007). It has been suggested that biofilm formation by *L. monocytogenes* have highlighted the need for additional cleaning and disinfection strategies to control persistent *L. monocytogenes* strains in the food processing environment (Holah et al. 2004).

1.3.2.3. Disinfectants

1.3.2.3.1. Classical disinfectants

There are a number of prerequisites for an efficient disinfection. Firstly, the disinfectant must have the right spectrum of activity and be able to eliminate the relevant contaminants in the production site. Generally, disinfectants have a very broad spectrum of targets, since they are efficient against bacteria, viruses and fungi. However, Gram-negative bacteria tend to be less susceptible than Gram-positive bacteria (McDonnell and Russell, 1999). Secondly, it is important to use the right concentration, pH, temperature, and exposure time to obtain sufficient elimination of bacteria. Finally, cleaning of the surface prior to disinfection is necessary to remove organic compounds. Otherwise, the disinfection will be useless. A wide range of chemical disinfectants are available for the food-industry, and they can be divided into the following seven groups (Asselt and Giffel, 2005): alcohols, aldehydes, biguanides, (bis)-phenols, halogen-releasing agents (HRA), peroxygens and quaternary ammonium compounds (QACs). The most well-known disinfectant agents of each group, including their applications and restrictions in use, are summarized in **Table 1.3.3**. Generally, disinfectants act on multiple targets on a specific cell, which make these compounds highly active against microorganisms, but also potentially harmful to humans. Bactericidal activity of a disinfectant is well-known, but the mechanism of action is rarely fully understood (McDonnell and Russell, 1999).

Table 1.3.3: Biocide groups applications and restrictions in use.

Group	Important types	Application	Action on bacteria	Comments
Alcohols	Ethanol	Small spots.	Membrane damage and rapid denaturation of proteins. Interference with metabolism. Cell lysis	Not for large industrial application due to need of high concentrations (60-90%). Flammable.
	Isopropanol	Quick wipe-downs.		
Aldehydes	Formaldehyde	Decontaminate romos.	Extremely reactive. Reacts un-specifically with functional groups of proteins	Used less frequently in food production and processing due to toxicological considerations.
	Glutaraldehyde	Instrument. Disinfectant.	Strong membrane association. Reacts unspecifically with functional groups of proteins. Inhibition of transport	
Biguanides	Polymeric biguanides	Used in particular by the food industry	Membrane active. Damage of intracellular membrane. Leakage of intracellular components.	Polyhexamethylene biguanides (PHMB) is a superior biocide due to lack of toxicology, colour, taste and surfactancy**.**
	Alexidine			
	Chlorhexidine	Most widely applied biocide in hand-washing and oral products.		
Bis-phenols	Triclosan	Soap, toothpaste, packing material, conveyer belts.	Membrane-active Inhibits a specific enzyme in lipid biosynthesis	The action at a specific target increases the risk of resistance.
Halogen-releasing agents	Hypochlorite	Frequently applied disinfectant in food industry.	Highly oxidizing agents Irreversible change and disruption of DNAprotein synthesis.	It is cheap.
	Iodine		Penetrates bacteria and attacks keygroups of proteins.	Expensive. Staining of skin and plastic parts.
Peroxygens	Peracetic acid	Frequently applied disinfectant in the food industry.		Mixture of water, hydrogen peroxide and acetic acid. Less sensitive to organic loads.
	Hydrogen peroxide		Oxidation of essential cell components as lipids, proteins and DNA**.**	Corrosive on some materials.
Quaternary ammonium compounds		Frequently applied disinfectant in the food industry.	Absorption to cell wall Perturbation of lipid bilayer. Cytoplasmic protein aggregation. Leakage of cytoplasmic materials.	More expensive than chlorine, but have residual action. Non-corrosive and non-tainting monocationic agents.

Chlorine-based compounds, peroxygens, and compounds based on quaternary ammonium compounds (QAC) are the most frequently applied disinfectants in the food industry. Each of these three groups have some advantages and disadvantages (**Table 1.3.4**), and therefore the choice of the disinfectant to be used depends on the production site.

Table 1.3.4: Advantages and disadvantages of some chemical disinfectants widely used in the food industry.

Disinfectant	Advantages	Disadvantages
Chlorine compounds	Cheap and effective. -Kills most types of microorganisms. -Less affected by hard water than some. -Does not form films. -Effective at low temperatures. -Relatively inexpensive. -Concentration easily determined by test strips.	-Inactivated in presence of organic material. -May corrode metals and weakenrubber. -Irritating to skin, eyes and throat. -Unstable, dissipates quickly. -Liquid chlorine loses strength in storage. -pH sensitive.
Iodophors	-Kills most types of microorganisms. -Less affected by organic matter than some. -Less pH sensitive than chlorine. -Concentration determined by test strips. -Solution colour indicates active sanitiser.	-May stain plastics and porous materials. -Inactivated above 50°C. -Reduced effectiveness at alkaline pH. -More expensive than hypochlorites. -May be unsuitable for CIP1 due to foaming.
Peroxy compounds	-Effectiveness. -Best against bacteria in biofilms. -Kills most types of microorganisms. -Relatively stable in use. -Effective at low temperatures. -Meets most discharge requirements. -Low foaming; suitable for CIP.	-Inactivated in presence of organic material. -More expensive than some. -Inactivated by some metals/organics. -May corrode some metals. -Not as effective as some against yeasts and moulds.
QAC	-Non corrosive -Less affected by organic matter than some -Residual antimicrobial activity if not rinsed -Can be applied as foam for visual control -Effective against *Listeria monocytogenes* -Effective for odour control -Concentration determined by test strips	-Expensive. -Inactivated by most detergents. -May be ineffective against certain organisms. -May be inactivated by hard water. -Effectiveness varies with formulation. -Not as effective at low temp. as some. -May be unsuitable for CIP due to foaming.
Acid-Anionic	- Sanitize and acid rinse in one step - Very stable - Less affected by organic matter than some - Can be applied at high temperature - Not affected by hard water	-Effectiveness varies with microorganism. -More expensive than some. -pH sensitive (use below pH 3.0). -Corrode some metals. -May be unsuitable for CIP due to foaming.
Carboxylic Acid	-Kills most types of bacteria -Sanitize and acid rinse in one step -Low foaming, suitable for CIP -Stable in presence of organic matter -Less affected by hard water than some	-Inactivated by some detergents. -pH sensitive (use below pH 3.5). -Less effective than chlorine at low temp. -May damage non-stainless steel materials. -Less effective against yeasts and moulds than some.
Chlorine Dioxide	-Kills most type of microorganisms -Stronger oxidiser (sanitizer) than chlorine -Less affected by organic matter than some -Less corrosive than chlorine -Less pH sensitive than some	-Unstable and cannot be stored. -Potentially explosive and toxic. -Relatively high initial equipment cost.
Ozone	-Kills most type of microorganisms -Stronger oxidiser (sanitizer) than chlorine and chlorine dioxide	- Unstable and cannot be stored. -May corrode metals and weaken rubber. -Potentially toxic. -Inactivated by organic matter (similar to chlorine).
Hot Water / Heated Solutions	-Kills most types of microorganisms -Penetrates irregular surfaces -Suitable for CIP -Relatively inexpensive	-May form films or scale on equipment. -Burn hazard. -Contact time sensitive.

1.3.2.3.2. Other compounds with potencial application in cleaning and disinfection protocols

Enzymes

A promising strategy may be the use of enzymes that can dissolve the biofilm matrix (e.g. DNase and alginate lyase) as well as quorum-sensing inhibitors that increase biofilm susceptibility to antibiotics (Høiby et al. 2010). Several works have shown the potential applicability of enzymes against biofilms: Gamble and Muriana (2007) tested three enzyme preparations against single-species biofilms of 6 "strongly adherent" strains previously isolated from meat and meat processing plants. They found that protease, lipase, and cellulase were effective in releasing adherent cells from microplates. Longhi et al., (2008) examined the effects of a protease treatment on the ability of *L. monocytogenes* to form biofilms and to invade tissues. They have chosen serratiopeptidase (SPEP), an extracellular metalloprotease produced by *Serratia marcescens.* Treatment of *L. monocytogenes* with sublethal concentrations of SPEP reduced their ability to form biofilms and to invade host cells. Zymograms of the treated cells revealed that Ami4b autolysin, internalin B, and ActA were sharply reduced. These cell-surface proteins are known to function as ligands in the interaction between these bacteria and the host cells, and their data suggest that treatment with this natural enzyme may provide a useful tool in the prevention of the initial adhesion of *L. monocytogenes* to the human gut. More recently, Harmsen et al. (2010) have shown that DNase I treatment resulted in dispersal of biofilms, not only in microtiter tray assays but also in flow cell biofilm assays.

Essential oils

In vitro and *in vivo* studies have demonstrated that essential oils act as antioxidants and show antibacterial activities (Viuda-Martos 2010). Considering the large number of chemical substances that make up essential oils (EOs), it is most probable that their antibacterial activity cannot be explained by a single specific mechanism but rather by several different mechanisms. Nevertheless, phenolic compounds are generally considered to be responsible for the antibacterial properties of EOs. Several studies that have demonstrated the effectiveness of EOs, alone or combined with other antimicrobials, as antilisterial agents in foods are recopilated in **Table 1.3.5.**

Table 1.3.5: Some examples of sucessful applications of essential oils (EOs) as antilisterial agents in foods

Essential oil	Food	Reference
Thyme´s (0.08%)	Minced pork	Aureli et al., 1992
Rosemary (0.3%) and encapsulated rosemary oil (5%)	Pork liver sausage	Pandit and Shelef 1994
"DMC base natural" preservative comprising 50% Eos of rosemary, sage and citrus.	Soft cheese	Mendoza-Yepes et al., 1997
Eugenol	Cooked chicken breast, pieces	Hao et al., 1998a
Eugeniol	Roast beef sirloin, sliced	Hao et al., 1998b
Pelargonium oil	Quiche	Lis-Balchin et al., 1998
Mint oil (Mentha piperita)	Tzatziki (yogurt and cucumber salad pH 4.5), taramosalata (cod´s roe salad pH 5.0) and pâté (pH 6.8)	Tassou et al., 1995
Oregano	Packed meat	Tsigarida et al., 2000
Clove oil	Mozzarella cheese	Vrinda et al., 2001
Carvacrol	Semi skimed milk	Karatzas et al., 2001
Cilantro oil (6%)	Vacuum packed ham	Gill et al., 2002
Oils and vapours of lemon (Citrus limon), sweet orange (Citrus sinensis) and bergamot (Citrus bergamia)	Chicken skin	Fisher and Philips 2006
Oregano or savory	Pork meat	Ghalfi et al., 2007
Spanish oregano (O; Corydothymus capitatus), Chinese cinnamon (C; Cinnamomum cassia), or winter savory (S; Satureja montana)	Bologna and ham	Oussalah et al., 2007
Carvacrol	Steak tartare	Veldhuizen et al., 2007
Thyme	Minced beef during refrigerated storage	Solomako et al., 2008
Oregano	Barbecued chicken	Firouzi et al., 2007
Clove and cinnamon	Pasteurized milk	Cava et al., 2007
Cinnamon, bark	Melon and waterlemon juices	Mosqueda-Melgar et al., 2008
Basil, lemon balm, marjoram, oregano, rosemary, sage and thyme	Model media included potato starch (0, 1,5 or 10%), beef extract (1.5, 3, 6 or 12%), sunflower oil (0, 1, 5 or 10%).	Gutierrez et al., 2008
Satureja montana	Minced pork	Carramiñana et l., 2008
Basil, caraway, fennel, lemon balm, marjoram, nutmeg, oregano, parsley, rosemary, sage, and thyme	Ready-to-eat vegetables	Gutierrez et al., 2008
Citron	Fruit-based salads	Belletti et al., 2008
Lemon balm, marjoram, oregano and thyme	Model media based on lettuce, meat and milk	Gutierrez et al., 2009
Allspice, garlic, and oregano	tomato films	Du et al., 2009
Garlic, bay, black pepper, origanum, orange, thyme, tea tree, mint, clove, and cumin	Apple-carrot juice	Irkin and Korukluoglu, 2009
Oregano and rosemary	Fresh-cut vegetables	Scollard et al., 2009
Thyme verbena, thyme red, Spanish oregano, ajowan, tea tree, clove, and sage oils tested at 1%, as well as with 2% rosemary oil	Ready-to-eat salad	Cobo Molinos et al., 2009
Clove (Syzygium aromaticum L.), fennel (Foeniculum vulgare Miller), cypress (Cupressus sempervirens L.), lavender (Lavandula angustifolia), thyme (Thymus vulgaris L.), herb-of-the-cross (Verbena officinalis L.), pine (Pinus sylvestris) and rosemary (Rosmarinus officinalis)	Films for fish preservation	Gómez-Estaca et al., 2010

There are few studies on the effects of essential oils in biofilms (Chorianopoulus et al., 2008; Oliveira et al., 2010; Sandasi et al., 2008; Sandasi et al., 2010). Sandasi et al., 2010 studied the *in vitro* antibiofilm activity of selected culinary herbs and medicinal plants against *L. monocytogenes* biofilms. The majority of extracts tested prevented cells adhesion to polyvinyl chloride (PVC) surface. In contrast, inhibition of preformed biofilms (during 4

hours) was more difficult to achieve, with only three extracts (*Rosemarinus officialis*, *Mentha piperita* and *Melaleuca alternifolia*) inhibiting the growth of both strains by at least 50%. Previously, Sandasi et al., 2008 showed that some essential oils at a concentration of 1 mg/ml components promote the growth and development of a preformed *L. monocytogenes* biofilm (6 h) *in vitro*. However, Chorianopoulus et al., 2008 observed that the essential oil of *S. thymbra* (1%), as well as its hydrosol fraction (100%), presents sufficient bactericidal effect on bacterial biofilms formed on stainless steel after 5 days. And also, Oliveira et al., 2010 studied the effect of disinfectant and essential oils (*Cymbopogon citrtus Stapf.* and *Cymbopogon nardus*) in different phases of biofilm formation (3 hours of biofim formation) of *Listeria monocytogenes* on stainless steel surface and found that essential oils have effectively reduced the number of surface-adhered cells, especially after 60 min of contact.

Biosurfactants

A few studies have confirmed the effectiveness of biosurfactants produced by gram-negative (*Pseudomonas fluorescens*) and Gram-positive (*Lactobacillus helveticus*) bacteria against the adhesion of *Listeria monocytogenes* on stainless steel (Meylheuc et al., 2001 and 2006). Zeraik and Nitschke, 2010 studied the adhesion of different pathogenic bacteria (*Staphylococcus aureus*, *Listeria monocytogenes*, and *Micrococcus luteus*) to polystyrene surfaces previously condicioned with surfactin and rhamnolipid , whereas Nitschke et al. (2009) to stainless steel and polypropylene previously adsorbed with surfactin.

Bacteriocins

The effectiveness of nisin and pediocin against planctonic *L. monocytogenes* cells has been demonstrated in numerous studies (Nisin: Scannell et al., 2000; Szabo, and Cahill. 1999; Harris et al., 1991; Coma et al., 2001; Nilsson et al., 2000; Benkerroum and Sandine. 1988; Fang and Lin. 1994, Zhang and Mustapha. 1999. Pediocin: Degnan et al., 1993, Goff et al., 1996; Liao et al., 1993; Scannell et al., 2000). In fact, biocontrol of *L. monocytogenes* in refrigerated storage through co-culture with bacteriocin-producing lactic acid bacteria has been proposed by several authors (Yousef et al., 1991.). However, both nisin-producing lactococci and pediocin producing pediococci are hardly effective because they do not grow well at refrigeration temperatures. The psychrotrophs *C. piscicola* L103 (Schobitz et al., 1999), *Leuconostoc carnosum* 4010 (Budde et al., 2003), and several strains of *Lb. bavaricus* (Winkowski et al., 1993) were proposed as alternative bacteriocin producers to prevent

Listeria to grow at refrigeration temperature. Growth of the pathogen may also be retarded in ready-to-eat, fresh-cut vegetables inoculated with *Lb. delbrueckii* (Harp and Gilliland. 2003).

On the contrary, studies about the effectiveness of bacteriocins against *L. monocytogenes* biofilms are really scarce. Only three were found in the bibliography: Leriche et al., 1999; Minei et al., 2008 and Guerrieri et al., 2008. The first one studied *L. monocytogenes* biofilm growth in association with a nisin-producing strain of *Lactococcus lactis*. Both when *L. monocytogenes* was combined with *Lc. lactis* and inoculated onto stainless steel coupons or was inoculated onto a preexisting *Lc. lactis* biofilm, the antilisterial activity of the biocontrol was effective. Minei et al., 2008 studied the influence of nisin (3 h) on *L. monocytogenes* biofilms and observed that although treatment decreased the number of cells 4.6 log CFU/cm^2, a renewed biofilm was detected after 24 h of incubation. However, coculture with a bacteriocin produced by *E. faecium* reduced efficiently the biofilm formation. Guerrieri et al., 2008 studied the use of lactic acid bacteria (LAB) biofilms bacteriocin producers (*Lactobacillus plantarum* 35d, *Enterococcus casseliflavus* IM 416K1) and by two non-producers (*L. plantarum* 396/1, *Enterococcus faecalis* JH2-2) for the control of *Listeria monocytogenes* in a small-scale model. They found the bacteriocin producers showed the best antilisterial potentiality. Comparing the antilisterial activity of LAB biofilm against both planktonic and adherent cells, the *L. monocytogenes* adherent cells showed a higher resistance. And also in the studies performed with *L. monocytogenes* in co-culture with a *Pseudomonas putida* strain it is produced a reduction of the antilisterial activity in the lactobacilli biofilms added with *P. putida*, probably due to the pH increase and to a better survival of *Listeria* in the presence of *P. putida*.

More recently, it has been showed that the presence of model resident *Lactococcus lactis* biofilms reduced the initial adherence of *L. monocytogenes*. Moreover, significant differences were seen in *L. monocytogenes* settlement as a function of the genetic background of resident lactococcal biofilm cells (Habimana et al. 2009).

1.3.2.4. Resistance of L. monocytogenes biofilms to disinfectants

It is generally accepted that biofilm formation implies an increase in the resistante to biocides. (Aarnisalo et al., 2000; Aarnisalo et al., 2007; Amalaradjou et al., 2009; Ammor et al., 2004; Blackmann and Frank, 1996, Bremer et al., 2002, Chavant et al., 2003; Gram et al.,

2007, Kastbjerg and Gram, 2009; Holah et al., 2002, Leriche et al., 1999; Minei et al., 2008, Frank et al., 2003; 48 h: Pan et al., 2006, Yang et al., 2009).

According with Costerton et al. (1999) and Donlan and Costerton (2002), some mechanisms responsible for that resistance may be one or more of the following:

i. A delayed penetration of the antimicrobial agent through the biofilm due to the polymeric substances that make up the matrix of a biofilm (Ammor et al., 2004; Bourion and Cerf 1996; Davies, 2003; Fatemi and Frank, 1999; Gilbert et al, 2002,; Pan et al., 2006). Extracellular polymeric substances surrounding the cells may hinder diffusion. Additionally, some authours have also pointed out that the abiotic part of the biofilm plays a part in resistance because of its potent neutralization effect on many compounds (Campanac et al. 2002).
ii. An altered growth rate of biofilms (Chavant et al., 2004, Fah and O´Toole).
iii. Other physiological changes due to the biofilm mode of growth. This suppose a more speculative hypothese than the preceding one, in which at least some of the cells in a biofilm adopt a distinct and protected biofilm phenotype (Chavant et al., 2004; Fah and O´Toole; Davies, 2003).
iv. Formation of multispecies biofilms: *L. monocytogenes* is more resistant when grown in multi-species biofilm than when grown as monospecies biofilm (Ammor et al., 2004; Fatemi and Frank 1999; Saá Ibusquiza et al., 2010).

1.3.2.5. Cross-resistance responses of L. monocytogenes

1.3.2.5.1. Cross-resistance of acid-adapted L. monocytogenes.

Acid-adapted *L. monocytogenes* (pH 5.2, 2 h) had increased resistance to heat shock (52 °C), osmotic shock (25–30% NaCl) and alcohol stress, suggesting that acid adaptation also provides cross-protection against other stress factors (Phan-Thanh et al., 2000). Cross-resistance of acid-adapted cells to other stresses such as high temperatures, sodium chloride, hydrogen peroxide, hypochlorite, crystal violet, ethanol, surfactants and the bacteriocin nisin (Begley et al., 2010; Bonnet and Montville, 2005) has important implications for the food industry, particularly since foods commonly encounter sublethal acidic treatments during processing (Phan-Thanh et al., 2000; van Schaik and Abee, 1999). Also, acid-tolerant strains of *L. monocytogenes* are more resistant to CO_2 (Francis et al. 2007; Jydegaard-Axelsen et al.,

2004). Specifically with bacteriocins, acid tolerance response (ATR) confers cross-resistance to nisin (Badaoui Najjar, et al., 2009; Begley et al., 2010; Bonnet and Montville, 2005; Ivanek et al., 2006). So the cross-resistance induced by the ATR should be considered for the safety of foods fermented with bacteriocin-producing cultures.

1.3.2.5.2. Cross-resistance between bacteriocins in L. monocytogenes

Song and Richard (1997) studied the antilisterial activity of three bacteriocins used at sub-minimal inhibitory concentrations and cross-resistance of the survivors. They obtained that the survivors displayed increased resistance not only toward the bacteriocin they were in contact with, nisin, but toward the two other bacteriocins under study, pediocin AcH and enterococcin EFS2. As the survivors to nisin are resistant to the other two bacteriocins, one can expect that combined use of nisin and one of these bacteriocins would not result in better inhibition. This conclusion is contradicted by the results of Hanlin et al. (1993) who showed that nisin and pediocin AcH had greater antibacterial activity in combination than individually.

1.3.3. Control of *Listeria monocytogenes* in foods

In general, there is not a unique strategy or technology to control *L. monocytogenes* in foods. Preservation technologies were not designed exclusively to eliminate *L. monocytogenes* so the application of multiple hurdles is necessary. However, in those foods were *L. monocytogenes* has been identified as a frequent pathogenic bacterium, several specific strategies have been developed:

1.3.3.1. Bacteriocins

Nisin and pediocin are the most investigated bacteriocins against *L. monocytogenes*. Bacteriocins are suitable as processing aids, complementing other preservation methods. Previous works have demonstrated that heat, freezing–thawing, acid (Muriana, 1996), high hydrostatic pressure (Kalchayanand et al., 1994), and pulsed electric fields were more listericidal in presence of bacteriocins than they were in their absence.

1.3.3.2. Application of active packaging

Active packaging that contains antilisterial agents is increasingly investigated as a means to improve food safety after processing. Listeriostatic or listericidal agents of active packaging are applied as coatings to surfaces in contact with food (Cowan et al., 2003; Hoffman et al., 2001) or incorporated into packaging materials (Brody et al., 2001). As an example, we can cite: organic acids, bacteriocins, spice extracts, lysozyme, chitosan, listeriophages, or EDTA.

1.3.3.3. Ozone (O_3)

Ozone has been used in European countries for decades and has recently been approved in the United States by the FDA for treatment, storage, and processing of foods, including meat and poultry, unless useis precluded by a standard of identity (Food and Drug Administration. 2003). Although ozone treatment inactivates *L. monocytogenes,* listericidal activity of ozone varies with temperature, medium composition, strain, and physiological status.

1.3.3.4. Electrolyzed water (EO).

The effectiveness of EO water against *L. monocytogenes* has been demonstrated in suspension and in foods, like salmon (Ozer and Demirci 2005), eggs, fruits, and vegetables (Sharma and Demirci 2003; Singh et al., 2003; Stan and Daeschel 2003; Bialka et al., 2004; Koseki et al., 2004; Wang et al., 2004, Rahman et al., 2010; Park et al., 2004).

1.3.3.5 Phages

Bacteriophages have a potent antimicrobial activity by inducing the lysis of their host, are ubiquitous in nature including food ecosystems, and are harmless to mammalian cells. Their host specificity allows proper starter performance in fermented products and keeps the natural microbiota undisturbed. Moreover, there are also several phage-encoded proteins with antimicrobial activities, which may be useful for biological intervention approaches. *Listeria* phages have already been succesfully used as processing aids to avoid *Listeria c*ontamination during the manufacturing of soft cheeses (Carlton et al., 2005). However, limited work is reported regarding phages to control foodborne pathogens. In this context, EFSA has submitted a recommendation about more research on the use of phages in food (http://www.efsa.europa.eu/en/scdocs/scdoc/1076.htm) and their possible use in RTE

products products as it was recomended in the recent Scientific Opinion of the Panel on Biological Hazards on "The use and mode of action of bacteriophages in food production" (Anonymus 2009c).

1.3.3.6. Nanotechnology

The application of nanotechnology have been developed in the last few years. It is applied to detect and combat *Listeria* in foods. With the first objective, a nanoparticle inmunoassay to detect low concentrations of *L. monocytogenes* in food and environmental samples (Jaakohuhta et al., 2007) and a highly stable biosensor material that mimics existing whole-cell assays for detecting listeriolysin O (Zhao et al., 2006) have been developed, among others. To combat *L. monocytogenes* in foods, Hong et al. (2008) studied the antimicrobial activity of organically modified nano-clays and they observed that two of the three tested caused rupture of cell membrane and inactivation of *L. monocytogenes* and *Staphylococcus aureus.*

1.3.4. *Listeria monocytogenes* as a tool.

But *Listeria* has been also applied as a tool in medicine. Sawosz et al. (2010) tried to construct bacteria-nanoparticle vehicles of gold and platinum with *Listeria* and *Salmonella enteritidis* although in the case of *Listeria monocytogenes* they did not penetrate or were removed. In the same line, Powell et al., 2010 used ovalbumin-derived designed peptides of *Listeria monocytogenes* as a surrogate antigen to generate nanoparticle vaccines. All these multiple applications constitute a promising field in biotechnology

Moreover, Tangney and Gahan (2010) described the potencial of *Listeria monocytogenes* as a vector for anti-cancer therapies. Several recent studies about this medical application were published: Paterson et al. (2010) designed *Listeria* and *Salmonella* bacterial vectors of tumor-associated antigens for cancer immunotherapy. Shahabi et al., 2010 developed a live and highly attenuated *Listeria monocytogenes*-based vaccine for the treatment of Her2/neu-overexpressing cancers in human. Finally, Wood, et al. (2010) described *Listeria*-derived ActA as an effective adjuvant for primary and metastatic tumor immunotherapy.

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2. Justificacion and objectives

2. JUSTIFICATION AND OBJECTIVES

Listeria monocytogenes is one of the most important food-borne pathogens, since it has a high incidence in different kinds of food products, including fish products, and can cause severe illness and even death in susceptible individuals. In general terms, this work is therefore justified by the need of reducing the risk of the presence of *L. monocytogenes* in food. Within this global objective three main reasons support the appropriateness of the specific objectives raised in this Ph.D. thesis:

1. That the formation of biofilms is one of the principal strategy for *L. monocytogenes* to persist in food processing plants. Persistence implies a high risk of cross-contamination of food by biotransfer (contact, aerosols).
2. That the persistence of *L. monocytogenes* biofilms in the food industry may be related to the resistance to external physical or chemical stimuli, including biocides.
3. That the transfer of biocide-resistant cells from biofilms to food can imply cross-responses to preservation techniques.

The study of different aspects related to these three matters was considered to be useful to prevent the persistence of biofilms formed by *L. monocytogenes* in the food environment and thus improve the control of this bacterial pathogen.

Additionally, experimental systems were developed with the aim of simulating realistic industrial conditions, which is not taken into consideration in many previous studies. Biofilms were thus formed under experimental conditions simulating conditions found in mussel processing plants. The use of mussels and mussel soils as experimental systems is justified by the fact that Spain, particularly Galicia, is the number one mussel-producing European country. In second term, single and dual species mature biofilms were used in this work to assess the biocide activity of widely-used disinfectants as well as the potential of further biocides as new disinfectants, whereas planktonic cells and young biofilms are commonly used as experimental systems in previous works. Lastly, the structural complexity of biofilms was measured in terms of the resistance to biocides instead of the number of cells.

Taking this into account, the main objectives of this work were:

1. To compare the adhesion kinetics of *L. monocytogenes* (strains CECT 5873, CECT 936, CECT 911 and CECT 4032, representing serotypes 1/2a, 1/2b, 1/2c and 4b, respectively) in polypropylene (PP) and stainless steel (SS) under two surface contamination conditions (with and without conditioning film) simulating those found in mussels processing plants. This permitted to select for subsequent studies those scenarios that give rise to the most risky conditions.

2. To develop of a new method to obtain benzalkonium chloride adapted *L. monocytogenes* by exposing exponential-phase cells once to a sub-lethal concentration of the biocide. This permitted to obtain a BAC-adapted strain derived from *L. monocytogenes* CECT 5873, to explore and discuss the effect of the inoculum size and the BAC concentration in the exposition in the level of adaptation of *L. monocytogenes* to BAC and to compare the proteomic pattern of BAC-adapted and non-adapted *L. monocytogenes*.

3. To compare the increase of resistance to biocides (BAC, peracetic acid and nisin) and the changes in microscopic structure during biofilm formation at 25 ºC by three strains of *L. monocytogenes* (CECT 911, CECT 4032, CECT 5873 and BAC-adapted CECT 5873) in those scenarios selected in 1. This objective will permit to compare the effectiveness of the checked biocides, to identify some cross-responses derived from BAC adaptation and to highlight the importance of the structure in biofilm formation.

4. To compare the resistance to BAC and the microscopic structure of mixed-species biofilms formed by different strains of *L. monocytogenes* and *Pseudomonas putida CECT 845* under different scenarios and that obtained by the corresponding mono-species *L. monocytogenes* biofilms. Obtained results in this section could give us an idea of how important is to consider multispecies biofilm in food safety.

5. To compare the viability of BAC adapted and non-adapted *L. monocytogenes* CECT 5873 biofilm cells in modified atmospheres rich in CO_2 and O_2 during storage at 2.5 ºC once they had been transferred by contact to cooked and live mussels, respectively. In addition, in cooked mussels the combined effect of CO_2 and nisin against the survival of *L. monocytogenes* was also studied by using a first order factorial design.

3. Results and discussion

3.1. Effects of mussel processing soils on the adherence of *Listeria monocytogenes* to polypropylene and stainless steel

3.1. ADHERENCE OF *LISTERIA MONOCYTOGENES*: EFFECTS OF MUSSEL PROCESSING SOILS ON THE ADHERENCE OF *LISTERIA MONOCYTOGENES* TO POLYPROPYLENE AND STAINLESS STEEL

A comparative study of adhesion kinetics of *L. monocytogenes* (strains CECT 5873, CECT 936, CECT 911 and CECT 4032, representing serotypes 1/2a, 1/2b, 1/2c and 4b, respectively) to polypropylene (PP) and stainless steel (SS) under two surface contamination conditions in plants processing cooked mussel was carried out. Either: i) contamination of clean surfaces with mussel cooking juice carrying *L. monocytogenes*, or ii) contamination with *L. monocytogenes* after soiling with mussel cooking juice, i.e. conditioning film (CF).

The kinetics of adhesion were successfully described by a modified logistic model. Adhesion to polypropylene was higher than to stainless steel in all strains, except CECT 5873. Adhesion was initially higher in the presence of CF, but numbers of adherent cells decreased sharply in the late phase of study in 3 out of 8 cases as a result of cell detachment.

Combinations of strain, surface material and surface conditioning where adhesion was most enhanced were defined as worst case scenarios (CECT 911-PP, 4032-PP-CF, 5873-SS, and 4032-SS-CF). Subsequently, adhesion in worst case scenarios was compared with a similar contamination event taking place in plants processing live mussels, using intervalvar water of mussel as food residues.

Adhesion levels were higher in intervalvar water than in cooking juice, especially in both cases with no conditioning film; this was attributed to more space available for adhesion or physico-chemical conditions enhancing cells to adhere.

3.1.1. Introduction

Listeria monocytogenes is a potencially pathogenic bacterium widely distributed in nature and in food industries. Therefore it has been found in a wide range of sources, including vegetables, processed foods, silage and soil. (Cox et al., 1989; Ivanek et al., 2006). *L. monocytogenes* grows at temperatures ranging from 1 to 45 ºC, pH between 4 to 9, and high salt concentrations (up to 10%) (Gandhi and Chikindas, 2007). Food are the primary route of transmission of *L. monocytogenes*, which has been involved in numerous outbreaks and sporadic cases of foodborne diseases in humans (Farber and Peterkin, 1991; McLauchlin et al., 2004; Denny and McLauchlin, 2008).

L. monocytogenes comprises 13 distinct serological groups, but 95% of human isolates belong to serotypes 1/2a, 1/2b, 1/2c and 4b (Kathariou, 2002). Although serotype 1/2a is more frequently isolated from food and the environment, most outbreaks are caused by serotype 4b (Borucki et al., 2003). In fact, serotype 4b strains were found in 59% of human cases of listeriosis (Rocourt and Cossart, 1997).

L. monocytogenes can attach to food industry surfaces and evolve a biofilm (Kim and Frank 1995). Biofilms can allow *L. monocytogenes* to persist in the food processing environment (Frank and Koffi 1990; Pan et al., 2006). Although extensive research has been conducted on *L. monocytogenes* biofilms, the control of biofilms is still a major problem for the food industry (Chmielewski and Frank, 2003).

Bacterial adhesion to surfaces is the initial step in biofilm formation. The effects of different environmental factors on the adherence of *L. monocytogenes* to different surfaces have been the subject of several studies (Bonaventura et al., 2008, Chavant et al., 2002; Mai and Conner, 2007; Moltz and Martin, 2005; Rodríguez et al., 2008*;* Smoot and Pierson, 1998). However, conflicting results have been obtained, and it has recently been stated that biofilm formation is still poorly understood (Harvey et al., 2007). In fact, two main aspects can be criticized in many studies. Firstly, adhesion was only determined during the first hours of study, so the kinetics of adherence are not defined and it makes comparisons difficult. Secondly, commercial or synthetic media were commonly used and these media do not reproduce the actual conditions found in food processing plants.

This study aimed to examine the adhesion kinetics of different *L. monocytogenes* strains on stainless steel and polypropylene, which are widely used in the food industry. First, two different surface contamination possibilities in plants processing cooked mussel were compared: i) contamination of clean surfaces with food residues carrying *L. monocytogenes* and ii) contamination of surfaces with *L. monocytogenes* after conditioning with food residues. Following this, worst case scenarios, i.e. combinations of strain, surface material and surface conditioning where adhesion was most enhanced, were further compared with a similar contamination event which may occur *in situ* in plants processing live mussels i.e. use of intervalvar water as a food residue matrix.

3.1.2. Materials and methods.

Bacterial strains

Listeria monocytogenes CECT 5873, CECT 911, CECT 4032 and CECT 936 were provided by the Spanish Type Culture Collection (Valencia). Strains were stored at -80ºC in nutrient broth containing 50% glycerol (v/v) until use.

Food processing surfaces

Stainless steel (AISI-304, 2B finish, 0.8 mm thickness) and polypropylene sheets (compact 00226) were cut into coupons (20 mm x 20 mm). Each polypropylene (PP) coupon was used only once. Prior to being used, PP coupons were cleaned with alcohol and left overnight in distilled water. In contrast, stainless steel coupons were used repeateadly. Prior to being used, SS coupons were soaked in 2 M NaOH to remove any grease or food residues and then rinsed several times with distilled water.

After cleaning, a circular shape (15 mm diam) was drawn on each coupon with a correction pen (Tipp-Ex) to define the working area.

Mussel cooking juice (MCJ) and intervalvar water of mussel (IWM) were used as culture media to simulate contamination of surfaces in plants processing cooked mussel and live mussel, respectively.

MCJ was prepared by cooking live mussels (ca. 1 kg) during 1 min at 100ºC in a pot with no water added. Mussels prepared in this manner open and release liquid. About 400 ml MCJ were obtained and stored in 50 ml aliquots at -20ºC (**Table 3.1.1**).

Table 3.1.1: Composition of mussel cooking juice (MCJ) and intervalvar water of mussel (IWM)

	MCJ	IWM
Carbohydrate (g/l)	9,19	2,7
Nitrogen (g/l)	1.70	1.85
pH	7.74	8,80

IWM was directly collected by opening live mussels (ca. 1 kg) with a knife. About 250 ml IWM were obtained and similarly stored (**Table 3.1.1**). The pH of MCJ and IWM was measured using a pHmeter.

Whenever required, aliquots of either MCJ or IWM were thawed and used either for preparing the inocula or forming the conditioning film on coupons.

A conditioning film was formed on the working area of approximately half of the coupons by drying 250 µl of mussel cooking juice (MCJ) or intervalvar water (IWM) under a stream of air. Then coupons were sterilized by overnight exposure to ultraviolet light and subsequently placed into sterile glass petri plates.

Preparation of inocula

Whenever required, stock strains were thawed and subcultured twice in Tryptone Soy Broth (TSB; Difco, Spain) at 37 ºC prior to use.

Inocula were then prepared by adjusting the absorbance at 700 nm of each strain in TSB to a value of 0.100, corresponding to a cell density of 10^8 CFU/ml previously, calibration curves.

Cells were then harvested by centrifugation (6000 rpm, 10 min, 25 ºC) and finally resuspended in MCJ or in IWM. Two hundred and fifty µl-aliquots of each inoculum were pipetted onto the working area of each coupon. In the case of the inocula dispensed on

coupons with conditioning film, cells were resuspended in 1/2 x PBS instead. It was used 10 ml of MCJ and 12.5 ml of IMW in each experiment.

Two hundred µl of sterile distilled water were dispensed around the coupons placed in Petri dishes to maintain humidity. Mass determinations initially and at each sampling time indicated that this procedure was sufficient to prevent evaporation of inoculum.

Experimental design

During the first stage, the kinetics of adhesion of *L. monocytogenes* strains on polypropylene and stainless steel were examined under two surface contamination possibilities with different nutrient availability in plants processing cooked mussels:

- Contamination of clean surfaces with food residues, i.e. mussel cooking juice, carrying *L. monocytogenes*. In this case, nutrients were freely available.
- Contamination of surfaces with *L. monocytogenes* after conditioning with dried mussel cooking juice. In this case, inocula were prepared in 1/2 strength PBS and nutrients were initially much less available.

During the second stage, the kinetics of adhesion of the worst case scenarios defined in the first stage (combination of strain, surface material and surface conditioning showing most adhesion), were examined in a similar contamination event which may occur in situ in plants processing live mussel. Intervalvar water was used as matrix of food residues during these experiments.

These studies were carried out during a period of 60-80 h at 25 ºC, which is a usual temperature in the food processing environment. Two replicate coupons were examined at each sampling time. Each experiment was carried out on 3 separate ocasions.

Determination of the number of adhered cells

The number of adhered cells was determined according to Herrera et al. (2007). Briefly, coupons were drained and immediately immersed in 10 ml of PBS for 10 s to release non-adhered cells. Adhered cells were collected by sequentially rubbing coupon surfaces with two peptone water-moistened swabs for 30 s each. Both swabs were transferred to 10 ml peptone water and subjected to vigorous vortexing for 1 min. The number of adhered cells was

determined by plating appropriate serial dilutions on Triptone Soy Agar after incubation at 37 ºC for 24 h. Peptone water was always used as dilution medium. Results were expressed as CFU per mm^2.

Nutrient analysis

The liquid medium poured from coupons was centrifuged (2000 x *g*, 10 min) and the supernatant was collected. In the case of coupons with conditioning film, the nutrients remaining behind in the various food residues were analysed during biofilm growth. The conditioning film was detached from the coupon surface by pipetting 10 ml of distilled water up and down several times. Once detached, it was mixed with the liquid medium and the whole volume was centrifuged. In both cases, supernatants were stored for subsequent carbohydrate and nitrogen analysis.

The content of total carbohydrates was determined according with Dubois et al. (1956). Total nitrogen was determined by the Kjeldahl method with a kjeltec 2300 analyser unit (Foss Tecator, Barcelona, Spain).

Statistical analysis.

The fits of the models to the experimental data were performed according to a least-squares method (quasi-Newton).

A Student´s test (α=0.05) was used to test the significance of the differences between means of the total number of adhered cells.

3.3. Results and Discussion

Effects of mussel cooking juice on the kinetics of adhesion of L. monocytogenes on polypropylene and stainless steel under different nutrient availability

A study on the effects of the presence of mussel cooking juice on the adhesion kinetics of four *L. monocytogenes* strains to polypropylene and stainless steel surfaces, which are widely

used in the food industry, was carried out first. The strains chosen for this study belonged to the major foodborne serotypes.

The kinetics of adhesion obtained for each of the cases are shown in **Figure 3.1.1**. Two different profile patterns could be distinguished:

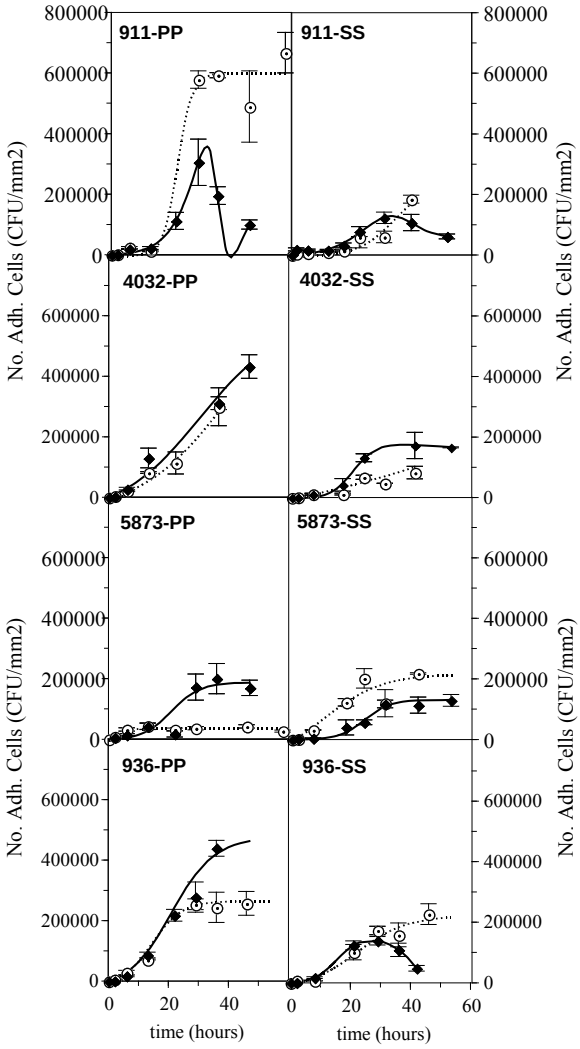

Figure 3.1.1: Adhesion kinetics of different strains of *Listeria monocytogenes* to polypropylene (PP) and stainless steel (SS) coupons in the presence of mussel cooking juice either in liquid state **(Θ)** or as a conditioning film **(♦)**. Lines represent estimates obtained using eq**.[3.1.1]** or **[3.1.2]**.

- A sigmoidal type pattern, which is most frequent. This profile was described by a logistic equation modified according to Cabo et al. (1999):

$$NAC = \left(\frac{a_{ad}}{1 + e^{r_{ad}(m_{ad} - t)}} - \frac{1}{1 + e^{r_{ad} m_{ad}}} \right) \qquad [\mathbf{3.1.1}]$$

- A pattern showing a sharp decrease after approximately 24 h. It appeared in 3 out of the 16 experimental cases under study, namely, CECT 911 on polypropylene and stainless steel and CECT 936 on polypropylene (**Fig. 3.1.1**), all of them in the presence of conditioning film. An empirical additive model represented by two logistic terms was used to describe this second pattern:

$$NAC = \left(\frac{a_{ad}}{1 + e^{r_{ad}(m_{ad} - t)}} - \frac{1}{1 + e^{r_{ad} m_{ad}}} \right) - \left(\frac{a_D}{1 + e^{r_D(m_D - t)}} - \frac{1}{1 + e^{r_D m_D}} \right) \qquad [\mathbf{3.1.2}]$$

where,

- NAC: number of adhered cells (CFU/mm^2).
- a_{ad}: maximum number of adhered cells (asymptote).
- r_{ad}: specific adhesion coefficient (t^{-1}).
- m_{ad}: time at which the number of adhered cells is half of the maximum.
- a_D: total number of adhered cells which detach or die (CFU/mm^2).
- r_D: specific detachment/death coefficient (t^{-1}).
- m_D: time at which the number of detached/death cells is half of the maximum.

The values obtained for these parameters in each case are shown in **Tables 3.1.2** and **3.1.3**. Fits of eq [**3.1.1**] and eq [**3.1.2**] to experimental data are shown by solid lines in **Figure 3.1.1**.

The values of parameters r_{ad} and m_{ad} have clearly shown that the strains CECT 911 (serotype 1/2c) and CECT 4032 (serotype 4b) had the highest adhesion ability ($P < 0.05$) to both stainless steel and polypropylene, and that the adhesion of *L. monocytogenes* CECT 911 to polypropylene was the highest (as judged especially by the values of r_{ad}). On the contrary, *L. monocytogenes* CECT 5873 (serotype 1/2a) showed the lowest adhesion ($P < 0.05$) in all

the cases, except on stainless steel in the absence of conditioning film, a case in which the adhesion of this strain was higher than all other strains.

Table 3.1.2: Values obtained for the parameters of eq [3.1.1] and eq [3.1.2] of adhesion to polypropylene in the presence of mussel cooking juice

	911	911-CF	4032	4032-CF	936	936-CF	5873	5873-CF
a_{ad}	603302	1134524	788373	650000	285000	500000	45000	190000
r_{ad}	0,420	0,171	0,068	0,075	0,198	0,140	0,550	0,211
m_{ad}	21,727	34,270	40,012	29,886	14,000	21,000	3,200	21,000
a_D	-	898804	-	-	-	-	-	-
r_D	-	0,573	-	-	-	-	-	-
m_D	-	35,67	-	-	-	-	-	-

CF: presence of conditioning film.

Table 3.1.3: Values obtained for the parameters of eq [3.1.1] and eq [3.1.2] of adhesion to stainless steel in the presence of mussel cooking juice

	911	911-CF	4032	4032-CF	936	936-CF	5873	5873-CF
a_{ad}	250000	342779	1116597	204055	240993	169052	300000	131004
r_{ad}	0,122	0,109	0,017	0,252	0,122	0,253	0,102	0,242
m_{ad}	45,761	39,377	117,579	20,457	21,887	15,744	10,000	24,171
a_D	-	281047	-	198133	-	737975	-	-
r_D	-	0,146	-	0,014	-	0,141	-	-
m_D	-	50,27	-	43,302	-	53,76	-	-

CF: presence of conditioning film

Although studies relating serotype to adhesion on polypropylene were not found, some studies have shown that serotype 1/2c strains have higher adhesion ability on stainless steel than serotype 1/2a and 4b strains (Norwood and Gilmour, 1999; Norwood and Gilmour 2001). This could be related to a different composition of flagella. The presence of flagella and the consequent mobility have been shown to be determinant for initial adhesion (Vatanyoopaisarn et al., 2000; Lemon et al., 2007). On the contrary, no significant differences in initial adhesion were detected among different serotypes by Kalmokoff et al (2001). Even

taking into account the variability in initial adhesion among different strains, discrepancies between different studies are in some cases the result of differences in the experimental design. It seems thus clear that sampling has to be carried out at several time points to define adhesion kinetics and distinguish worst case scenarios, which would be defined by maximum adhesion in minimum time, that is, by maximum values for a_{ad} and r_{ad}. (Herrera et al., 2007). Otherwise, comparisons can be difficult to make. Additionally, it is disregarded that a time limit between initial adhesion and mature biofilm formation cannot be fixed a priori.

In the present study, the adhesion to polypropylene was significantly higher ($P < 0.05$) than to stainless steel in most of the scenarios and this is reflected in higher values of r_{ad} and m_{ad}. The exception was *L. monocytogenes* CECT 5873, which adhered better to stainless steel in the absence of conditioning film. This strain may exhibit a higher affinity towards hydrophilic surfaces than the other strains tested.

Lower numbers (CFU/mm^2) of adherenced CECT 911 and CECT 4032 on stainless steel in the absence of conditioning film questioned the validity of the values for the asymptote of eq. **[3.1.1]**. However, very similar values were obtained (a_{ad}=1116597 vs. a_{ad}real=1115190; and r_{ad}=0.018 vs. r_{ad}real=0.017 for CECT 4032) in later studies, in which the incubation period was extended to 11 days ($P < 0.05$) (results not shown).

The present results agree with several studies showing that the physico-chemical properties of surface materials (electric charge, hydrophobicity, roughness, etc.) are contributing determinants of the initial adhesion of *L. monocytogenes* (Palmer et al., 2007; Rodríguez et al., 2008; Sinde and Carballo, 2000). However, they may not be the only determinants. A previous study by Sinde and Carballo (2000) had found that *L. monocytogenes* adhered in higher numbers to polytetrafluoroethylene and rubber -hydrophobic substrates- than stainless steel -a hydrophilic substrate-. Additionally, hydrophilic passivating groups were suggested to be the best method for preventing cell attachment to synthetic substrates (Cunliffe et al., 1999). On the contrary, other studies showed that *L. monocytogenes* formed biofilms faster on stainless steel than polytetrafluoroethylene (strain LO28), (Chavant et al., 2002; Meylheuc et al., 2001) or Buna-N rubber (strain Scott A), (Smoot and Pierson, 1998). In this latter work, however, changes in cell hydrophobicity could not be correlated to differences in adhesion, or to attachment of 21 *L. monocytogenes* strains to glass (Chae and Schraft, 2000). As Teixeira et al. (2008) pointed out, a correlation between surface hydrophobicity and the extent of

adhesion of *L. monocytogenes* is difficult to be established and other factors, such as surface charge or roughness, should also be taken into account.

Adhesion was slightly higher ($P < 0.05$) under low nutrient availability conditions, i.e. in the presence of conditioning film, particularly on polypropylene. However, it seems more important that the number of cells adhered in the presence of conditioning film decreased in the late phase of study in 3 out of 8 cases, that is, CECT 911 on both polypropylene and stainless steel, and CECT 936 on stainless steel. Although the presence of conditioning film enhanced initial adhesion, it hindered direct adhesion of cells to surface material. The conditioning film plastified gradually over time. As a result, the system became more labile, as the conditioning film tended to detach from the coupon surface, carrying the adhered cells. This would be prevent a mature biofilm to be formed, but detachment involves a potential risk of food contamination (Chavant et al., 2002; Norwood and Gilmour 1999).

With regard to the uptake of nutrients, it was approximately constant (1-2-g/l) in all the cases, except for the strain CECT 5873 in the absence of conditioning film, in which a higher carbohydrate content was consumed (ca. 4 g/l), despite lowers numbers of adhered cells was low. Previous studies had shown that nutrient consumption was inversely proportional to exopolysaccharide production, and therefore to adhesion (Laspidou et al., 2002).

Effects of intervalvar water of mussel on the kinetics of adhesion of L. monocytogenes

After this, the worst case scenarios were defined, i.e. combinations of strain, surface material and surface conditioning showing most adhesion in plants processing cooked mussel. These are:

- CECT 911 on polypropylene in the absence of conditioning film (PP-ACF).
- CECT 4032 on polypropylene in the presence of conditioning film (PP-CF).
- CECT 5873 on stainless steel in the absence of conditioning film (SS-ACF).
- CECT 4032 on stainless steel in the presence of conditioning film (SS-CF).

Subsequently, the kinetics of adhesion of these scenarios were compared with a similar contamination event which may occur in situ in plants processing live mussels. Intervalvar water was used as matrix of food residues in these subsequent comparative studies.

As shown in **Table 3.1.4** and **Figure 3.1.2** adhesion was significantly higher ($P < 0.05$) in the presence of intervalvar water, especially in the cases 1 and 3, both with no conditioning film. However, only slight differences were observed in the presence of conditioning film. In fact, practically no differences were found between IWM and MCJ in case CECT 4032 PP-CF

Table 3.1.4: Values obtained for the parameters of eq [1] and eq [2] of adhesion in the presence of intervalvar water of mussel

	911-PP	4032-PP-CF	5873-SS	4032-SS-CF
a_{ad}	1444459.618	571898	809482	152976.856
r_{ad}	0.424	0.100	0.115	0.115
m_{ad}	25.517	23.087	41.286	33.36
a_D	-	-	-	1068974
r_D	-	-	-	35.63
m_D	-	-	-	0.650

PP: polypropylene
SS: stainless steel
CF: presence of conditioning film

Intervalvar water of mussel is nutritionally poorer than mussel cooking juice (see **Table 3.1.1**), but cells were not starved. In fact, the consumption of nutrients was low so nutrients do not seem to play an important role.

At least three non-mutually exclusive factors could account for the higher adhesion found in cases **3.1.2 A** and **3.1.2.C** ($P < 0.05$): i)

- Higher probability of contact between cells and surface due to a lower nutrient concentration,
- Intervalvar water was not an adequate medium for cells to survive in the planktonic state so they would tend to adhere,
- pHs of intervalvar water and mussel cooking juice were different (7.74 in MCJ, 8.80 in IWM and about 7.50 in coupons with conditioning film). However, with respect to the latter, it was shown that the lower the pH (in the range 6.0-9.0), the higher the adhesion rate of *L. monocytogenes* Scott A to Buna N-rubber (Smoot and Pierson, 1998).

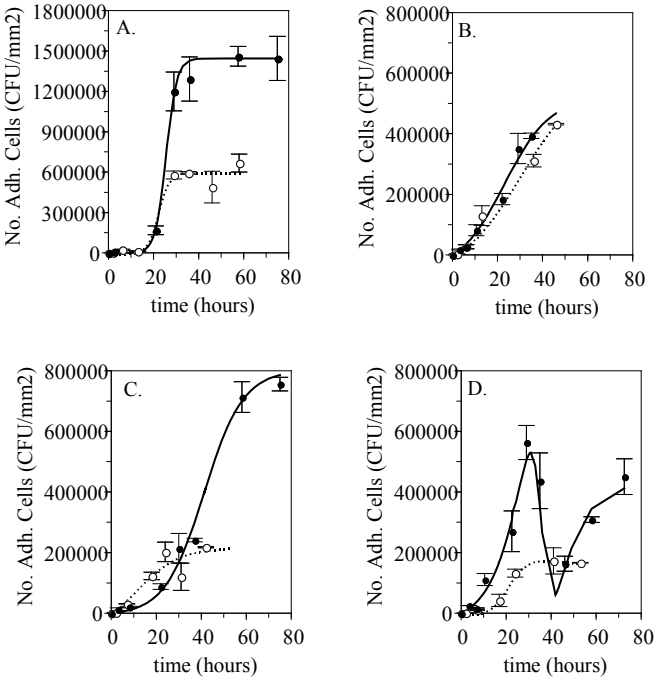

Figure 3.1.2: Comparison between the adhesion kinetics of *Listeria monocytogenes* under four different conditions (worst-case scenarios A-D) in the presence of mussel cooking juice (O) and intervalvar water of mussel (●). Lines represent estimates obtained using eq**.[3.1.1]** or **[3.1.2]**.

A.:CECT 911 on polypropylene in the absence of conditioning film (PP-ACF).

B.:CECT 4032 on polypropylene in the presence of conditioning film (PP-CF).

C.:CECT 5873 on stainless steel in the absence of conditioning film (SS-ACF).

D.:CECT 4032 on stainless steel in the presence of conditioning film (SS-CF).

Additionally, the distinct adhesion ability of strains in MCJ or IWM should not be forgotten. A high variability ($P < 0.05$) among strains had already been observed (Borucki et al., 2003; Folsom et al., 2006; Tresse et al., 2006; Tresse et al., 2007). Also, Folsom et al (2006) found that serotype 4b strains were better biofilm-formers than serotype 1/2a strains in a high nutrient medium, whereas the latter were better biofilm-formers in a low nutrient medium, and reported it to be a possible cause of the predominance of serotype 1/2 strains in food processing plants.

Biofilm formation: a cyclical dynamic?

Results from the present study showed a decrease in numbers of cells adhered to the various surfaces after 40 h presumably because cell detachment became higher than adhesion. This decrease was also observed by Herrera et al. (2007). This effect was observed in all cases in the presence of conditioning film (CECT 911-PP, CECT 911-SS, CECT 936-PP, CECT 4032-SS) (**Fig. 3.1.1, Tables 3.1.2, 3.1.3**). However, in the cases of CECT 911-PP and CECT 4032-SS, the kinetics of adhesion showed a further increase subsequent to each decrease ($P < 0.05$) (**Figure 3.1.1** and **Figure 3.1.2**, respectively). This may indicate a cyclic nature in *L. monocytogenes* biofilm development. However, further studies would be needed to confirm that a cyclical model is followed.

In conclusion, the initial adhesion of *L. monocytogenes* seems to depend mainly on the interactions between cells and surfaces. The interactions are in turn mainly determined by their physicochemical properties and possible space restrictions (hence the effects of the conditioning film), along with environmental conditions. Adhesion is the initial step in biofilm formation. However, subsequent biofilm growth was not dependent on initial attachment (Chae and Schraft, 2000). Nonetheless, preventing the presence of pathogenic bacteria on surfaces of food processing plants requires the control of environmental conditions enhancing adhesion on a day to day basis. And this makes essential to carry out studies allowing such conditions to be defined.

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3.2 An efficient method to obtain benzalkonium chloride-adapted cells of *Listeria monocytogenes* CECT 5873.

3.2. AN EFFICIENT METHOD TO OBTAIN BENZALKONIUM CHLORIDE-ADAPTED CELLS OF *LISTERIA MONOCYTOGENES* CECT 5873.

This section presents a new method for obtaining BAC-adapted *L. monocytogenes* cells. A factorial design was used to assess the effects of the inoculum size and BAC concentration on the adaptation (measured in terms of lethal dose 50 -LD_{50}-) of exponential *L. monocytogenes* 5873 cells after only one exposure. A significant empirical equation was obtained showing the positive effect of the inoculum size and the positive interaction between the effects of BAC and inoculum size on the level of adaptation achieved (LD_{50}). However, a slight negative effect of BAC, due to it being a biocide, was also significant. By applying the new procedure, it was possible to increase BAC adaptation 4.17-fold in only 33 hours whereas 5 days were necessary in the classical procedure based on successive stationary phase cultures in sublethal BAC concentrations. Lastly, we present preliminary results of the comparison between the proteomic patterns of non-BAC adapted and BAC-adapted cells.

3.2.1. Introduction

Listeria monocytogenes is a pathogenic bacteria widely found in nature and food industry (Cox et al. 1989, Ivanek et al. 2006). The frequent use of disinfectants in food environments can lead to the development of disinfectant resistant isolates due to inadequate cleaning and disinfection protocols (Walton et al., 2008). Benzalkonium chloride (BAC) is a biocide belonging to the group of quaternary ammonium compounds (QACs) that is commonly used in the food industry. Previous works have found that persistent *L. monocytogenes* strains are QAC resistant (Aase et al. 2000; Mullapudi et al., 2008). Changes in the cell membrane and efflux pumps lead to this resistance (Mereghetti et al., 2000, Romanova et al., 2006). This could be partly related to inadequate cleaning and disinfection protocols that result in long-term exposure of microbial communities to subinhibitory concentrations of QACs, which causes the emergence of more resistant clones with changes in their susceptibility to other antimicrobials (Langsrud et al. 2003; Hegstad et al., 2010). Consequently, a lot of research in applied microbiology is focused on studying the physiological consequences of BAC and other biocides on bacterial resistance. To carry out this research it is necessary to generate BAC-adapted bacterial cells in the experimental protocols.

Classical methods for obtaining bacterial cells adapted to antimicrobial agents are based on successive exposures of stationary-phase cells (Aase et al. 2000; Soumet et al., 2005; Taormina et al., 2001; To et al. 2006). Although this classical method is time-consuming and does not ensure that the maximum level of adaptation is achieved, it is the procedure most widely used in bacterial physiology experiments. However, some authors have found that *L. monocytogenes* only begins to show gradual adaptation to BAC after several successive subcultures in increasing BAC concentrations during 1 week (To et al. 2002), whereas others have found it necessary to wait 5 (Aarnisalo et al. 2007) or 7 days (Aase et al. 2000) with no growth to obtain BAC-adapted cells.

It is known that exponential-phase cells are metabolically more active than stationary-phase cells. This implies that exponential cells are more able to activate a change in the ongoing metabolic pathways in response to an external stimulus (Whistler et al., 1998; Zotta et al., 2009). Stationary phase cells have already activated phase-associated resistance mechanisms (Davies et al., 1996; Kang et al. 2004; Hengge-Aronis et al., 1991; Lange and Hengge-Aronis, 1991; Hu et al., 1998; Munro et al., 1995). However, no previous studies in

which *L. monocytogenes* exponential-phase cells were exposed to biocides to generate adaptation were found in the literature. Therefore, in the present work we studied the effects of the concentration of bacterial cells (inoculum size) in exponential phase and the concentration of BAC on the level of adaptation reached by *L. monocytogenes* after only one exposure. Based on the results obtained we propose an alternative procedure for obtaining cells with a higher level of adaptation in a shorter period of time.

3.2.2. Materials and methods

Bacterial strains, synthetic media and biocide

Listeria monocytogenes CECT 5873, CECT 911 and CECT 4032 were provided by the Spanish Type Culture Collection (Valencia). Strains were frozen and stored at -80ºC in TSB containing 50% glycerol (v/v) until use. Whenever required, one cryovial was thawed and strains were subcultured twice in Triptone Soy Broth (TSB, Cultimed, S.L., Spain) at 37 ºC before use. All subsequent cultures were grown in TSB at 25 ºC. In the adaptation experiments, TSB with different concentrations of benzalkonium chloride was used. Benzalkonium chloride was purchased from Sigma Chemical Co.

Experimental method

Inocula preparation

One ml aliquot of an overnight culture of *L. monocytogenes* CECT 5873 in stationary phase was transferred to 49 ml of TSB (final volume: 50 ml) in a 250-ml erlenmeyer flask (1:5) (v:v) and incubated at 25 ºC until the required growth phase according to the experimental procedure applied (see below). Cultures were incubated until 1.0 optical density (700nm) when inoculum in stationary phase was required and to 0.4-0.5 optical density (700 nm) when an exponential phase culture was needed, corresponding to cell densities of $2x10^9$ CFU/ml and 10^9 CFU/ml respectively according with previous calibration.

Experimental procedures

i. *Classical multi-step* (successive cultures) experimental design: 1 ml aliquots of successive stationary-phase subcultures of *L. monocytogenes* were inoculated into 49 ml of TSB containing increasing concentrations of BAC (**Figure 3.2.1.A**). The cultures were also carried out in a 250 ml erlenmeyer flask (1:5) (v:v) at 25ºC and shaking (100 rpm).

Figure 3.2.1. Diagram outlining experimental protocols to enhance adaptation to BAC. **A**: Classical multi-step method. **B**: Single-step method. S1-S4: cultures after exposure to a number of successively increasing concentrations of BAC. E1-E4: cultures after exposure to several combinations of inoculum size and BAC concentration as defined by a first-order factorial design. Double-line circles represent non-BAC adapted cultures.

ii. *Single-step exposure procedure*: Several non-adapted inocula of the exponential-phase cultures of *L. monocytogenes* (**Figure 3.2.1.B**) were exposed only once to several concentrations of BAC. Both the inoculum size and the BAC concentrations used during exposures were set according to a factorial design (**Table 3.2.1**.). Culture conditions as in i).

Table 3.2.1: Natural and codified values used in the first order ortogonal design

	Natural values	
Codified values	**BAC (mg/l)**	**Inoculum (ml)**
1;1	9	25
1;-1	9	5
-1;1	3	25
-1;-1	3	5
0;0	6	15

Collection of BAC-adapted cells of L. monocytogenes

After each experimental assay described above, 0,5 ml of BAC-adapted cells were collected by centrifugation at 2000 g during 5 minutes at 25 ºC. Obtained pelled pellet was washed with PBS to eliminate residual BAC concentration by centrifuging and resuspending twice in 0.05 M sterilized phosphate buffer. Cells were then transferred to a cryovial and stored at -80ºC in TSB containing 50% glycerol (v/v) until their resistance to BAC was determined.

Determination of the resistance to BAC in L. monocytogenes collected cells.

Dose-response assays in microtiter plates

One hundred and twenty five µl of the activated inoculum diluted (1:10) twice of *Listeria monocytogenes* was added to microplate wells containing 125 µl solutions of different BAC concentrations (0 to 15 mg /l, step: 1 mg/l). 3 replicates of each BAC concentration were prepared and the microtiter plate was incubated at 37 ºC for 24 h. Absorbance at 700 nm was measured using a spectrophotometer (iMark^{TM}, Biorad). Series with sterile water instead of BAC was used as a control. The inhibition (I) of each BAC concentration was calculated from the absorbance values obtained in the dose-response assay according to the following equation [**3.2.1**]:

$$I\ (\%) = [1-(A_X/A_0)]*100 \qquad [\mathbf{3.2.1}]$$

- I: Inhibition (%)
- A_x: average of the absorbance values obtained for each concentration of BAC
- A_0: average of the absorbance values obtained in absence of BAC.

Determination of resistance

Two different methods were used to quantify the bacterial resistance to benzalkonium chloride:

i) In terms of the minimum inhibitory concentration (MIC), defined as the minimum value of dose were the absence of growth in detected. Used in the screening of the mutants carried out in the genetic studies.

ii) In terms of LD_{50}, that was obtained by adjusting the inhibition values calculated according with Eq. [**3.2.1**] to the modified logistic equation **[3.2.2.]** (Cabo et al. 1999) and the Solver application of Excel. Used in the rest of experiments.

$$I\,(\%) = k\left(\frac{1}{1+e^{r(LD50-D)}} - \frac{1}{e^{rLD50}+1}\right) \qquad [\mathbf{3.2.2}]$$

- LD_{50}: Dose that kills 50% of initial population
- k: Maximum inhibition BAC value (asymptote)
- r: Specific inhibition coefficient (dimensions: inverse of the dose)
- D: Dose

Proteomic assays

Cytosolic proteins were extracted from controlled exponential-phase cultures (OD. O.4-0.5) and then purified using the ReadyPrep™ 2-D Cleanup Kit (Biorad, S.L.). First- and second-dimension electrophoresis were carried out according to Sánchez et al. 2010. In the proteomic experiments, BAC-adapted cells (LD_{50}=7.22 mg/l) obtained after one exposure of 7.5 ml of exponential cells of *L. monocytogenes* to 5.25 mg/l of BAC were used.

Statistics

Factorial designs were carried out according to Box and Hunter (1989). A Student's *t* test (α=0.05) was used to test for significant differences between means of the adaptation level and between the coefficients of the equations obtained in the factorial design. A Fisher test (α=0.05) was used to test the consistency of the models.

Genetic procedures

Information about bacterial strains, plasmids, culture conditions, nucleic acid manipulations, mutagenesis methods and cloning methods employed can be checked in Sleator et al., 2000 and Stack et al., 2005.

3.2.3 Results

Adaptation of L. monocytogenes CECT 5873, 911 and 4032 to benzalkonium chloride by the classical multi-step procedure after the exposure to different BAC concentrations

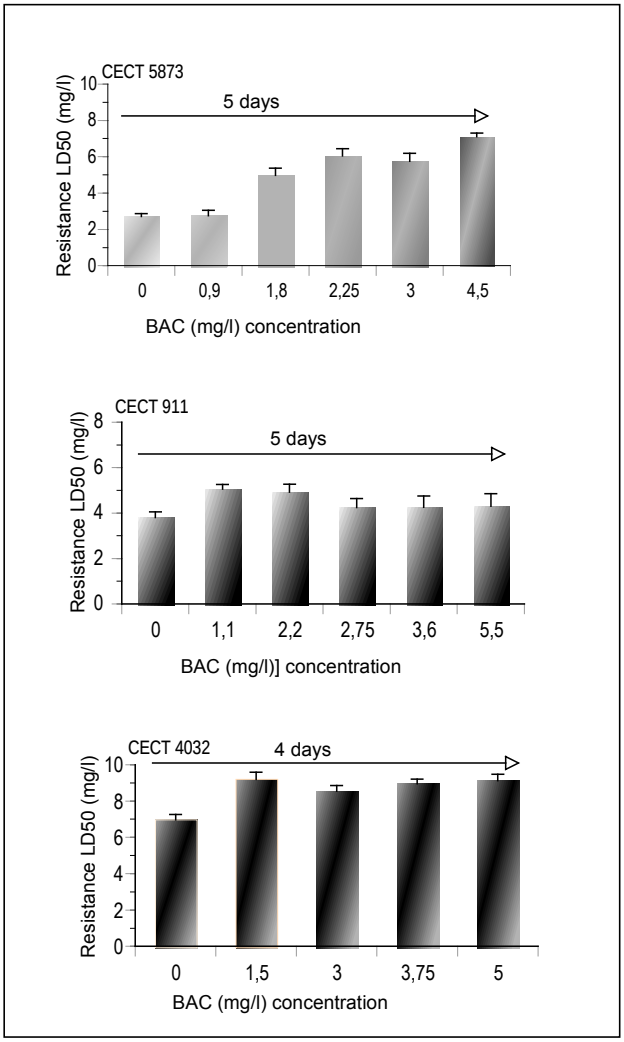

Figure 3.2.2: Increase in BAC-resistance of *L. monocytogenes* CECT 5873, 911 and 4032 according to a classical multi-step procedure

Stationary phase cells of each strain were tested for growth under a sequence of successively increasing sublethal concentrations of BAC and their adaptation capacities were compared (**Figure 3.2.2**).

The adaptation level of each strain was found to be related to its susceptibility to BAC. Following the classical procedure, the LD_{50} of CECT 5873 – the most susceptible strain – increased from 2.71 mg/l to 7.1 mg/l (2.61-fold) after five successive cultures in 5 days. However, the maximum adaptation of CECT 911 and CECT 4032 – both more resistant to BAC – was achieved after only one culture, with LD_{50} values of 5.06 mg/l (from 3.80, increasing 1.33-fold) and 9.19 (from 6.96, increasing 1.32-fold), respectively.

It was thus shown that, using stationary phase cells and the classical experimental protocol, some *L. monocytogenes* strains can reach the maximum level of adaptation to BAC in only one culture (**Figure 3.2.1**). This means that it is possible to reduce the experimental time needed to achieve BAC-adapted cells, which in previous studies were obtained after days or even weeks of culture (Aase et al. 2000; To et al. 2002; Romanova et al. 2006).

Although this experimental protocol is probably the most frequently used system for obtaining BAC-adapted-cells, the present results indicate that it is not optimum for all strains; therefore, each strain should be considered independently. In fact, the maximum adaptation capacity of *L. monocytogenes* CECT 5873 was not reached even after 5 successive cultures, whereas CECT 4032 and CECT 911 seemed to reach it after only one culture. This also indicates a higher adaptation potential of the strain CECT 5873. This strain was therefore chosen for subsequent studies in which the possibility of increasing the adaptation level was explored with exponential phase cells submitted to a single-step experimental design (see **Figure 3.2.1 B**).

An alternative strategy for obtaining BAC-adapted L. monocytogenes cells

Supported by the theory that exponential cells are metabolically more active than stationary-phase cells, in the present work we propose a new method for obtaining *L. monocytogenes* cells with a high degree of BAC adaptation in a short period of time based on only one exposure of exponential phase cells to sublethal concentrations of BAC. For this, the effects of the inoculum size and the concentration of BAC in the exposure on the level of adaptation achieved by *L. monocytogenes* CECT 5873 were explored by using a first-order

factorial design that allows both the individual and combined effects of these two variables to be determined.

The experimental results obtained were adequately described by the following empirical equation (r^2= 0.925), graphically shown in **Figure 3.2.3** (data are showed in the supporting information chapter):

$$LD_{50}=7,96-0,91B+1,84I+2,46BI \qquad [\mathbf{3.2.3}]$$

In which a significant positive individual effect of the *L. monocytogenes* inoculum size and a significant effect of the interaction between it and the concentration of BAC in the exposure wasfound. However, a negative individual effect of the benzalkonium chloride used in the exposure was also significant in the equation.

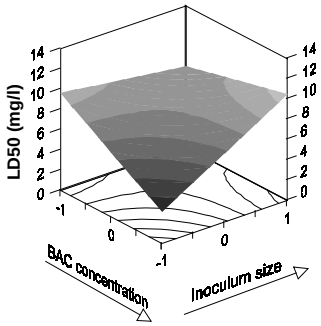

Figure 3.2.3. Effects of the Inoculum size and BAC concentration during the exposures on the resistance (LD_{50}) of non-adapted inocula of *L. monocytogenes* CECT 5873. Independent variables are expressed in coded values.

The significant first order negative term describing the individual effect of BAC reflects the biocide effect of BAC. This which exposing to low inoculum concentrations, could imply the absence of adaptation. Therefore, over [BAC: inoculum] ratios, *L. monocytogenes* does not grow. This is the case of the combination [1,-1], in which the basal resistance level (LD_{50} value of 2.71 mg/l) was used for calculation purposes. Therefore, it is recommended to determine the growth limits of the strain before setting the BAC concentrations to be used.

In the experimental ambit, the maximum resistance obtained by non-adapted CECT 5873 cells (LD_{50}=2.71 mg/l) was LD_{50}=10.0 mg/l, whereas using the classical multi-step procedure, the maximum value obtained for LD_{50} was 7.1 mg/l. In addition, adapted wild-type cells were obtained in only 33 hours, whereas 5 days were necessary with the classical procedure.

Proteomic patterns of non-BAC adapted and BAC adapted exponential-phase cells of L. monocytogenes* CECT 5873. *Preliminary results.

Proteomic patterns of non-BAC adapted (LD_{50}=2.71 mg/l) and BAC-adapted cells were compared (**Figure 3.2.4**). The statistical analysis of spots showed that 7 proteins (red circles) were down-regulated and 19 proteins with more than 0.1% non-BAC adapted cells were repressed (blue circles) as a result of adaptation to BAC. In contrast, only 4 proteins (green circles) were clearly up-regulated. It thus seems that the adaptation of *L. monocytogenes* to BAC leads to important protein repression. We are currently in the process of identifying these proteins, and the consequences of these changes will be studied in the future.

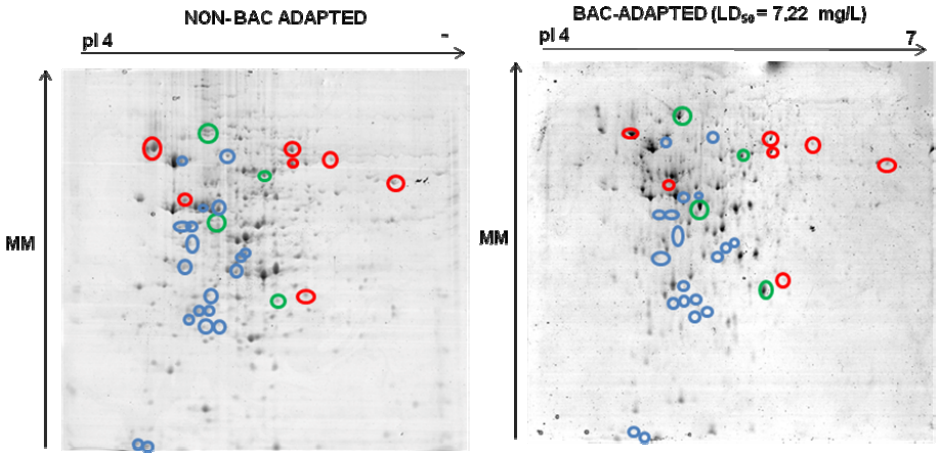

Figure 3.2.4: Proteomic patterns of non BAC-adapted and BAC-adapted exponential-phase cultures of *L. monocytogenes* CECT 5873

Isolation of BAC mutants of Listeria monocytogenes. Identification of genes and proteins associated with BAC resistance in L. monocytogenes

A bank of transposon mutants of *Listeria monocytogenes* EGDe and LO28 (Cao et al. 2007) was screened for mutants with respect to their BAC sensitivity or resistance, Several BAC mutants were isolated and confirmed by their MIC values. The region of transposon insertion of the resistant mutants was determined by sequencing the PCR products obtained using the appropriate Marq primers (Cao et al. 2007). The proteins predicted to be encoded by the disrupted genes were deduced from the ListiList website (http:/genolist.pasteur.fr/ListiList/). The MIC's of the selected mutants and the proteins encoded by the disrupted genes are shown in **Table 3.2.2**.

Tabla 3.2.2: MIC values (mg/l) of BAC in the wild type and isolated mutants of *L. monocytogenes.*

Strain	MIC of BAC (mg/l)	Identified protein
***L. monocytogenes* EGDe**	2.5	
1 sensitive mutant	2.0	
1 resistant mutant	6.5	lmo 2277: unknown protein
1 resistant mutant	6	stress response protein encoding DNA
***L. monocytogenes* LO28**	2.5	
1 resistant mutant	7.0	lmo 601: cell surface protein

The role of the protein encoded by *lmo2277* in the BAC resistance of *L. monocytogenes* EDGe is currently under investigation using further mutagenesis and cloning strategies.

4.2.4. Discussion

In this work it has been shown that the exposure of high concentrations of exponential cells of *L. monocytogenes* to sublethal BAC concentrations is a more efficient protocol than the classical method used in applied microbiology for obtaining cells with a high level of BAC adaptation. To achieve this, the individual and combined effects of both variables, the inoculum size and the concentration of BAC during exposure were studied using a first order factorial design.

The equation [**3.2.3**] highlights the relevant positive effect of the inoculum size, with two significant terms (individual and combined with BAC) in the empirical equation. There are some previous studies that have considered the inoculum effect to initiate growth in bacteria (Bidlas et al., 2008; Skandamis et al. 2007; Koutsoumanis and Sofos, 2005; Pascual et al. 2001; Robinson et al. 2001). Most of these works agree that increasing the inoculum levels allows *Listeria monocytogenes* to grow under more stressful environmental (pH, temperature, salt, temperature, a_w) or chemical and antimicrobial conditions (bacteriocins, sodium lactate, sodium diacetate). Two possible explanations for the inoculum effect are considered: 1) that it is due to the heterogeneity of the bacterial population, which means that the larger the inoculum size the longer the resistant fraction of the population (Skandamis et al, 2007; Koutsoumanis and Sofos, 2005; Pascual et al. 2001); and 2) that a co-operative effect due to cell-cell signalling (*quorum sensing*) occurs at high cell densities. However, this last explanation was only partially demonstrated by Robinson et al. (2001), who found that using spent medium caused a decrease in the lag phase of *L. monocytogenes*. However, these authors did not observe any significant changes in the probability of growth. Moreover, Bidlas et al. (2008) proposed a "composite model" that demonstrated in five different pathogenic bacteria (one of which was *Listeria innocua*) According to this model, the effect of the inoculum size is only the consequence of the time required for a specific inoculum size to start growing (TTD, time to detection of growth) and it is directly related to microbial number. This is in agreement with the present study, the cooperative effect associated with the inoculum size was checked by exposing *L. monocytogenes* adapted cells (LD_{50}=6.62) to different inoculum sizes and a concentration of BAC that allowed the BAC:inoculum size ratio to be constant. No significant differences were obtained in the resistance obtained after exposure (**Table 3.2.2**). The equation **3.2.3** ecuation reflects the larger the inoculums size the higher BAC concentration needed to kill the cells.

So, one evident consequence of the described inoculum effect is that increasing the microbial inoculum exposed to a set biocide concentration (BAC in our case) will decrease the time to growth of the exposed strain, thus facilitating adaptation and increasing cell resistance (measured as LD_{50}). This is precisely reflected by the significant I-containing terms of the equation [**3.2.3**].

The proposed procedure significantly reduces the time needed to obtain BAC-adapted *L. monocytogenes* cells in batch cultures. This is especially important considering that all the

methods in the literature are based on successive exposures to increasing BAC concentrations without increasing the inocula, which makes them to be tedious and time consuming.

Table 3.2.3: Effects of the inoculum size of the level of adaptation achieved by *L. monocytogenes* exposed to the same ratio BAC:cell.

Inoculum (ml)	[BAC] (mg/l)	µg[BAC]/cell.	LD_{50}
1	0.88	0.000045	5.58
5	4.09	0.000045	5.00
5	4.55	0.00005	6.62
10	7.5	0.000045	5.72
10	8.33	0.00005	6.39
15	11.54	0.00005	No growth

The results obtained are in agreement with two previous works which demonstrated that the metabolic activity of exponential cells allows them to develop adaptation mechanisms quicker than stationary phase cells exposed to sublethal concentrations of a biocide: Davies et al. 1996 studied the acid tolerance response (ATR) of *L. monocytogenes* and demonstrated that this response is growth-phase dependent and that the low metabolic activity of stationary phase cells implies a phase-specific resistance mechanism; and McMahon et al. (2000) found that *L. monocytogenes* has a higher adaptive response to heat-shock when working cultures were obtained after two successive cultures until the exponential phase instead of culturing twice untill stationary phase or one till stationary and the second one untill exponential phase. However, exponential-phase cells are more susceptible to biocides than stationary-phase cells (Luppens et al., 2001; Hayman et al. 2007; Weeks et al. 2006; Pascual et al. 2001). This could be a disadvantage of the proposed method, as biocide concentration during the exposure could not increase over the growth limit unless the inoculum concentration is increased sufficiently. But this fact will be also present in the classical method. This susceptibility of the cells to BAC is reflected in the negative first order term for the benzalkonium chloride, which indicates that it is important to control the concentration of the disinfectant carefully during the exposures.

In addition, we found significant differences in the expression of proteins between BAC non-adapted and semi-adapted (LD_{50}= 7.22 mg/l) *L. monocytogenes* CECT 5873 cells. Although such proteins should be identified further, the balance of protein expressed proteins showed that adaptation to BAC seems to imply a repression. There is little previous

information on this subject for *L. monocytogenes*. In fact, only two proteomic works were found with proteomics in *Listeria monocytogenes* (Mbandi et al., 2007; Folsom et al, 2007); however, only in the first changes in the protein profiles associated with the exposure to biocides were studied. They studied protein variations when the bacteria were exposed to the organic acids. Sodium diacetate treatment produced a higher number of unmatched proteins than sodium lactate (124 versus 53 in lactate), and a increase in protein expression (20 versus 5 in lactate) and in the number of novel proteins (90 versus 45 in lactate). The combination of the treatments involved a higher number of repressed proteins (41 versus ~30 in the single salt treatment). Six proteins were further investigated.

There have been few proteomic studies carried out on the resistance of bacteria to chemicals**.** Bore et al. (2007) also studied adapted tolerance to benzalkonium chloride *Escherichia coli* K-12 using transcriptome and proteome analyses. Changes in the expression levels of adapted cells were shown for porins, drug transporters, glycolytic enzymes, ribosomal subunits and several genes and proteins involved in protection against oxidative stress and antibiotics. However, they found that genes related to these functions were mainly up-regulated, which is in contrast with the present results.

In summary, the results obtained permitted us to propose a new method for obtaining *Listeria monocytogenes* cells with a higher level of BAC adaptation in a shorter period of time than the classical method based on successive cultures of stationary phase cells in sublethal BAC concentrations. This method consists in only one exposure of a high concentration of exponential phase cells to sublethal BAC concentrations, always under the growth limit of the strain for BAC.

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3.3. Resistance to benzalkonium chloride, peracetic acid and nisin during formation of mature biofilms by *Listeria monocytogenes.*

3.3. RESISTANCE TO BENZALKONIUM CHLORIDE, PERACETIC ACID AND NISIN DURING FORMATION OF MATURE BIOFILMS BY *LISTERIA MONOCYTOGENES*

Increase of resistance to the application of benzalkonium chloride (BAC), peracetic acid (PA) and nisin during biofilm formation at 25 ºC by three strains of *L. monocytogenes* (CECT 911, CECT 4032, CECT 5873 and BAC-adapted CECT 5873) in different scenarios was compared. For this purpose, resistance after 4 and 11 days of biofilm formation was quantified in terms of lethal dose 90% values (LD_{90}), determined according with a dose-response logistic mathematical model. Microscopic analyses after 4 and 11 days of *L. monocytogenes* biofilm formation were also carried out. Results demonstrated a relation between the microscopic structure and the resistance to the assayed biocides in matured biofilms. The worst cases being biofilms formed by the strain 4032 (in both stainless steel and polypropylene), which showed a complex "*cloud type*" structure that correlates with the highest resistance of this strain against the three biocides during biofilm maturation. However, that increase in resistance and complexity appeared not to be dependent on initial bacterial adherence, thus indicating mature biofilms rather than planctonic cells or early-stage biofilms must be considered when disinfection protocols have to be optimized. PA seemed to be the most effective of the three disinfectants used for biofilms. We hypothesized both its high oxidising capacity and low molecular size could suppose an advantage for its penetration inside the biofilm. We also demonstrated that organic material counteract with the biocides, thus indicating the importance of improving cleaning protocols. Finally, by comparing strains 5873 and 5873 adapted to BAC, several adaptative cross-responses between BAC and nisin or peracetic acid were identified.

3.3.1. Introduction

Listeria monocytogenes is a psychotrophic pathogenic bacterium (O'Driscoll et al., 1996) that is found throughout the food industry (Kastbjerg and Gram, 2009). The ability of *L. monocytogenes* to adhere to surfaces and form biofilms has been demonstrated in numerous studies (Adrião et al., 2008; Beresford et al., 2001; Blackman and Frank, 1996; Palmer et al., 2007; Smoot and Pierson, 1998; Rieu et al., 2008; Somers and Wong, 2004, Takahashi et al., 2010). The formation of *L. monocytogenes* biofilms is frequently evaluated by quantifying the biomass (Bonaventura Di, 2008; Djordjevic et al., 2002; Harvey et al., 2007; Folsom et al., 2006), using sometimes *in situ* microscopic analyses (Borucky et al., 2003; Chae and Scraft, 2000; Chavant et al., 2004; Kalmokoff et al., 2001; Rodríguez et al., 2008; Moltz and Martin, 2005). However, quantifying the total biomass does not permit to determine the consequences of different biofilm structure and composition.

One consequence of biofilm formation is the acquisition of (adaptive) resistance to cleaning and disinfection agents, which can lead to serious economic and health problems (Aarnisalo et al., 2007; Gram et al., 2007; Leriche et al., 1999; Minei et al., 2008). Therefore, the efficacy of different biocides against *L. monocytogenes* biofilms has been examined in various studies (Aarnisalo et al., 2000; Amalaradjou et al., 2009; Ammor et al., 2004; Chavant et al., 2002; Rieu et al., 2010; Tyh-JenK and Frank, 1993; Yang et al., 2009). Industrial disinfectants including quaternary ammonium compounds, alcohols, chlorinated compounds, and other oxidizing agents such as peracetic acid, ozone and peroxide derivatives among others, have been tested in most of these studies (Aarnisalo et al., 2007; Frank et al., 2003; Pan et al., 2006; Krysinski and Brown, 1992; González-Fandos et al., 2005). In contrast, there are few studies on natural antimicrobials, and only some essential oils (Chorianopoulus et al., 2007; Oliveira et al., 2010; Sandasi et al., 2008; Sandasi et al., 2010; Tajkarimi et al., 2010) and lactic acid bacterial biofilms (Leriche et al., 1999; Minei et al., 2008; Guerrieri et al., 2009) have been tested for controlling *L. monocytogenes* biofilms.

However, immature biofilms were used in most studies, which greatly affects the efficacy of disinfectants (72 h: Aarnisalo et al., 2007; 24 h: Bonaventura Di et al., 2008; 40 h: Borucki et al., 2003; 28 h: Frank et al., 2003; 48 h: Pan et al., 2006). Only Gram et al. (2007) and Kastbjerg and Gram, (2009) simulated real conditions in their studies by allowing the biofilms to mature for up to seven days. In addition, the experimental methods

used to quantify the sensitivity of biofilms to disinfectants involve determining the minimum inhibitory concentration (MIC), which can lead to underestimating the efficacy of the disinfectant under study, as noted by other authors (Kastbjerg and Gram, 2009).

Therefore, this study aimed to compare the mature biofilms formed by three strains of *L. monocytogenes* in different scenarios by determining the increase in resistance to benzalkonium chloride (BAC), peracetic acid (PA) and nisin. This will allow us to elucidate some of the real repercussions of *L. monocytogenes* biofilm formation in different scenarios. BAC belongs to the quaternary ammonium chloride (QUACs) family and was chosen due to its widespread use as an industrial disinfectant. PA was chosen due to its efficacy and degradability (Briñez et al 2006), and nisin due to the lack of studies on it and because it is a natural antimicrobial agent widely used in foods (Cabo et al., 2009; Zhao et al., 2004). In addition, microscopic analysis was used to determine whether the increase in resistance to disinfectants is related to any significant changes in the structure of the biofilms formed by *L. monocytogenes*.

3.3.2. Material and methods

Bacterial strains and synthetic media

Listeria monocytogenes CECT 5873, CECT 911, CECT 4032 were provided by the Spanish Type Culture Collection (Valencia). Strain 5873 adapted to BAC (5873A) was obtained in a previous experiment though sequential exposure of the strain 5873 to sub-lethal concentrations of BAC (LD_{50}: 2.71 mg/l; BAC-adapted strain: 7.22 mg/l) (see **section 3.2**). Strains were frozen and stored at -80º C in TSB containing 50% glycerol (v/v) until use. Whenever required, one cryovial was thawed and subcultured twice in TSB (Cultimed, S.L., Spain) at 37 ºC before use for activation.

Food soils media

Mussel cooking juice (MCJ) and intervalvar water of mussel (IWM), used as culture media to simulate contamination of surfaces in plants processing cooked mussel and live mussel, were prepared as it was previously described (Saá et al. 2009). The composition of

MCJ and IWM are shown in **Table 3.3.1**. pH was adjusted to 7 in both media and autoclaved to 121 ºC for 15 min for sterilization.

Table 3.3.1: Composition of mussel cooking juice (MCJ) and intervalvar water of mussel (IWM)

	MCJ	IMW
Carbohydrate (g/l)	9,19	2,7
Nitrogen (g/l)	1.70	1.85

Biocides

Benzalkonium chloride was purchased from Fragon Iberica S.A.U. Terrassa (Barcelona). Pure nisin was obtained from Applin and Barrett (Terrassa, Spain). Peracetic acid (40% in acetic acid/water) was obtained from Fluka (S.L. purum from disinfection). Before each assay, real peracetic acid concentrations were checked by using a colorimetric test (Merkoquant Peressigsäure-test Merck).

Food processing surfaces

Stainless steel (AISI-304, 2B finish, 0.8 mm thickness) and polypropylene sheets (compact 00226) were cut into coupons (20 mm x 20 mm). Prior to being used, PP coupons were cleaned with alcohol and left overnight in distilled water. New and reused SS coupons were soaked in 2M NaOH to remove any grease or food soils left and then rinsed several times with distilled water. Coupons were dried before use in a laminar flow cabinet.

Inocula preparation

Inoculum was prepared by adjusting the activated culture to an absorbance 0.1 at 700 nm, which correspond to a cell density of 10^8 CFU/ml according with previous calibration. Once centrifuged at 6000 rpm, 25 ºC during 10 min, collected cells were resuspended in the same volume of TSB (planctonic system) or in food soils media (MCJ, IWM) for the sessile system. As the biofilms were washed up (see below) before the application of the biocides, it was considered more adequate for comparison to use TSB instead of residual media in the experiments with planctonic cells. This cell suspension was directly used as inoculum in

experiments with sessile cells. In planctonic cells, cell suspension was diluted (1:10) to achieve approximately the same number of initial cells (**Table 3.3.2**) in both systems, according with previous experiments.

Table 3.3.2: Average of total planctonic and matured biofilms cells number (CFU) of *L. monocytogenes* exposed to disinfectans for each experiment.

	911-PP-IWM	4032-SS-MCJ	4032-PP-MCJ	5873-PP-MCJ	5873A-PP-MCJ
PLANT	2,95E+07	4,28E+07	4,28E+07	3,72E+07	1,78E+07
BM.4D	1,11E+07	3,20E+08	2,78E+07	1,30E+07	1,39E+07
BM.11D	1,74E+07	2,14E+07	1,68E+08	2,49E+07	3,83E+07

BM 4D: Mature biofilms obtained after 4 days of incubation at 25º C
BM 11D: Mature biofilms obtained after 11 days of incubation at 25 ºC
PP: polypropylene. SS: stainless steel
MCJ: mussel cooking juice. IWM: intervalvar mussel water.
PLANT: Planctonic cells

Experimental systems

- Planctonic system*:* 0.5 ml of inoculum was added in each tube and immediately used in the dose-response assays.
- Sessile system*:* cleaned coupons were placed into mini glass Petri plates for the assessment of biofilm formation. Polypropylene (PP) coupons were fixed by photo stickers to the Petri plates. Then, stainless steel and polypropylene (PP) coupons were packed 5 by 5 with foil and autoclaving for 20 min at 121 ºC. Once autoclaved 9 ml of inoculum was added in Petri plates and incubated at 25 ºC for biofilm formation. After 4 and 11 days, two replicate coupons were taken out of the incubator at each sampling time and rinsed with 10 ml of PBS for 10 s to release non-adhered cells and used for antimicrobial assays and microscopic analyses. Each experiment was repeated three times.

According with previous studies in which different strains, media, surface material and surface conditioning were compared (Saá et al., 2009), the following experimental cases were selected for this study. They pretend to be representative of different scenarios that could occur when *L. monocytogenes* adheres in plants processing mussel giving rise to biofilms with different thickness:

- CECT 911 on polypropylene and intervalvar mussel water (PP-IMW). That corresponded with the conditions that gave rise to the highest level of adherence.
- Strain 4032 in polypropylene and stainless steel using MCJ as medium. This strain showed the highest adhesion ability in both materials.
- Strain 5873 in polypropylene in MCJ. It showed the lowest level of adherence out from 16 experimental cases assayed. In this work we use this scenario to compare the biofilms formed by this strain (5873 wild type) and the physiologically-BAC-adapted (5873A)**.**

Dose-response assays

In the planctonic system: 0.5ml aliquots of the biocides (benzalkonium chloride, peracetic acid and nisin) at various doses were added to 0.5 ml of the culture.

In the case of sessile cells: after 4 and 11 days of incubation, coupons were drained and washed as previously described before the biocide dosification. 0.5ml aliquots of the biocides were added to the formed biofilms at various doses covering the total coupon surface. Doses were as follows:

- Benzalkonium chloride (mg/l): 2.5, 5,10, 17, 25, 50, 100, 250, 500, 1000
- Peracetic acid (mg/l): 1, 2.5, 5, 10, 17, 25, 50, 100, 250, 500
- Nisin (UI/ml): 5, 10, 15, 20, 50, 75, 100, 125, 150, 165, 185, 200, 400, 1500.

Several blanks with distilled water instead of antimicrobial were included in each experimental series. All experiments were carried out at 25 ºC.

After dosage and a time of exposition of 10 minutes, the biocides had to be neutralised. BAC and peracetic acid were neutralized by adding to a ratio 9:1 (v/v), a solution comprising 10 ml of a buffer containing 34 g/l KH_2PO_4 adjusted to pH 7.2 with NaOH, 3 g/l soybean lecithin, 30 ml/l Tween 80, 5 g/l $Na_2S_2O_3$ and 1 g/l L-histidine. This solution was applied during 1 min at room temperature, which was considered sufficient according with preliminary experiments. Nisin was neutralized by culturing surviving cells in Nutrient Agar previously adjusted to pH=9. The lack of effect of this pH on the growth of all the strains of *L. monocytogenes* was checked in preliminary experiments.

After neutralization, in the sessile system, adhered cells were collected by thoroughly rubbing with two moistened swabs and resuspended by vigorously vortexing swabs for 50 s in 9 ml of peptone water according to Herrera et al., 2007. Then adhered cells were determined by plating appropriate serial dilutions of this suspension on Triptone Soy Agar and subsequent incubation at 37 ºC for 24 h. Peptone water (Cultimed, S.A.) was always used as dilution medium.

Percentage mortality was calculated according to equation **[3.3.1]**:

$$M\ (\%) = 100 * \left(\frac{NTVC - NVC}{NTVC} \right), \text{ where:} \qquad \textbf{[3.3.1]}$$

- M: Percentage of death (%)
- NTVC: number of total viable cells (CFU/ml) in the planctonic system or total adhered cells (CFU/mm^2) in the sessile system.
- NVC: number of surviving planctonic or adhered cells after treatment with the corresponding doses of biocides (in CFU/ml or CFU/mm^2).

Determination of the resistance: calculation of Lethal Dose 90 (LD_{90})

LD_{90} values were used as a direct measurement of the biofilm resistance to the biocides. Values for this parameter were obtained by adjusting experimental data of mortality obtained in the dose-response assays to the following modified logistic equation obtained according with previous works (Cabo et al., 1999; Cabo et al., 2009):

$$M\ (\%) = k\left(\frac{1}{1 + 0.11e^{r(LD90 - D)}} - \frac{1}{0.11e^{rLD90} + 1} \right) \qquad \textbf{[3.3.2]}$$

- LD_{90}: Dose that kills 90% of initial population
- k: Maximum inhibition value
- r: Specific inhibition coefficient (dimensions: inverse of the dose)
- D: Dose

Microscopic analyses

Sessile cells were fixed in 2% glutaraldehyde with 0.1 M cacodylate buffer (pH 7.4) for at least 2 h at 4º C. The fixing solution was washed out with PBS, and samples were dehydrated with a series of increasing concentrations of ethanol: 30, 50, 70, 80 and 95% for 30 min each, and pure ethanol for 1 h. Ethanol was progressively replaced with amyloacetate and then samples were subjected to critical point drying with CO_2 (73 atm; 31.3º C). Afterwards, samples were coated with gold (10-20 nm), and later observed by using a Philips XL30 Scanning Electronic Microscope.

Statistical analysis

The fits of the models to the experimental data were performed according to a least-squares method (quasi-Newton). Significant differences between the obtained LD_{90} values were identified by a one-way analysis of variance (Anova).

3.3.3. Results

Resistance to benzalkonium chloride (BAC) of planctonic cells and mature L. monocytogenes biofilms

Values of the parameters (r and k) obtained after the mortality values were adjusted to equation **[3.3.2]** are shown in **Table 3.3.3**.

We compared the LD_{90} values obtained for planctonic cells and maturebiofilms (4 and 11 days old) formed by different strains of *L.monocytogenes* (911-PP-IWM, 4032-SS-MCJ and 5873-PP-MCJ) after the exposition to BAC (**Figure 3.3.1**). To study whether the physiological adaptation of *L. monocytogenes* to BAC results in the formation of more-resistant mature biofilms, the strain CECT 5873A was included in this and subsequent studies.

LD_{90} obtained for the planctonic cells in TSB (**Table 3.3.2**) were significantly ($p < 0.05$) higher for the CECT 4032 strain, which was the most resistant to the disinfectants tested, followed by the wild 5873 and 911 strains.

Table 3.3.3:Values of the parameters (k and r) obtained after fitting of experimental data to eq [3.3.2]:

	911-PP-IWM		4032-SS-MCJ		4032-PP-MCJ		5873-PP-MCJ		5873A-PP-MCJ	
	BM. 4D	BM.11D	BM. 4D	BM.11D	BM. 4D	BM.11D	BM. 4D	BM.11D	BM. 4D	BM.11D
K_{BAC}	97.363	95.506	99.65	99.450	99.060	93.188	99.477	99.097	99.748	91.275
r_{BAC}	0.308	0.1746	0.053	0.033	0.235	0.007	0.256	0.231	0.138	0.404
K_{nisin}	99.595	99.056	86.413	91.913	84.872	87.189	69.412	83.274	76.843	89.048
r_{nisin}	0.013	0.007	0.215	0.006	0.255	0.023	0.028	0.327	0.0730	0.029
K_{PA}	99.342	99.810	99.704	99.736	99.998	97.457	98.976	98.560	94.353	99.883
r_{PA}	0.330	0.419	0.169	0.069	0.480	0.097	0.439	0.263	2.816	2.255

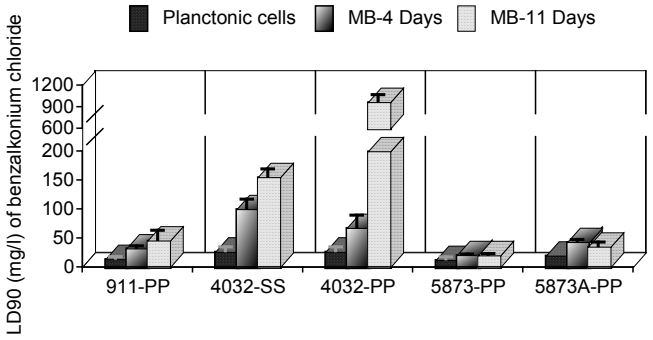

Figure 3.3.1: Values of LD_{90} (mg/l) obtained after the exposure of planctonic cells and mature biofilms of *L. monocytogenes* to benzalkonium chloride (BAC).

A significant increase ($p<0.05$) in LD values (i.e. resistance) between planctonic cells and biofilms was observed. However, whereas no significant difference was observed between the resistance to BAC of biofilms formed by 5873 on polypropylene (PP) after 4 and 11 days, the resistance of biofilms formed by the strains 911 and 4032 increased significantly ($p<0.05$) as they matured, and was quantitatively higher in the case of 4032 on both stainless steel and polypropylene. On stainless steel (4032-SS-MCJ), the resistance (LD_{90} values) in

biofilms incubated for 4 and 11 days was multiplied by factors of 3.7 and 6 respectively with respect to the planctonic system. On polypropylene (4032-PP-MCJ), the resistance increased by a factor of 36 after 11 days with respect to the planctonic system.

Resistance to nisin of planctonic cells and mature L. monocytogenes biofilms

The results obtained for planctonic cells showed that strain 911 were the most sensitive to nisin, followed by the strain 5873 adapted to BAC. Statistically similar results were obtained for the other two strains ($p < 0.05$).

As for BAC, a significant increase ($p<0.05$) in resistance to nisin was found at both 4 and 11 days of biofilm maturation for the planctonic cells in all the assayed scenarios (**Figure 3.3.2**). No significant differences in LD_{90} values obtained for the biofilms formed in the different scenarios after 4 days were observed. However, after 11 days of incubation, biofilms formed by strain 4032 on stainless steel were significantly more resistant than those formed by the same strain on polypropylene and by those formed by strain 911.

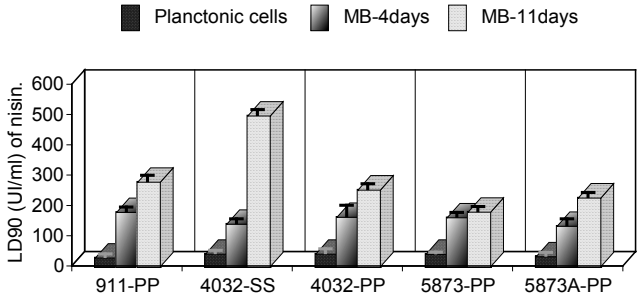

Figure 3.3.2: Values of LD_{90} (mg/l) obtained after the exposition of planctonic cells and matured biofilms of *L. monocytogenes* to nisin.

Biofilms formed by the strain 5873 were the most sensitive and did not show significant differences during biofilm maturation. However, a significant increase in resistance ($p < 0.05$) in the BAC-adapted strain with respect to biofilms formed by the non-adapted strain was observed after 11 days of incubation.

Beside LD_{90}, k and r parameter values obtained from the model (**Table 3.3.3**) could also be helpful for comparative purposes. In the particular case of nisin, two important

observations can be made: 1) the lower maximum inhibition values (parameter k) respecting to those obtained for the other antimicrobial agents, and 2) the very low specific growth rate (parameter r), especially in the scenario 4032-SS.

Resistance to peracetic acid of planctonic cells and mature L. monocytogenes biofilms

Surprisingly, in the case of peracetic acid, the LD_{90} values were significantly higher for planctonic cells than for mature biofilms at both 4 and 11 days of incubation (**Figure 3.3.3**).

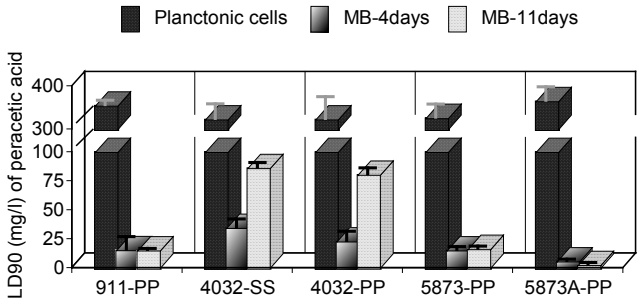

Figure 3.3.3: Values of LD_{90} (mg/l) obtained after the exposition of planctonic cells and matured biofilms of *L. monocytogenes* to peracetic acid (PA).

No significant differences in PA sensitivity were observed between any of the three strains in their planctonic state ($p > 0.05$).

During biofilm formation, a significant increase in resistance ($p < 0.05$) between 4 and 11 days of maturation was only observed for strain 4032. However, in the case of peracetic acid this increase was independent of the host material used. Finally, a lower LD_{90} value was obtained for mature 4 and 11 day old biofilms of the BAC-adapted strain 5873 with respect to the corresponding wild strain

Microscopic analysis of L. monocytogenes biofilms formed under the different scenarios studied

The images obtained in all assayed scenarios after 4 and 11 days of maturation are showed in **Figure 3.3.4**. Three levels of biofilm thickness could be observed in the three different *L. monocytogenes* strains:

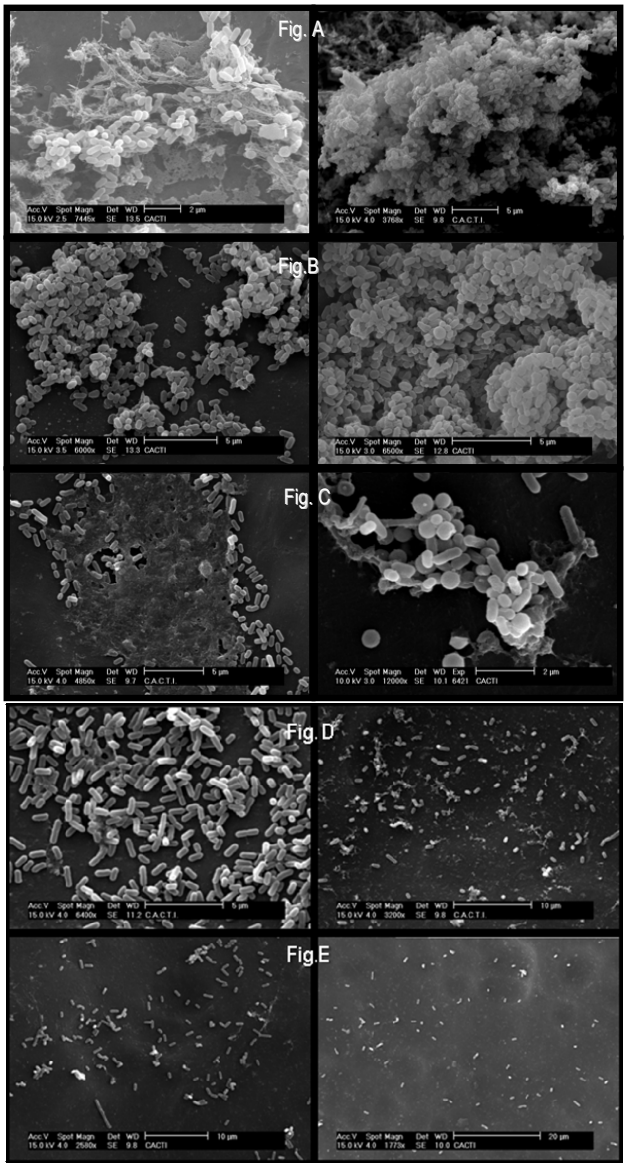

Figure 3.3.4: Scanning electronic microscopy (SEM) images of strains CECT 4032 (4b) in SS (**A**) and PP (**B**), CECT 911 (1/2 c) in PP (**C**)and CECT 5873 (**D**) and 5873A in PP (**E**) after 4 (right side) and 11 days (left side) .

- Strain CECT 4032 (SS and PP) has the most complex structure, with a cloud-type cell distribution that makes contact between the cells and the biocide more difficult.

- Strain CECT 911 shows a structure of intermediate complexity. The cells form a monolayer at 4 days and some isolated microcolony-type formations are visible after 11 days.

- Strain CECT 5873, both the wild type and BAC-adapted (5873A), forms the simplest structure, namely a monolayer, and shows the least resistance to the biocides tested.

Study of the relationship between the adherence kinetics of L. monocytogenes and mature biofilm formation

The adherence kinetics of the different *L. monocytogenes* strains in all assayed scenarios are represented in **Figure 3.3.5**. Results showed that the strain 4032, which forms the thickest biofilms according to the microscopic images, has a slightly (but not significant) higher maximum level of adherence than the other two strains, with no significant differences between polypropylene and stainless steel. No significant differences were found between the maximum number of adhered *Listeria* cells in the rest of the scenarios.

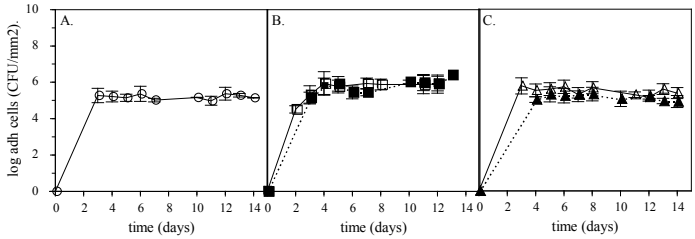

Figure 3.3.5: Adherence kinetics of *Listeria monocytogenes* in different experimental cases.
A.: CECT 911 on polypropylene (○) in the presence of intervalvar water (IWM).
B.:CECT 4032 on stainles steel (□) in the presence of cooked mussel water (MCW) and on polypropylene (■) in MCW.
C.: CECT 5873 (Δ) and CECT 5873A (BAC-adapted) (▲) on polypropylene in MCW.

3.3.4. Discussion

The mature biofilms formed by the three strains of *L. monocytogenes* in different scenarios were compared by studying the increase in resistance to three biocides (benzalkonium chloride, peracetic acid and nisin) with SEM analysis of their microstructures after incubation for 4 and 11 days at 25 °C.

The results showed a clear relation between the structure of the biofilms formed and the acquisition of resistance to BAC and nisin from the planctonic state, with strain 4032 (serotype 4b) developing the thickest ("*cloud shape*") and most biocide-resistant structure, presumably as a result of reduced access to the bacterial cells. In contrast, the increase in resistance does not appear to be correlated with the adherence levels during biofilm formation or with each strain's sensitivity in the planctonic state to the biocides tested. Concerning the first, when comparing obtained results with the adherence levels previously obtained during the initial stages of biofilm development (72 hours) (Chae et al., 2006; Saá et al., 2009), it can be seen that the highest adherence levels were obtained by strain CECT 911 after 40 hours of incubation, whereas the initial adherence of strain CECT 4032 to stainless steel was surprisingly low. On the contrary, the material has a specific effect on the biofilm formed for BAC with higher resistance of biofilms formed on stainless steel, although the opposite effect (higher resistance of biofilms formed on polypropylene) was obtained for nisin.

Furthermore, this structure-resistance relation supports the theory that the increased resistance associated with the formation of biofilms is due more to the population structure in the sessile state than to the associated metabolic and physiological changes (including quorum sensing) that occur at cellular level (Folsom et al., 2006; Kalmokoff et al., 2001). Indeed, this hypothesis could explain the results reported by other groups (Kastbjerg and Gram, 2009; Stopforth et al., 2002), who found no significant differences in disinfectant sensitivity between the planctonic and sessile stages of *L. monocytogenes*, as is the case with strain 5873 and BAC here.

Biofilm maturation does not, however, have the same effect on the action of peracetic acid, with the lethal doses obtained being higher for planctonic cells in the presence of organic matter (present in TSB). This is likely due to interference by the organic material found in TSB, which reduces the effective concentration of this compound due to its high

reactivity and non-specificity. Indeed, it was found that using PBS as the culture medium led to LD values similar to those obtained for biofilms at 4 days (data not shown).

These counterproductive effects of the organic matter highlight the importance of effective cleaning protocols prior to disinfection to optimize the action of disinfectants, as has been previously reported in other experimental systems (Briñez et al., 2006; Fatemi and Frank, 1999; Gram et al., 2007; González-Fandos et al., 2005).

Moreover, the results obtained suggest that PA is more effective against biofilms than BAC and nisin, as reported previously (Fatemi and Frank, 1999; Stopforth et al., 2002). In fact, no significant increase in resistance (LD_{90} values) was observed in the 911 and 5873 scenarios during biofilm maturation, and the increase in resistance during biofilm formation of strain 4032 was not significantly different on either of the materials. This could be due to the physicochemical properties of PA: 1) its high reactivity and oxidizing ability, together with its higher decomposition rate, facilitate its entry into the biofilm matrix and greatly reduce its effective concentration in systems containing large amounts of organic matter (such as that containing planctonic cells); and 2) its small size, which also greatly increases its ability to penetrate the biofilm network. In fact, previous studies performed with ozone (a molecule that also has high reactivity and a small size) in our laboratory showed that it is more effective on *Staphylococcus aureus* biofilms than on planctonic cells (Cabo et al., 2009). In contrast, the structure of the biofilm presents a significant physical barrier to the entry of BAC and nisin, thereby reducing their ability to diffuse into the matrix and reach the cells, although with some differences. Indeed, the results obtained highlight two reasons why nisin is less effective than BAC for disinfecting surfaces potentially contaminated with *L. monocytogenes*: 1) it acts at a slower rate, as shown by the lower r values (**Table 3.3.3**) and has lower efficacy which indicates that nisin has less capacity to eliminate the entire bacterial population, as shown by the maximum inhibition values (k parameter, **Table 3.3.3**), which in most cases are lower than 90%.

The different mechanisms of action of BAC and nisin (destabilization of the cytoplasmic membrane) and PA (it is intracellular and much more non-specific) probably explain why mature biofilms formed by the BAC-adapted strain 5873 are more resistant to BAC and nisin than the wild strain (particularly after 11 days of incubation) and more sensitive to PA at both 4 and 11 days of incubation. Furthermore, these results also highlight why the cross-

responses to adaptations acquired upon exposure of a strain to sub-lethal concentrations of industrial disinfectants should be studied: First, studies would allow risk situations that could arise from the use of a particular disinfectant, and which may generate positive cross-responses (as is the case with BAC and nisin), to be identified. Second, effective treatments, which generate a negative cross-response (as is the case with BAC and PA) could be developed that could be used if resistance to a disinfectant used in a processing plant is detected. Only one similar study (Stopforth et al., 2002), in which the authors found no cross-response between the adaptation of *L. monocytogenes* biofilms to acid and subsequent exposure to PA, BAC and sodium hypochlorite, has been reported in the literature.

Assuming that the increased biocide resistance is related to the ability of the adhered cells to form three-dimensional cloud-type (strain 4032-SS-11D) or flat structures (911-4D), or microcolonies (5873) consisting of a complicated network of bacterial cells and secreted extracellular polysaccharides (EPSs), it becomes clear that an understanding of the composition and structure of the extracellular matrix is essential in order to improve the efficacy of the disinfection strategies currently used. Despite this, and although several studies appear to indicate that the structure of *L. monocytogenes* biofilms differs from the classical fungus-type model (Dubravka et al., 2009; Blaschecket al., 2007) the chemical composition of these EPSs and their spatial distribution is unknown. Most of the studies reported are focused on the factors that affect the cell's ability to form biofilms, such as variations in the fatty acid composition (Gianotti et al., 2008) or the role of N-acylated homoserine lactones (Belval et al., 2006). Such an understanding, and the application of treatments prior to disinfection that ensure disintegration of the extracellular matrix, would allow current cleaning protocols to be improved, thereby increasing the efficacy of those disinfectants that are unable to penetrate the extracellular matrix of biofilms.

The experimental procedures considered here are also an improvement on those frequently used as they use pure active ingredients that avoid multi-resistance responses. Moreover, setting out the results in terms of microbial kinetics equations allows the bacterial resistance (LD_{90}) to be quantified appropriately and an objective comparison can be made between different test cases. The majority of studies reported in the literature use semi-quantitative methods (MIC) and compare commercial disinfectants with unknown compositions or mixtures of different active ingredients, which makes determining a cause-effect relationship more difficult (Frank et al., 2003; González-Fandos et al., 2005; Jacquet

and Reynaud, 1994). Other studies only determine the response of the population on a short timescale and with two or three biocide concentrations (Aarnisalo et al., 2000; Thy-Jenq et al., 1993).

In conclusion, we have demonstrated that 1) the acquired resistance to disinfectants during maduration of *L. monocytogenes* biofilms reflects the possible consequences of biofilms rather than adherence, 2) the microscopic structure of mature biofilms correlates with resistance to disinfectants, and 3) biofilms formed by BAC-resistant *L. monocytogenes* CECT 5873 are more resistant to BAC and nisin but more sensitive to PA.

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3.4. Adherence kinetics, resistance to benzalkonium chloride and microscopic analysis of mixed biofilms formed by *L. monocytogenes* and *Pseudomonas putida*

3.4 ADHERENCE KINETICS, RESISTANCE TO BENZALKONIUM CHLORIDE AND MICROSCOPIC ANALYSIS OF MIXED BIOFILMS FORMED BY *LISTERIA MONOCYTOGENES* AND *PSEUDOMONAS PUTIDA*.

Comparison between the resistance to BAC and the microscopic structure of mixed-species biofilms formed by different strains of *L. monocytogenes* and *Pseudomonas putida* CECT 845 under different scenarios and these obtained by the corresponding mono-species *L. monocytogenes* biofilm was carried out. The association of *P. putida* with *L. monocytogenes* quickens biofilm formation and increases significantly ($p<0.05$) the BAC-resistance of the biofilm after 4 days of incubation at 25 ºC respect to monospecies biofilms. According with the adherence profiles of *P. putida*, two different patterns of association (A and B) were observed, being type A pattern much more resistant to BAC. After 11 days of incubation, in 2 out of 5 experimental cases (4032 and BAC-adapted 5873 on polypropylene) a destructuration of mixed biofilms occurred, being accompanied by a sharp decrease in the number of adhered cells. Microscopic analyses demonstrated that the most complex three-dimensional structure showed the highest resistance to BAC (4032-SS). Results clearly highlight that it is necessary to consider natural bacterial association for improving the safety effectiveness of disinfection protocols.

3.4.1. Introduction

Listeria monocytogenes is a pathogenic bacterium implicated in numerous foodborne outbreaks (Denny et al., 2008; Gilmour et al., 2010). It is widely recognized that one of the most frequent contamination routes of *L. monocytogenes* in foods is through cross contamination from surfaces in food processing plants (Ammor et al., 2004; Norwood and Gilmour, 1999; Porsby et al., 2008), where it is able to attach and form resistant biofilms (Aase et al., 2000; To et al., 2002; Takahashi et al., 2009). This resistance is related to the three-dimensional structure of the biofilm, which defines the interactions between the bacterial species (Wuertz et al., 2004) and determines the degree to which chemicals can access the cells (Bourion and Cerf, 1996, Qu et al., 2010). Moreover, it has been demonstrated that biofilm cells are phenotypically different from the corresponding planktonic cells (Nadell et al., 2008).

But the situation is even more complicated, as it is known that more than two species coexist in real biofilms and it has been demonstrated that this could increase their resistance to biocides (Sharma and Anand, 2002; Kastbjerg and Gram, 2009). Some studies on *L. monocytogenes* have analyzed the attachment of multispecies biofilms involving this bacterium, with different results. It has been found that in some cases the association with other bacteria increases the number of adhered cells in the mixed biofilm: *L. monocytogenes* with *Pseudomonas* (Sasahara and Zottola, 1993; Marshall and Smimidt, 1988; Hassan et al., 2003) or with *Flavobacterium* (Bremer et al., 2001). However, other studies have found that the number of adhered cells in the mixed-biofilm decreased: *L. monocytogenes* with *Pseudomonas fragi* and *Staphylococcus xylosus* (Norwood and Gilmour, 2001); with *Staphyloccocus* sciuri (Leriche et al., 2000); and with *E. faecium* (Minei et al., 2008). Lastly, some studies found that the number of adhered cells did not vary due to the presence of other species in the biofilm: *L. monocytogenes* with *Staphyloccocus aureus* (Rieu et al., 2008), except for one strain; and with *Salmonella enterica* and *Ps. putida* (Chorianopulos et al., 2007).

Although it is recognized that bacteria in nature form complex mixed-species communities, the European standard EN 13697 points out that adhered cells should be obtained by inoculation of the target microorganism and subsequent drying for testing the bactericidal activity of chemical disinfectants. However, following this procedure, the effectiveness of

disinfectants will be overestimated. Even though there are few studies on disinfection against mixed biofilms (Bourion and Cerf, 1996; Bremer et al., 2002; Chlorianopoulous et al., 2007; Fatemi and Frank, 1999; Lebert et al., 2009; Minei et al., 2007), thus, to improve the efficacy of disinfection systems commonly used it is necessary to continue studying the repercussions of the increased resistance to disinfectants related to the association between *L. monocytogenes* and other bacterial genera that are normally present in the environment, such as *Pseudomonas* species.

Thus, the main aim of this work was to study the association between *L. monocytogenes* and *Pseudomonas putida* by comparing the resistance to BAC and the microscopic structure of the dual-species biofilm with that of *L. monocytogenes* mono-species biofilms.

3.4.2. Material and methods

Bacterial strains

Listeria monocytogenes, CECT 911, CECT 4032, CECT 5873 and *Ps. putida* 845 were provided by the Spanish Type Culture Collection (Valencia). Strain 5873 was adapted to BAC in previous experiments after two exposures of the wild type strain 5873 to sub-lethal concentrations of BAC (wild strain LD_{50}: 2.71 mg/l; BAC-adapted strain LD_{50}: 7.22 mg/l, **see section 3.2**). Strains were frozen and stored at -80 ºC in TSB containing 50% glycerol (v/v) until use. Whenever required, one cryovial was thawed and subcultured twice in TSB (Cultimed, S.L., Spain) at 37 ºC or at 31º C for activation of *Listeria monocytogenes* and *Pseudomonas putida*, respectively.

Food soils media

In order to simulate contamination of surfaces in plants processing cooked mussels and live mussel, mussel cooking juice (MCJ) and intervalvar water of mussel (IWM) were used as culture media (Saá Ibusquiza et al. 2010, **see section 3.2**). The composition of MCJ and IWM is shown in **Table 3.3.1**.

Disinfectant

Benzalkonium chloride was purchased from Fragon Iberica S.A.U. Terrassa (Barcelona).

Food processing surfaces

Stainless steel (SS, AISI-304, 2B finish, 0.8 mm thickness) and polypropylene sheets (PP, compact 00226) were cut into coupons (20 mm x 20 mm). PP coupons were cleaned with alcohol and left overnight in distilled water. New and reused SS coupons were soaked in 2M NaOH to remove any grease or food soils left and then rinsed several times with distilled water. Coupons were dried in a laminar flow cabinet.

Inocula preparation

Inoculum of each strain (*Listeria* and *Pseudomonas*) was prepared by adjusting the activated culture to an absorbance 0.1 at 700 nm, centrifuged (6000 rpm, 10 min, 25 ºC) and the pellet was re-suspended in the same volume of the corresponding residual media (MCJ or IWM). To form mixed-species biofilms, obtained pellets of each strain were resuspended in half of the total centrifuged volume, thus achieving the same final concentration of *P. putida* and *L. monocytogenes* cells, which correspond to a cell density of 10^8 CFU/ml of each species according to previous calibrations.

Experimental systems

Cleaned coupons were placed into mini glass Petri plates for the assessment of biofilm formation. Polypropylene (PP) coupons were fixed by photo stickers to the Petri plates. Then, cleaned stainless steel or polypropylene (PP) coupons were packed 5 by 5 with foil and autoclaved for 20 min at 121 ºC. Once autoclaved, 9 ml of cell suspension was added to Petri plates and incubated at 25 ºC for biofilm formation. After 4 and 11 days, two replicate coupons were taken out of the incubator at each sampling time and rinsed with 10 ml of PBS for 30s to release non-adhered cells and used for antimicrobial assays and microscopic analyses. Each experiment was repeated three times.

According with previous studies in which different strains, media, surface material and surface conditioning were compared (Saá et al., 2009, Saá Ibusquiza et al., 2010, **see sections 3.1 and 3.2** respectively of this thesis) the following experimental cases were assayed. They

pretend to be representative of different scenarios that can occur when *L. monocytogenes* adheres in plants processing mussel giving rise to biofilms with different thicknes:

- CECT 911 on polypropylene and intervalvar mussel water (PP-IMW). It corresponds with the conditions that gave rise to the highest level of adherence.
- Strain 4032 on polypropylene and stainless steel using MCJ as medium. This strain showed the highest adhesion ability on both materials.
- Strain 5873 on polypropylene in MCJ. It showed the lowest level of adherence out of the 16 experimental cases assayed. In this work we use this scenario to compare the biofilms formed by this strain (5873 wild type) and the physiologically-BAC-adapted (5873A)**.**

Determination of the number of adhered cells

First of all, coupons were drained and immediately immersed in 10 ml of PBS during 10s to release non-adhered cells. The number of adhered cells was determined according to Herrera et al. 2007. They were collected by thoroughly rubbing with two moistened swabs and resuspended by vigorously vortexing swabs for 50 s in 9 ml of peptone water (Cultimed, S.A.) and subsequent plating. The number of adhered cells of *P. putida* was determined by plating the prepared dilutions in Violet red bile agar with glucose (VRBG, Cultimed S.L.) and incubation at 37 ºC during 24 hours. Number of adhered cells of *L. monocytogenes* was determined by plating in Palcam Agar (Liofilchem, S.L.R., Italia) and incubation at 37 ºC during 48 hours before counting.

Inactivation assays

After 4 and 11 days of incubation, coupons were drained and inmediately inmersed in 10 ml of PBS for 10 s to release non-adhered cells before the addition of biocide.

After PBS was drained, 0.5 ml aliquots of benzalkonium chloride were added to the formed biofilms at various doses (mg/l): 2.5, 5, 10, 17, 25, 50, 100, 250, 500, 1000.

Several blanks with distilled water instead of BAC were included in each experimental series. All experiments were carried out at 25 ºC.

After the addition of BAC and a time of exposure of 10 minutes, the effect of BAC was neutralized by adding a solution comprising 10 ml of a buffer containing 34 g/l KH_2PO_4 adjusted to pH 7.2 with NaOH, 3 g/l soybean lecithin, 30 ml/l Tween 80, 5 g/l $Na_2S_2O_3$ and 1 g/l L-histidine , to a ratio 9:1 (v/v). Neutralizing solution was applied during 1 min at room temperature according with preliminary experiments. After neutralization, adhered cells of *P. putida* and *L. monocytogenes* were collected and determined as previously described.

Finally, percentage of mortality was calculated according to equation **[3.3.1]**:

$$M\ (\%) = 100 * \left(\frac{NTAC - NAC}{NTAC} \right), \text{ where:} \qquad \textbf{[3.3.1]}$$

- M: Mortality (%)
- NTAC: number adhered cells (CFU/mm^2) in biofilms without treatment.
- NAC: number of adhered cells after treatment with the corresponding doses of BAC (CFU/mm^2).

Determination of the Lethal Dose 90 (LD_{90}).

LD_{90} values were obtained by fitting experimental data of mortality obtained in the dose-response assays to the following modified logistic equation obtained according with previous works (Cabo et al., 1999; Cabo et al., 2009):

$$M\ (\%) = k\left(\frac{1}{1 + 0.11e^{r(LD90 - D)}} - \frac{1}{0.11e^{rLD90} + 1} \right) \qquad \textbf{[3.3.2]}$$

- LD_{90}: Dose that kills 90% of initial population.
- k: Maximum BAC inhibition value (asymptote).
- r: Specific inhibition coefficient (dimensions: inverse of the dose).
- D: dose.

Microscopic analyses

Cells were fixed in 2% glutaraldehyde with 0.1 M cacodylate buffer (pH 7.4) for at least 2 h at 4 ºC. The fixing solution was washed out with PBS, and samples were dehydrated with a series of increasing concentrations of ethanol: 30, 50, 70, 80 and 95% for 30 min each, and pure ethanol for 1 h. Ethanol was progressively replaced with amyloacetate and then samples were subjected to critical point drying with CO_2 (73 atm; 31.3 ºC). Afterwards, samples were coated with gold (10-20 nm), and finally observed by using a Philips XL30 Scanning Electronic Microscope.

Statistical analysis

Fits of the model to the experimental data were performed according to a least-squares method (quasi-Newton). A Student´s test (α=0.05) was used to test the significance of the differences between means of the total number of adhered cells and between the lethal doses values obtained in each experimental case.

3.4.3. Results

Comparison between the adherence of L. monocytogenes in monoculture and in the presence of Pseudomonas putida CECT 845

Adherence kinetics of 3 strains of *L. monocytogenes* (CECT 911, 4032 and 5873) in monoculture and co-cultured with *P. putida* were compared in different scenarios, which were selected according to previous studies (Saá et al. 2009; Saá Ibusquiza et al. 2010 see **sections 3.1 and 3.3** respectively of this thesis).

Slightly, higher adherence levels were reached by *L. monocytogenes* in a monoculture compared with a mixed culture of *L. monocytogenes* and *P. putida* at the beginning of incubation (compare open and closed symbols in **Figure 3.4.1**). Whereas adherence of *L. monocytogenes* in monoculture remained constant or even increased (as in 4032) after the *plateau* phase was reached, a sharp decrease in the number of adhered cells was detected for strains 4032 and BAC-adapted 5873 on polypropylene in the presence of *P. putida*. In the latter strain, adherence decreased to almost undetectable values after 14 days of incubation.

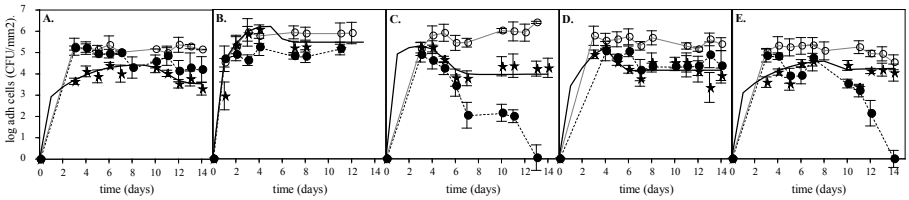

Figure 3.4.1: Adhesion kinetics of different *Listeria monocytogenes* in monoculture (**O**) and in the presence of *Pseudomonas putida* 845 (●)**.**

A.: CECT 911 on polypropylene in the presence of intervalvar water (IWM),

B.: CECT 4032 on stainless steel in the presence of cooked mussel water (MCW),

C.: CECT 4032 on polypropylene in MCW, **D:** CECT 5873 on polypropylene in MCW and

E.: CECT 5873A (BAC-adapted) on polypropylene in MCW.

Observed (⋆) and expected (continuous line) adhesion kinetics of *Pseudomonas putida* in mixed biofilms are also included in these graphs.

Although modelling the experimental results could be helpful for comparing the adherence of *L. monocytogenes* in the different scenarios it was not feasible, because there were no experimental points before 4 days of incubation. However, in the case of *P. putida*, a satisfactory fit (r^2=0,995) to a two-term additive logistic-type model **[3.4.3]** that adequately describes the increase and decrease of biofilm population (Herrera et al. 2007; Saá et al. 2009, see **section 3.1**) was obtained (**Figure 3.4.1, Table 3.4.1**).

Table 3.4.1: Values for the parameters obtained after fitting experimental results of adhesion of *Ps. putida* in presence of different *L. monocytogenes* strains to equation [3.4.3]

	911-PP-IWM	4032-SS-MCJ	4032-PP-MCJ	5873-PP-MCJ	5873-PP-MCJ
a_{ad}	30499	1800000	370723	199237	45000
r_{ad}	5,014	2,974	1,101	3,204	5,014
m_{ad}	0,700	1,843	0,998	1,881	0,700
a_D	26244	1497876	268936	184311	26244
r_D	10,425	5,500	4,173	4,965	8,500
m_D	1,676	4,500	1,820	51,632	8,000

PP: polypropylene. SS: stainless steel
MCJ: mussel cooking juice. IMW: intervalvar water.

$$NAC = \left(\frac{a_{ad}}{1 + e^{r_{ad}(m_{ad} - t)}} - \frac{1}{1 + e^{r_{ad} m_{ad}}} \right) - \left(\frac{a_D}{1 + e^{r_D (m_D - t)}} - \frac{1}{1 + e^{r_D m_D}} \right) \qquad \textbf{[3.3.3]},$$

where,

- NAC: number of adhered cells, log (CFU/mm^2).
- a_{ad}: maximum number of adhered cells (asymptote).
- r_{ad}: specific adhesion coefficient (t^{-1}).
- m_{ad}: time at which the number of adhered cells is half of the maximum.
- a_D: total number of adhered cells which detach or die, log (CFU/mm^2).
- r_D: specific detachment/death coefficient (t^{-1}).
- m_D: time at which the number of detached/death cells is half of the maximum.

Analysis of the adjusted experimental data permitted to distinguish two different adherence patterns for *P. putida* that gave rise to two different patterns of association with *L. monocytogenes*:

- Type A.: This pattern was observed in the associations between *P. putida* and strains 4032 (in both materials) and the wild type strain 5873 (**Fig. 3.4.1.B., C., D.** respectively) This type has a sharp **initial** increase in the adherence level that permits higher **maximum** adherence levels (compare the parameters a_{ad} and r_{ad} shown in **Table 3.4.2**) to be obtained compared to type B association. The increase in adherence of *P. putida* was especially clear with strain 4032 on SS, in which a 2-log increase in the maximum level with respect to that obtained for the type B pattern was observed.
- Type B: This type has a flatter adherence profile that implies a 1-log decrease in the maximum adherence levels of *P. putida* with respect to those obtained in pattern type A. This profile occurs between *P. putida* and strains 911 and BAC-adapted 5873 (**Fig. 3.4.1.A., E.** respectively). Conversely, in these experimental cases a slightly higher adherence level of *L. monocytogenes* was observed.

As we will see further, both types of association patterns give rise to mature mixed-species biofilms (after 4 days) with different levels of resistance to benzalkonium chloride.

Comparison between the resistance to BAC of monospecies L. monocytogenes biofilms and dual-species biofilms formed from the association between L. monocytogenes and P. putida

The comparison of the LD_{90} of BAC obtained in all assayed scenarios demonstrated a significant variation in the resistance to BAC of the dual-species biofilms after 4 and 11 days of incubation with respect to the single-species biofilms. This indicates that the presence of *P. putida* modifies the *L. monocytogenes* biofilm structure, but in different ways after 4 and 11 days of incubation:

After 4 days a significant increase ($p<0.05$) in the resistance to BAC was observed in the dual-species biofilms formed by *L. monocytogenes* and *P. putida* in all experimental cases (**Figure 3.4.2**). However, quantitative differences were observed depending on the type of association pattern between the strains. Thus, in the biofilm formed by *P. putida* and *L. monocytogenes* 4032-SS (type A), the LD_{90} value (i.e. resistance to BAC) increased until a factor of around 6; however, in the cases that showed a type B pattern, the resistance was multiplied by a maximum factor of 2. Specially significant is the case of *L. monocytogenes* 5873 (WT), whose association with *P. putida* permits the formation of resistant biofilms at 25 ºC.

After 11 days of incubation the situation was completely different: a significant reduction in BAC resistance was shown in all mixed biofilms formed by *L. monocytogenes* and *P. putida.* Moreover, a significant decrease in the resistance of mixed-species biofilms compared with that obtained for mono-species biofilms was observed in all cases except for 4032-SS and 5873 (wt).

It seems, then, that destabilization of mixed-species biofilms occurred in all experimental assays. However, only for 4032 and BAC-adapted 5873 strain on polypropylene this destructuration is accompanied by a sharp decrease in the number of adhered cells (**Figure 3.4.1.C.** and **E.** respectively). Furthermore, for 5873, the results obtained demonstrated that BAC-adaptation hindered its association capacity with *P. putida,* giving rise to a mixed-species biofilm that was significantly ($p<0.05$) less resistant to BAC and which had a higher biofilm destabilization rate than the corresponding wild type. Lastly, in the case of 911-IWM, a 1-log decrease in the number of adhered cells between 4 and 11 days of incubation was observed (**Figure 3.4.1**).

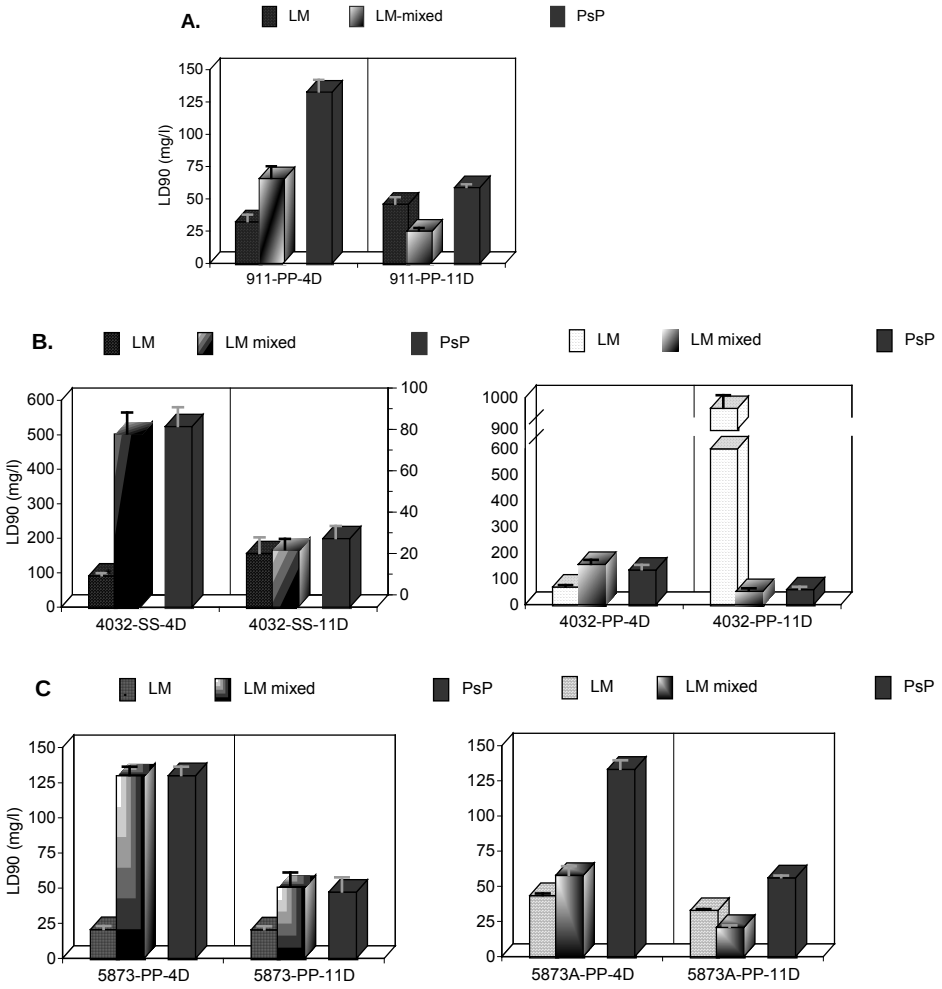

Figure 3.4.2: Comparison between the LD_{90} of BAC (mg/l) against monospecies biofilms and dual-species biofilms of *L. monocytogenes* formed from the association with *P. putida.*

A: CECT 911 on polypropylene in the presence of intervalvar water of mussel (IWM)

B: CECT 4032 on stainless steel and on polypropylene in the presence of cooked mussel water (MCW) and

C: CECT 5873 in MCW and CECT 5873A (BAC-adapted) on polypropylene in MCW.

Microscopic studies

Microscopic structures of mixed-species biofilms

The microscopic structure of 2 out of the 5 experimental cases was compared after 11 days of incubation (**Figure 3.4.3**):

- *L. monocytogenes* CECT 4032-SS in association with *P. putida*, which forms the biofilms with the highest resistance after 4 and 11 days of incubation (**Fig.3.4.3.B**)
- *L. monocytogenes* CECT 5873A-PP in association with *P. putida*, which forms the biofilms with the lowest resistance and highest level of destructuration among all the experimental cases (**Fig. 3.4.3.D**).

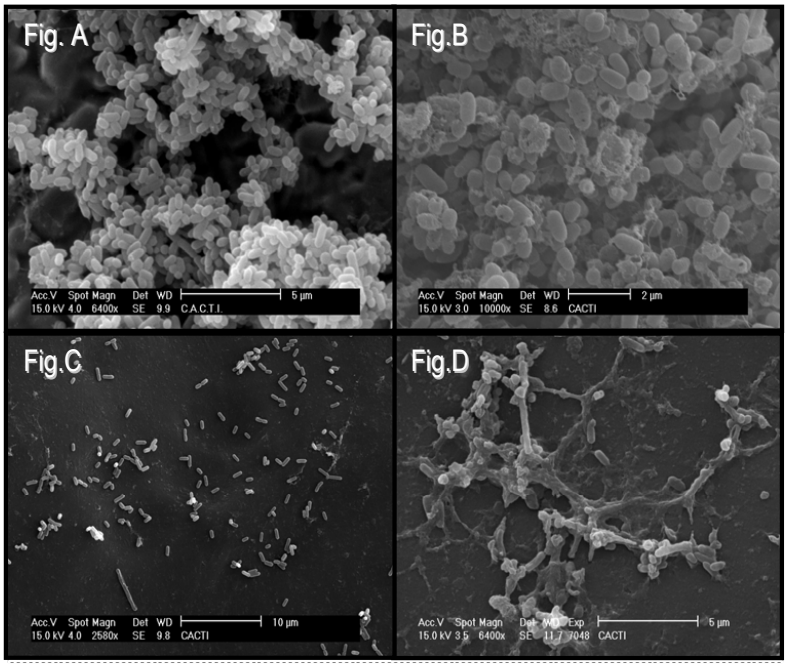

Figure 3.4.3: Scanning electronic microscopy (SEM) images of 11 days-mature biofilms formed by the strains CECT 4032 on SS (**A**) and CECT 5873 adapted to BAC (**C**) in PP in monospecies and associated with *P. putida* CECT 845 (**B** and **D**).

The SEM images obtained of the biofilm formed by *L. monocytogenes* CECT 4032-SS showed that it had a similar spatial cell arrangement in both the absence (**Figure 3.4.3.A**) and in the presence (**Figure 3.4.3.B**) (Saá Ibusquiza et al., 2010, **see section 3.3**) of *P. putida.* In fact, a *cloud-type* structure can be observed in both cases.

As a consequence, the LD_{90} values were similar (155.64 mg/l of BAC in the monospecies biofilms and 166.51 mg/l of BAC in the mixed-species biofilm). However, a monolayer-biofilm without a three-dimensional cell arrangement was formed by the BAC-adapted 5873 strain both alone (**Fig. 3.4.3.C**) and associated with *P. putida* (**Figure 3.4.3D**), and low values of LD_{90} were obtained (32.77 and 20.69 mg/l) in monospecies and mixed-species biofilms respectively). By the way, cells with a rough plasmatic membrane were observed in the mixed biofilms formed by BAC-adapted cells of *L. monocytogenes* CECT 5873.

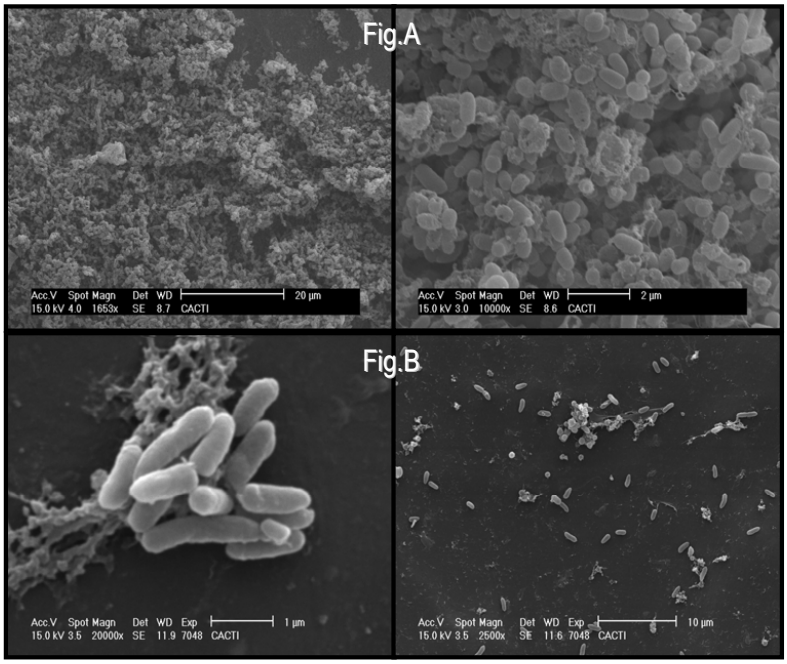

Figure3.4.4: Scanning electronic microscopy (SEM) images of 11 days-mature mixed species biofilms formed by CECT 4032 on SS (**A**) and in PP (**B**) with *P. putida.*

On the other hand, **Figure 3.4.4** showed a clear effect of material on the association between *P. putida* and *L. monocytogenes* CECT 4032, being the destructuration of the mixed biofilm after 11 days of maturation significantly higher in PP (**Figure 3.4.4B**) than on stainless steel (**Figure 3.4.4A**)

Microscopic analysis of the biofilm after disinfection

A picture of the mixed 11-day matured biofilm formed by strain 4032 was taken after it was exposed to 100 mg/l of BAC for 10 minutes (**Figure 3.4.5**). Curiously, whereas a low number of *L. monocytogenes* cells were observed after the treatment, exopolysaccharide matrix remained attached to the stainless steel

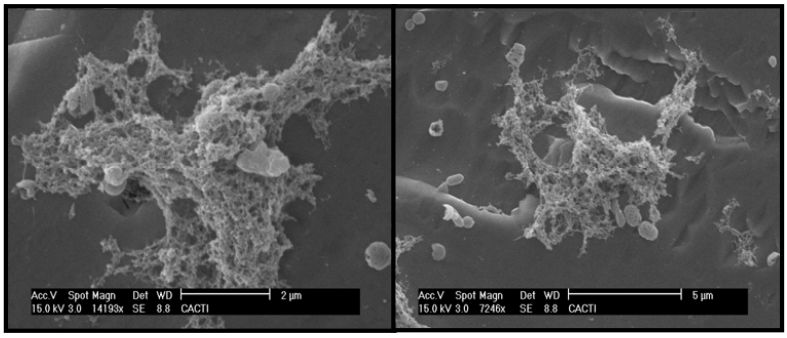

Figure 3.4.5: Scanning electronic microscopy (SEM) images of residues left after treatment of biofilms formed by the strain CECT 4032 and *P. putida* on SS with 100 mg/l of BAC.

3.4.4. Discussion

The results obtained demonstrate that the presence of *Pseudomonas putida* in *L. monocytogenes* biofilms significantly increases their resistance to BAC with respect to the resistance of monospecies *L. monocytogenes* biofilms after 4 days of incubation.

These results are in agreement with those obtained by previous authors, who found a smaller log decrease for mixed *L. monocytogenes* biofilms exposed to disinfectants compared to the corresponding monospecies biofilm (Ammor et al., 2004).

The presence of *Pseudomonas putida* quickens the biofilm maturation process compared to the corresponding monospecies *L. monocytogenes* biofilm. A possible explanation for this could be that the exopolysaccharide matrix secreted by *Pseudomonas* could facilitate the adhesion of *L. monocytogenes*, as has been previously stated by other authors (Nadell et al., 2009). However, lower adherence levels of *L. monocytogenes* in the mixed biofilm were observed in this study, which could be due to competitive effects (specific or non-specific, Jameson effects) between the two strains during biofilm formation (Mellefont et al., 2008). This has been previously demonstrated in other bacterial species (Chorianopoulos et al., 2007).

Nonetheless, although after 4 days of incubation a significant increase in the resistance to BAC is observed when *P. putida* is present in the biofilm, in those cases in which an initial sharp increase in the adherence of *P. putida* was observed (type A), 4032-SS-PP and 5873-PP, the increase in resistance was quantitatively higher than in the other two cases. It seems that when experimental conditions imply that *P. putida* has a higher affinity for the surface compared to *L. monocytogenes*, a favourable association between the two species occurs, at least with respect to resistance to BAC. Specially in the case of the wild type 5873 strain, in which the resistance to BAC increased from 20.51 mg/l to 130 mg/l in the absence and presence of *P. putida* respectively. However, the modifications derived from BAC adaptation in the same strain gave rise to a mixed biofilm with *P. putida* that is considerably less resistant and has a higher detachment rate.

After 11 days of incubation, a significant decrease in the resistance to BAC with respect to that obtained after 4 days was observed. This was probably related to the destructuration of the mixed-species biofilm observed in all experimental cases. However, only in 2 (4032-PP and 5873A) out of the 5 experimental cases assayed was this destructuration accompanied by a drastic decrease in the number of adhered cells. In the case of 4032-PP, the decrease in the adhered *L. monocytogenes* cells is already observed from day 4 of incubation (**Figure 3.4.1**). This contrasts with its good capacity to form biofilms and its BAC resistance value. This

could be explained by the detachment of the cells in clusters and the high resistance of the remaining adhered cells (Stoodley et al., 2001).

Although detachment of cells from monospecies biofilm has been previously described by several authors (*S.aureus*: Herrera et al., 2007, *P. aeruginosa*: Boles et al., 2005, *B. cereus*: Wijman et al., 2007), the reasons are still unclear. Some authors related it to nutritional (Marshall, 1988) and gas transference restrictions (Applegate and Bryers, 1991). This does not agree with our results, in which the low level of destructuration observed in 4032-SS and 5873-PP contrast with the high biomass concentration and the consequent nutritional and respiratory restrictions. Others authors believed that EPSs can be used as nutrients by the cells, making it easier for starving cells to detach from the biofilm (Takhistov and George, 2004). However, it could also be the result of competitive interactions between *L. monocytogenes* and *P. putida* during biofilm formation (Simões et al., 2007). Also, Rodríguez et al., 2007 believe that after drying, *Listeria* biofilms weakens adhesion forces and it causes cell dettachment. In fact, in our experiments with mixed *L. monocytogenes*-*P.putida* biofilms, progressive water lost of the media that initially covered the coupons was also observed. Additionally, different detachment regulating systems have been demonstrated in various bacterial species that could specifically disrupt the external matrix (EPS) of the biofilms (Sauer et al., 2004; Liu et al., 2007; Boles et al., 2005).

In the other three cases, the decrease in BAC resistance (which probably reflects biofilm destructuration) was not accompanied by a drastic decrease in the number of adhered cells, which was only slight during the incubation time. One possible explanation for this could be that even a low detachment level during biofilm formation is enough to increase the accessibility of BAC to *L. monocytogenes* cells, but insufficient to be reflected in a decrease in the total number of adherent cells.

Another interesting observation is that LD_{90} values of BAC obtained for dual-species biofilms were close to those obtained for *P. putida* biofilms in the association type A. This suggests that when two or more strains are integrated in a single biofilm, the effectiveness of the disinfectant seems to depend mainly on the cell arrangement in the final structure, being less relevant the particular sensitivity of the individual species involved.

The microscopic analysis demonstrated the risk associated to the formation of a three-dimensional structure by adhered pathogenic bacteria. The best example is that biofilms

formed by the strain 4032 on SS, which showed similar cell arrangement (**Figure 3.4.3A** and **3.4.3B**) and similar values of BAC resistance, 155.64 mg/l and 166.51 mg/l, in monospecies and mixed-species biofilm, respectively (**Figure 3.4.2**).

Additionally, results clearly demonstrated an effect of the surface on biofilm formation of *L. monocytogenes* CECT 4032. If we compare the LDs (**Figure 3.4.2**) and the microscopic structure of strain 4032 cells attached in the presence of *P. putida* on SS and PP (**Figures 3.4.4A** and **3.4.4B**), it can be observed that "*cloud"* shaped structures formed by the mixed biofilm on stainless steel (LD_{90} =166.51 mg/l of BAC) appeared as microcolonies in the case of polypropylene (LD_{90}= 49.97 mg/l of BAC), which demonstrates that the two strains have a high affinity for stainless steel. In fact, *P. putida* reached a 1-log higher maximum adherence level on stainless steel than on a polypropylene surface (a_{AD} in SS: 1800000; a_{AD} in PP: 370323). Lastly for this strain, the microscopic analysis of the biofilm after disinfection showed that the exopolysaccharide matrix remained attached to the stainless steel whereas the number of remaining cells were low. This matrix could function as a trap for new colonizing cells that would recontaminate the surface and form another mature biofilm (Pan et al. 2006). Furthemore, rough plasmatic membrane observed in the mixed biofilms formed by BAC-adapted cells of *L. monocytogenes* CECT 5873 (See **Figure 4.4.3D**) could be due to the inactive cell metabolism as a consequence of nutrient starvation, membrane modifications associated with BAC-adaptation or both of these.

Although when the LD_{90} values of monospecies and mixed-species biofilms are compared it seems to be clear that each strain has an inherent capacity to form biofilms, the results obtained demonstrate that the association with *P. putida* is relevant. Similar conclusions were found by Simões et al., 2009 and Davies et al., 2003 that observed the importance of the biofilm architecture for metabolic cooperation where antimicrobial-resistant phenotypes are formed. Moreover Simões et al., 2009 stated that biofilm species association promotes community stability and functional resilience even after chemical and mechanical treatment.

Two facts show this clearly:

- The wild type 5873 in the presence of *Ps. Putida* forms a biofilm with a higher BAC resistance than when the biofilm is formed by a monoculture.
- The differences between the biofilms formed by 4032 on polypropylene and on stainless steel are related to the higher adherence capacity of *P. putida* on the latter

surface, on which the maximum adherence levels increased more than 1-log. As a consequence, biofilms with higher BAC-resistance were formed on SS, after 4 and 11 days of incubation. However, another explanation for these differences could be related to a decrease in the metabolic activity derived from the formation of a more dense and complex biofilm on SS (Rodrigues et al. 2009), which implies more stability and probably a delay in the beginning of the destructuration phase. Moreover, the presence of intercellular connections and the proximity between cells of different species in the biofilm could facilitate the transfer of genetic material, including genes associated with resistance to external stimuli (Ammor et al., 2004, Wuertz et al., 2004, Nadell et al., 2009).

Finally, the following conclusions and perspectives can be drawn:

i. The presence of *P. putida* increases the resistance to BAC of the biofilms formed by *L. monocytogenes*, giving rise to situations of higher microbiological risk. This highlights the need to consider microbial communities and not individual genera in food safety studies.
ii. Biofilm formation between *P. putida* and *L. monocytogenes* depends on the bacterial strains involved, their interactions and the interactions between them and the environmental conditions.
iii. The resistance of mixed-species biofilms of *L. monocytogenes* and *P. putida* to BAC seems to be related to their microscopic structure and to the association between the involved strains.
iv. This structure did not correlate with the number of adhered cells, which implies that indirect variables, such as the resistance to disinfectants, should be considered for biofilm evaluation.
v. The application of doses of industrial disinfectants, such as a benzalkonium chloride that kill practically 100% of cells in the mixed *P. putida-L. monocytogenes* biofilm does not ensure the absence of organic residues (probably from the external EPS matrix). Therefore, new disinfection methods or improvements of the current disinfection strategies should be developed to ensure that there are not biofilm residues left which could act as a trap for new cells that could recolonize the surface.

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3.5. Comparison between the resistance of benzalkonium chloride-adapted and non-adapted biofilms of *Listeria monocytogenes* to modified atmosphere packaging (MAP) and nisin once transferred to mussels

3.5. COMPARISON BETWEEN THE RESISTANCE OF BENZALKONIUM CHLORIDE-ADAPTED AND NON-ADAPTED BIOFILMS OF *LISTERIA MONOCYTOGENES* TO MODIFIED ATMOSPHERE PACKAGING (MAP) AND NISIN ONCE TRANSFERRED TO MUSSELS.

Benzalkonium chloride (BAC) adapted and non-adapted CECT 5873 *L. monocytogenes* biofilm cells were transferred by contact to cooked or live mussels and packed under CO_2 and O_2 rich atmospheres, respectively. Viability of transferred cells during storage of these packed samples at 2.5 ºC was compared. In addition, in cooked mussels the combined effect of CO_2 and nisin against the survival of *L. monocytogenes* was also studied by using a first order factorial design. Obtained results demonstrated that biofilms formed by BAC-adapted *L. monocytogenes* cells could be more resistant to the application of modified atmospheres rich in CO_2 and nisin once they have been transferred to cooked mussels by contact (simulating cross-contamination). This implies an increase in the risk associated with the presence of these cells in food processing plants. Significant empirical equations obtained after 7, 11 and 20 days showed an inhibitory effect of CO_2 and nisin against *L. monocytogenes*. However, a significant positive interaction between both variables highlights an incompatibility between CO_2 and nisin at high concentrations. Results also demonstrated that *L. monocytogenes* could persist after cross-contamination during the processing of live mussels, so *L. monocytogenes* is of concern as a contaminant in live mussels packaged in high O_2 atmospheres.

3.5.1. Introduction

The aim to maintain a high level of mussel (*Mytilus galloprovinciales*) commercialization in Galicia and the increase in international competitiveness have led to the development of alternatives of preservation that allow long distance markets to be reached such as modified atmosphere packaging (MAP). It has been applied extensively to fish products (Cabo et al., 2003; Sivertsvik et al., 2002; Ohlsson and Bengtsson, 2002; Skara et al., 2003). MAP is used for preservation of mussels with two objectives: 1) to increase the shelf life of cooked mussels in a CO_2-rich atmospheres; and 2) to increase the viability of live mussels in an O_2-rich atmosphere (Pastoriza et al., 2004).

According to Rutherford et al. (2007), *Listeria monocytogenes* is often found in processing environments as a post-processing contaminant. It is transferred to food through contact at food processing plants, where *L. monocytogenes* forms biofilms (Porsby et al., 2008, Rodríguez and McLandsborough 2007). Also it has been previously shown that persistent *Listeria monocytogenes* cells are resistant to Quaternary Ammonium compounds, such as benzalconium chloride (BAC) (Aase et al., 2000; Mullapudi et al., 2008), probably due to inadequate disinfection protocols that lead to residual sublethal concentrations of disinfectants (Langsrud et al., 2003).

It is possible for *L. monocytogenes* to grow in modified atmosphere packed mussels. In fact, thermal soft treatments (like those applied in boiled mussels) may not eliminate this bacterium (Doyle et al., 2001, Huang, 2004), it can resist high CO_2 atmospheres (Fang and Lin 1994; Jenssen and Lammert, 2002; Szabo and Cahill, 1999; Olarte et al., 2002) and the aerobic conditions of high O_2 atmospheres are not expected to damage *L. monocytogenes* cells. In this context, the application of antimicrobials, such as bacteriocins, with a specific effect against these opportunistic bacteria can be useful for ensuring food safety (Cabo et al., 2001; Cabo et al., 2005; Cabo et al., 2009¸ Cleveland et al., 2001, Delves-Broughton et al., 1996).

It is therefore possible that in a mussel processing plant, BAC-adapted biofilms of *L. monocytogenes* can be transferred by contact to the products during processing and it could survive refrigerated under MAP during storage. Nonetheless, no previous studies on the repercussions of BAC-adaptation of *L. monocytogenes* to the resistance to MAP were found. Therefore, the main objective of this work was to compare the viability of BAC adapted and

non-adapted *L. monocytogenes* biofilm cells in modified atmospheres rich in CO_2 and O_2 once they had been transferred by contact to cooked and live mussels, respectively. In addition, in cooked mussels an experimental factorial design allowed us to study the combined effects of CO_2 and nisin against the survival of *L. monocytogenes.*

3.5.2. Material and methods

Bacterial strains and synthetic media

Listeria monocytogenes CECT 5873 was provided by the Spanish Type Culture Collection (Valencia). Strain CECT 5873 adapted to BAC was obtained in previous experiments after two exposures of the wild type strain 5873 to sub-lethal concentrations of BAC (wild strain LD_{50}: 2.71 mg/l; BAC-adapted strain LD_{50}: 7.22 mg/l, **see section 3.2**). Strains were frozen and stored at -80 ºC in TSB containing 50% glycerol (v/v) until use. Whenever required, one cryovial was thawed and this strain was subcultured twice in TSB (Cultimed, S.L., Spain) at 37 ºC before use.

Method for biofilm formation

Food processing surfaces

Stainless steel sheets (AISI-304, 2B finish, 0.8 mm thickness) were cut into coupons (10 mm x 10 mm). New and reused SS coupons were soaked in 2M NaOH to remove any grease or food soils left and then rinsed several times with distilled water. Coupons were dried before use in the laminar flow cabinet.

Food soils media

Mussel cooking juice (MCJ, Carbohydrate 9,19 g/l, Nitrogen 1.70 g/l), used as culture media to simulate contamination of surfaces in plants processing cooked mussel, was prepared as it was previously described (Saá et al., 2009).

Inocula preparation

Working cultures of BAC-adapted and non-adapted strains of *L. monocytogenes* CECT 5873 were adjusted to an absorbance 0.1 at 700 nm, which correspond to a cell density of 10^8 CFU/ml according with previous calibration. Adjusted cultures were centrifuged at 6000 rpm during 10 min at 25 ºC and collected cells were resuspended in MCJ. This suspension was used as inoculum.

Biofilm formation

Mini glass Petri plates (5 cm diameter) containing cleaned coupons were packed 5 by 5 with foil and autoclaved for 20 min at 121 ºC. Once autoclaved, 9 ml of inoculum was added per mini Petri plate and incubated at 25 ºC during 7 days for biofilm formation.

Transference assays

Preparation of mussels

Fresh mussels from Galicia (*Mytilus galloprovincialis*) were always acquired in the same market. For cooking, fresh mussels were cooked during 1 minute at 100 ºC without adding water. After cooking, 10 g weight mussels were selected for the experiments.

Transference of biofilms from coupons to mussels

After 7 days of biofilm maturing, coupons were rinsed with 10 ml of PBS for 30 s to release non-adhered cells before transferring. Total number of adhered cells in the coupons after 7 days were 7,88 log CFU. The surface was rubbing with two moistened swabs which were resuspended by vigorously vortexing for 50 s in 9 ml of peptone water according to Herrera et al. 2007 and plating on Triptone Soy Agar and incubated at 37 ºC for 24 h. The cells of the upper superface were taking account in the counting (7.88 log CFU)the other superface was in touch with the glass of the petri plates. Biofilm cells were the transferred as follows:

- to cooked mussel: by contact between the coupon and the meat without additional pressure during 120 seconds according with previous kinetic experiments that

showed maximum levels of adherence after 100 s. As a consequence, 5.6 log CFU per gram of mussel were transferred.

- to live mussel: by contact between the coupons and the shells during 120 s according with previous kinetic studies. As a consequence, 7.2 log CFU of mussel were transferred. In this case transference was made always in a previously marked zone of the shell (**Figure 3.5.1**).

Figure 3.5.1. The white circle indicates the area of the mussel-shell where the transference of the biofilm was carried out.

Application of nisin

Pure nisin was obtained from DANISCO (A/S Denmark). Different doses of nisin were prepared in distilled water and applied to cooked mussel by pipetting uniformly on the meat after transference and before packaging.

Packaging

Cooked and live mussels were placed on polyspan trays and packed into high-density polyethylene barrier bags (Cryobac, S.L.). Once ready, gas mixtures (Carburos Metálicos, Barcelona, S.L.) set according with the experimental design (see below) were injected into the bags, which were heat-sealed with a modified atmosphere packaging machine VP-430-20-A (Dordalpack, S.L.). Nitrogen was used as a filler gas. Mussels were stored at 2.5 ºC and removed for analysis at each sampling time.

Experimental design

- In cooked mussels: a first order factorial design (Box et al., 1989) with 4 combinations of variables and 5 replicates in the center of the domain was carried out. The independent variables were the proportion of CO_2 (C) the gas mixture and the concentration of nisin applied on the meat of the mussel (N). Natural and coded levels for each variable are showed in **Table 3.5.1**.
- In live mussels: the effect of increasing concentrations of O_2 (30, 60 and 90%) on the survival of *L. monocytogenes* were assayed.

Table 3.5.1: Natural and codified values used in the first order ortogonal design

	Natural values	
Codified values	**CO_2 (%)**	**Nisin (UI/g)**
1;1	90	210
1;-1	90	21
-1;1	20	210
-1;-1	20	21
0;0	55	115

Microbiological analyses

Determination of the Number of Viable Cells of L. monocytogenes (VLM)

In cooked mussels, determination of VLM was carried out after 7, 11 and 20 days of storage. At each day of sampling, 5 mussels were minced with sterilized scissors and 10 g of this mixture was homogenized in 90 ml of sterile peptone water (Cultimed, S.A.) in a Lab-Blender-400 Stomacher. Number of VLM were determined by serially ten-fold diluting it in peptone water (Cultimed, S.A.), subsequent spread out in Palcam (Liofilchem, S.L.R., Italia) and Triptone Soy Agar (Cultimed, S.A.) and finally incubated at 37 ºC during 48h. TSA was used to assure sublethal damaged cells, that may have problems to growth in a selective media as PALCAM, were computed.

In live mussels, determination of VLM was carried out after 5, 8 and 12 days of storage. Adhered cells were collected by thoroughly rubbing with two moistened swabs in the marked area and resuspended by vigorously vortexing the swabs for 50 s in 9 ml of peptone water according to Herrera et al. 2007. Proper serial ten-fold dilutions were then plated on Triptone Soy Agar and Palcam and incubated at 37 ºC for 24 h and 48 h respectively. Peptone water was always used as dilution medium.

Biofilm contaminated mussels were packed in increasing concentrations of O_2 and stored at 2.5 ºC. The viability of the 5873 BAC adapted and non-adapted biofilm cells to the application of modified O_2-rich atmospheres was compared after 5, 8 and 12 days of storage.

Mortality test in mussels packaged in O_2-rich atmospheres

Mortality test were carried out according with previous studies (Pastoriza et al., 2004). Briefly, it was carried out after 2, 6, 8, 10, 12, 13, 14 days of storage at 2.5 ºC by tapping on gaping bivalve shelves and observing mussel closure. Any shells gaping after tapping were considered dead.

Statistics

A Student t-test (α=0.05) was used to test the significance of the differences between means of the total number of viable cells (log CFU), between the mortality (%) rate in live mussels and between the coefficients of the equations obtained in the factorial design. A Fisher Test (α=0.05) was used to test the consistence of the models.

3.5.3. Results

Studies on cooked mussels

Effects of CO_2 and nisin on the viability of BAC-adapted and non-adapted biofilms cells once transferred to cooked mussels.

A first order factorial design (Box et al., 1988) was used to compare the sensitivity of biofilms cells of 5873 BAC adapted and non-adapted strains to the combined application of CO_2 and nisin during storage at 2.5 ºC. Both CO_2 percentage and nisin concentration used

during the expositions were set following the factorial design (**Table 3.5.1**). Viable cells of *L. monocytogenes* (VLM, in log CFU/g) in cooked mussels after 7, 11 and 20 days of storage could be predicted satisfactory according to the following empirical equations **[3.5.1-3.5.6]**:

1) BAC non-adapted *L. monocytogenes* CECT 5873:

After 7 days:	VLM=4.25-1.18C-1.48N-1.18CN	**[3.5.1]**
After 11 days:	VLM=5.25-0.45C-0.74N+0.46CN	**[3.5.2]**
After 20 days:	VLM=5.72-1.43C-0.86N	**[3.5.3]**

2) BAC-adapted 5873 strain:

After 7 days:	VLM=3.65-0.57C-0.87N	**[3.5.4]**
After 11 days:	VLM=5.24-0.45C-0.94N	**[3.5.5]**
After 20 days:	VLM=5.47-0.37C-0.79N+0.54CN	**[3.5.6]**

Graphycal representations of all the empirical equations are shown in **Figure 3.5.2** and **3.5.3** (data are showed in the supporting information chapter). The comparison of the effects of the two variables on the wild type strain and the BAC adapted strains showed that the biggest differences occured after 7 and 20 days of storage. At these times of storage, it can be clearly observed that BAC adaptation gave *L. monocytogenes* a higher resistance to CO_2 and nisin. In fact, the CO_2 and nisin coefficients obtained for the non-adapted strain after 7 (eq. **[3.5.1]**) and 20 (eq. **[3.5.3]**) days were significantly higher ($p<0.05$) than those obtained at the same times of storage for the non-adapted strain (eq. **[3.5.4]** and eq. **[3.5.6]**, respectively). In **Table 3.5.2**, log reductions (LR) obtained at different combinations of CO_2 and nisin in adapted and non-adapted biofilms of *L. monocytoenes* are showed. At high concentrations of both variables, 90% CO_2 and 210 UI/ml of nisin, the model predicts log reductions (LR=log UFC/g transferred-log UFC/g after storage) of 3.39 and 5.12 in the number of viable *L. monocytogenes* cells per gram of the 5873 BAC adapted and non-adapted strains respectively after 7 days of storage of the contaminated cooked mussels. However, after 11 days of storage the differences between the effects of CO_2 and nisin decreased and similar polynomial equations were obtained to predict the VLM. There was one difference: there was a

significant positive interaction between CO_2 and nisin at this time of storage for the non-adapted strain, which is reflected in a decrease in the effect of CO_2. This positive interaction, also significant in equation [**3.5.6**], contrasts with the beneficial individual effects of the CO_2 and the nisin and can be interpreted as an increase in the viable *Listeria monocytogenes* biofilm cells in mussels preserved under high concentrations of both CO_2 and nisin.

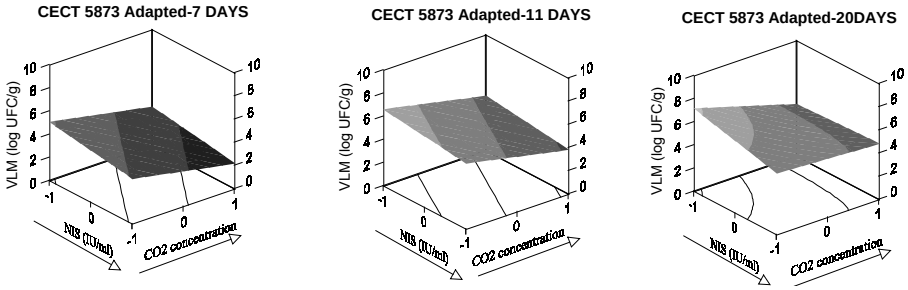

Figure 3.5.2. Response surfaces corresponding to the combined effects of CO_2 and nisin against viable *L. monocytogenes* CECT 5873 BAC-adapted biofilm cells (VLM, in log CFU/g) after 11 and 20 days of storage at 2.5 ºC. Variables are expressed as codified values (see **Table 3.5.1**).

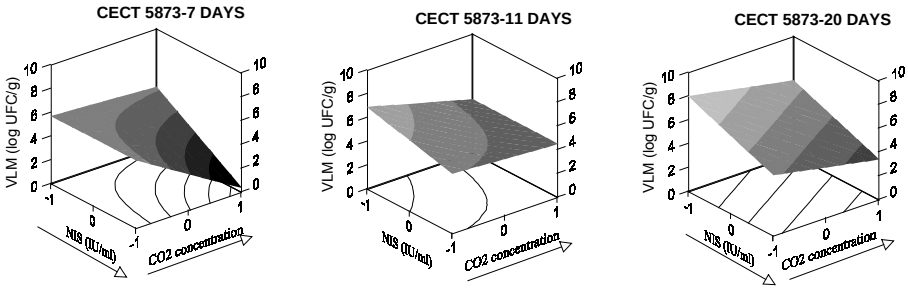

Figure 3.5.3. Response surfaces corresponding to the combined effects of CO_2 and nisin against viable *L. monocytogenes* CECT 5873 BAC non adapted biofilm cells (VLM, in log CFU/g) after 11 and 20 days of storage at 2.5 ºC. Variables are expressed as codified values (see **Table 3.5.1**).

Those results have also demonstrated that the effects of the variables change during the time of storage. In fact, if we calculate the log reductions in *L. monocytogenes* at different combinations of CO_2-nisin in the experimental design (see **Table 3.5.2**), it can be clearly observed that the effectiveness of the variables decrease with the time of storage, especially from 7 to 11 days.

Table 3.5.2: Log reduction (LR) in *L. monocytogenes* biofilm cells transferred to cooked mussels and stored under different combinations of CO_2 and nisin.

		Vc (CO_2, nis) (1,1)	Vc (CO_2, nis) (0,0)	Vc (CO_2, nis) (-1,-1)
Non adapted	**7D**	5.12	1.32	-0.18[a]
	11D	1.08	0.35	-1.3
	20D	2.17	-0.12	-2.41
BAC-adapted	**7D**	3.39	1.95	0.51
	11D	1.75	0.36	-1.03
	20D	0.75	0.13	-1.57

[a] Negative values indicate the model predicts the growth of *L. monocytogenes*

Studies on live mussels

Preliminary experiments: viability of live mussels packaged under O_2-rich atmospheres

In order to assure that mussels were viable during the set time of study, preliminary experiments to study the effect of the different O_2-rich atmospheres on the viability of live mussels without inoculation with *L. monocytogenes* were carried out.

Before 13 days of storage at 2.5 ºC, no significant differences were obtained between the mortality of mussels packed under different O_2 concentrations, being in all cases lower than 20%. At 14 days mortality decreased from 45% in air (21%O_2) till 10% in mussels packed under 90% O_2 (**Figure 3.5.4**). This is in accordance with previous results that demonstrated mortality of mussels decreased when packaging at high O_2 concentration (Pastoriza et al. 2004). According with those results and to assure mussels were alive during the experiments, sampling times were set at 5, 8 and 12 days of storage.

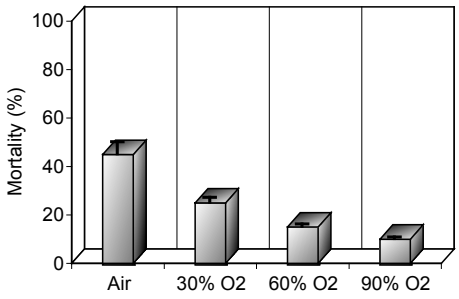

Figure 3.5.4. Mortality of live mussels packaged under different O_2-rich atmospheres (30%, 60% and 90%) and air after 14 days of storage at 2.5 ºC.

Comparison of the resistance to O_2 of BAC adapted and non-adapted, transferred to live mussels

The results did not show significant differences between the number of viable BAC adapted *L. monocytogenes* cells compared to the non-adapted cells during storage (**Figure 3.5.5**). In fact, the sensitivity of the BAC-adapted cells was only slightly higher in live mussels packed at 60% O_2 (0.25 log/g).

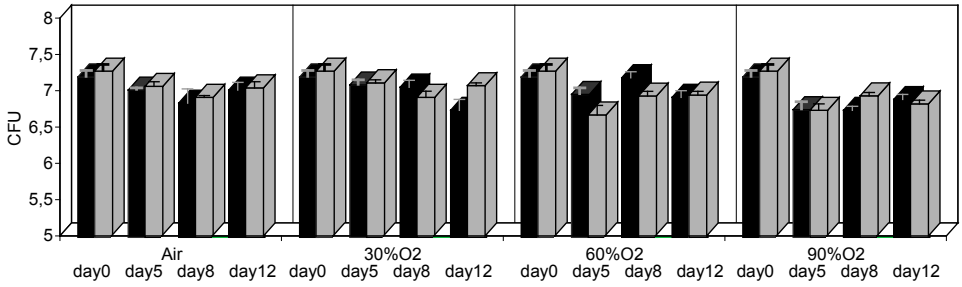

Figure 3.5.5. Viability of *L. monocytogenes* cells transferred to live mussels packaged under different O_2-rich atmospheres (30%, 60% and 90%) and air at 0, 5, 8 and 12 days of storage at 2.5 ºC. BAC non-adpated biofilm cells are showed in black columns and BAC adapted CECT 5873 in grey columns.

3.5.4. Discussion

Cooked mussels

The results obtained demonstrate that biofilms cells formed by BAC-adapted CECT 5873 *L. monocytogenes* cells were more resistant to the application of modified atmospheres rich in CO_2 and nisin once they have been transferred to cooked mussels by contact (simulating cross-contamination). This implies an increase in the risk associated with the presence of these cells in food processing plants. This was particularly significant after 7 days of storage, when the coefficients that quantitatively described the effects of CO_2 and nisin were significantly lower in the equation that describes the number of non-adapted *L. monocytogenes* cells (compare Eq. **[3.5.1]** and Eq. **[3.5.4]**). This is in agreement with other results recently published that demonstrated mature biofilms formed by BAC-adapted cells were more resistant to nisin compared to those formed by the non-adapted cells (Saá Ibusquiza et al., 2010).

However, those effects are not constant over the storage time. In fact, after 11 days a decrease in the effect of the two variables was observed both in the BAC adapted and non-adapted cells, which is reflected in the similar 3D surfaces shown in **Figure 3.5.2** and **3.5.3** respectively. Moreover, at this storage time nisin is more effective against the viability of BAC adapted *L. monocytogenes* biofilm cells than against the non-adapted cells. The variations observed in the effects of the variables over time reflect the kinetic conditions of the microbial system. And they are in agreement with those obtained by Minei et al., (2008), who found a significant decrease in the rate of *L. monocytogenes* biofilm formation after nisin had been added in the early stages of incubation, followed by the recommencement of biofilm formation after 12 hours.

Although it is very difficult to draw conclusions at this point without carrying out additional mechanistic experiments, several facts taken from previously published results could help us to explain the appearance of the identified adaptive responses:

- A confluence in the cross-responses of *L. monocytogenes* to acid (ATR) and BAC. It has been previously demonstrated that acid-tolerant strains of *L. monocytogenes* are more resistant to CO_2 (Francis et al., 2007, Jydegaard-Axelsen et al., 2004) and nisin (Badaoui Najjar et al., 2009; Begley et al., 2010; Bonnet and Montville 2005).

The confluence of the two cross-responses suggests that adaptation to BAC and acid could imply similar modifications in *L. monocytogenes*. However, we could not find any previous studies on the cross-resistance response between disinfectants and CO_2.

- A confluence of the membrane as a target for the CO_2, nisin and BAC action. It has been demonstrated that CO_2 modifies bacterial membrane permeability by changing the lipid composition (Jydegaard-Axelson et al. 2004, Lungu et al., 2009; Tassou et al., 2004; Nilsson et al., 2000). Nisin forms pores in the bacterial membrane of its targets by interacting with lipid II, a cell wall precursor (Christ et al., 2007; Dalmau et al., 2002; Wiedemann et al., 2007). It has been recently demonstrated that BAC induces membrane damage in *Bacillus cereus* (Ceragioli et al., 2010). In addition, a direct relation between the sensitivity to BAC and the increase in membrane permeability has been observed in *E. coli* and *L. monocytogenes* (Walton et al., 2008). Changes in the components in the outer membrane (QAC-resistance genes such as qacA, qacB, smr, qacG and qacH code for efflux membrane proteins) could also participate in the resistance of *L. monocytogenes* to BAC (Soumet et al., 2005).
- A relation between the resistance to nisin and the resistance to BAC could be related to the proton motive force (PMF)-efflux pump regulation system. In fact, it has been demonstrated that adding nisin can dissipate the basal PMF across the membrane (Bruno et al., 1992). However, Romanova et al., (2006) demonstrated that adding reserpine (an inhibitor of the efflux pump *mdrL* and *lde* caused by BAC) to physiologically adapted and BAC-sensitive *L. monocytogenes* implied a decrease in the MIC value of BAC, absent in those strains that are naturally resistant. Finally, alterations in the PMF caused by nisin and BAC could help CO_2 to act by preventing protons being expelled from the cytoplasm, as well as acidification (Garcia-Gonzalez et al. 2007).

Therefore, it seems that BAC, nisin and CO_2 have common points of action in the cell membrane, and it is biologically reasonable that some cross-resistances between them could appear after successive exposures.

Although quantitative differences between the effects of the variables against the BAC adapted and non-adapted *L. monocytogenes* transferred biofilms were identified, the signs of the individual and combined effects of CO_2 and nisin were very similar. CO_2 and nisin showed an inhibitor effect against both types of biofilms. This is in agreement with previous works with planktonic cells (effect of CO_2: Sheridan et al 1995, Olarte et al 2002; effect of nisin: Nisson et al. 1997, López- Mendoza et al. 2007). However, no previous studies on the effectiveness of CO_2 against *L. monocytogenes* biofilms were found. In the case of nisin, two types of studies were found: those that study controlling *Listeria monocytogenes* with nisin-producing biofilms (Guerrieri et al., 2009, Leriche et al., 1999) and only one article that assayed controlling *Listeria monocytogenes* biofilms with nisin (Minei et al., 2008). In this last, although bacterial growth was initially reduced up to 4.6 log CFU/cm^2 when compared with *L. monocytogenes* cultures on untreated coupons, after 24 h of incubation, a renewed biofilm was detected.

To elucidate the real repercussions of the identified cross-responses additional experiments with more strains should be carried out. However, the positive interaction between CO_2 and nisin, which reflects incompatibility at high concentrations of the two variables, contrasts with previous results in which a synergic effect between nisin and CO_2 against the survival of *L. monocytogenes* has been demonstrated (López-Mendoza et al. 2007; Nilsson et al. 2000; Szabo and Cahill, 1998). This discrepancy could be explained by an increase in incompatibility between the effects of CO_2 and nisin associated with the presence of an external polysaccharide matrix in the mature biofilm transferred to cooked mussels. In fact, a significant decrease in nisin effectiveness against mature *L. monocytogenes* biofilms compared with that observed against planktonic cells has been previously observed (Saá Ibusquiza et al., 2010). Moreover, the ineffectiveness of nisin in the presence of CO_2 against an exopolysaccharide producer, *Pseudomonas fragi*, has been demonstrated (Fang and Lin, 1994). Variations in the EPS composition during biofilm formation could explain the variations in the effect of nisin over the storage time.

However, the results of the individual and combined effects of CO_2 and nisin is that LR of *L. monocytogenes* decreased during the time of storage, thus demonstrating that *L. monocytogenes* biofilm cells (both adapted and non-adapted) can resist the preservation hurdles and growth in cooked mussels at this temperature.

Live mussels

No significant differences were found between the number of viable *L. monocytogenes* cells transferred to live mussels from BAC adapted and non-adapted biofilms over 12 days of storage at 2.5 ºC. This indicates that cellular modifications in *L. monocytogenes* related to BAC adaptation do not have any significant effects on its survival under high O_2 concentrations. Moreover, no significant differences were found in the number of viable *L. monocytogenes* cells recovered from the live mussels during storage, which indicates that *L. monocytogenes* could persist after cross-contamination during the processing of live mussels. This is in agreement with previous results obtained by Allende et al., (2002), who found no significant differences in the number of viable *L. monocytogenes* cells inoculated into a mixed vegetable salad during storage at 4 and 7 ºC under different O_2-rich atmospheres.

Overall, the results allow us to conclude that BAC adapted *L. monocytogenes* biofilms transferred to cooked mussels are more resistant to CO_2 and nisin than non-adapted biofilms but that this resistance can vary over the storage time. In addition, nisin and CO_2 rich atmospheres are not effective enough to control a hypothetical cross-contamination of *L. monocytogenes* biofilm cells transferred from industrial surfaces to ready-to-eat fish products, such as cooked mussels. Finally, it must be pointed out that *L. monocytogenes* is a pathogen of concern in live mussels packaged in high O_2 atmospheres.

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5. Conclusions

CONCLUSIONS:

A review of the results presented in this Ph.D. thesis has permitted to draw the following conclusions:

1. Initial adhesion kinetics of *L. monocytogenes* depends on environmental and nutritional conditions, bacterial strain, physico-chemical properties of the surface and the interactions between any of these factors. Initial adhesion was thus shown to be higher on polypropylene than on stainless steel. The presence of mussel soils as a conditioning film enhanced adhesion, the number of cells decreased sharply after 24 hours, likely due to a gradual plastification of the conditioning film leading to partial detachment. Additionally, reducing nutrient concentration increased significantly the maximum level of adhesion. Experimental data were successfully fitted to a logistic model which was shown to be a useful tool for comparative purposes, permitting the effect of independent variables to be differentiated and thus obtain more accurate conclusions.

2. Individual and combined effects of inoculum size and benzalkonium chloride concentration on adaptation of *L. monocytogenes* CECT 5873 to benzalkonium chloride were determined by means of an orthogonal factorial design which showed that adapted cells could be achieved in a short period by exposing a large inoculum of exponential-phase cells to sub-lethal concentrations only once. An alternative procedure to generate benzalkonium chloride-adapted cells is thus proposed. This procedure is more efficient and less time-consuming than classical methods based on successive exposure of stationary-phase cells to sub-lethal concentrations of biocide. Lastly, a significant protein repression resulting from adaptation to benzalkonium chloride was detected in proteomic studies.

3. From studies carried out on mature biofilms several conclusions are drawn:

    3.1. Resistance of mature biofilms to disinfectants (measured in terms of LD_{90}) increased with time in most of the cases, and it was correlated with biofilm thickness as observed by scanning electron microscopy. However, no correlation was found with cell adhesion (measured in terms of the number of adhered cells). These results underline the importance of the extracellular matrix, rather than the number of cells, to evaluate the repercussions of biofilm formation.

3.2. Mature biofilms formed by benzalkonium chloride-adapted *L. monocytogenes* CECT 5873 were more resistant to benzalkonium chloride and nisin (positive cross-adaptive response) than biofilms by the wild-type strain, which suggests some overlap between the effects of the two biocides. In contrast, biofilms by adapted cells were more sensitive to peracetic acid (negative cross-adaptive response). It seems therefore convenient to use peracetic acid for removal of persistent strains in processing plants where benzalkonium chloride has been routinely applied for disinfection.

3.3. Peracetic acid was significantly more effective than benzalkonium chloride or nisin against *L. monocytogenes* biofilms formed under different conditions. On the basis of the mechanism of action, it is hypothesized that a high reactivity and small size facilitate peracetic acid to enter and disrupt biofilms.

4. Dual-species biofilms formed by *L. monocytogenes* and *Pseudomonas putida* were more resistant to benzalkonium chloride than single-species biofilms by *L. monocytogenes*. This higher resistance can give rise to situations of higher microbiological risk and consequently highlights the need to consider microbial consortia and not individual strains in food safety studies. Also, it was demonstrated that the increase in benzalkonium chloride resistance depends on co-existing bacteria and biofilm structure, rather than on cell adhesion, which can not be used for assessment of biofilm structure.

5. Benzalkonium chloride-adapted *L. monocytogenes* biofilms transferred to cooked mussels are more resistant to CO_2 and nisin than non-adapted biofilms, and this resistance changed over the time of storage. Nisin and CO_2 were found not to be effective enough to control *L. monocytogenes* cross-contamination from biofilms on industrial surfaces in cooked mussels packed under CO_2-enriched atmospheres. *L. monocytogenes* was also found to be of concern as a microbial contaminant in live mussels packed under oxygen-enriched atmospheres.

6. Supporting information

SUPLEMENTARY MATERIAL section 3.2

Tables related to the equation [3.2.3]. Results of factorial designs and test of significance for models in equations [**3.2.3**], that describe the effects of BAC (B) and inocula (I) on the Viable Cells of non-adapted *L. monocytogenes* (VLM) CECT 5873 against the level of adaptation achieved.

Table related to equation 3.2.3: Effects of BAC (B) and inocula (I) on the Viable Cells of *L. monocytogenes* (VLM) CECT 5873 semiadapted cells against the level of adaptation achieved

B	I	VLM	VLM_e	Coefficients	t	Model
1	1	10.00	10.49	9.00	57.94	**7.82**
1	-1	2.71	3.19	3.00	4.68	**-0.99 B**
-1	1	7.5	7.99	-2.00	9.44	**1.42 I**
-1	-1	9.14	9.63	3.00	12.66	**2.24 BI**
0	0	9.00	7.82	Average value =	8.31	
0	0	8.24	7.82	Expected average value =	7.82	
0	0	8.00	7.82	Var (Ee) =	0.2244	
0	0	8.00	7.82	t(<0,05; υ=3) =	3.182	

	SS	υ	MS		
				MSM / MSE = 16.506	VLM_e (α=0,05) = 6.590
Model	31.871	3	10.624	MSMLF / MSM = 0.795	VLM_e (α=0,05) = 9.120
Error	2.574	4	0.644	MSE / MSEe = 2.868	VLM_e (α=0,05) = 9.010
Experim. error	0.673	3	0.224	MSLF / MSEe = 8.473	VLM_e (α=0,05) = 9.550
Lack of fitting	1.901	1	1.901	r^2 =	0.925
Total	34.445	7	4.921	corrected r^2 =	0.869

VLM (in log CFU/g); VLMe: expected response; NS: not significant coefficients; SS: sum of squares; υ: degrees of freedom; MSE, MSEe and MSLF: mean squares for total error experimental error and lack of fitting, respectively.

SUPLEMENTARY MATERIAL section 3.5

Tables related to equations [3.5.1-3.5.6]. Results of factorial designs and test of significance for models in equations [**3.5.1-3.5.6**], that describe the effects of CO_2 (C) and nisin (N) on the Viable Cells of BAC-adapted and non-adapted *L. monocytogenes* (VLM) CECT 5873 after 7, 11 and 20 days of storage at 2.5 ºC.

Table related to equation 3.5.1: Effects of CO_2 (C) and nisin (N) on the Viable Cells of *L. monocytogenes* (VLM) CECT 5873 after 7 days of storage after 2.5 ºC.

C	N	VLM	VLM_e	Coefficients	t	Model
1	1	0	0.41	9.00	33.01	**4.25**
1	-1	5.330	5.74	3.00	6.46	**-1.18 C**
-1	1	4.719	5.13	-2.00	8.14	**-1.48 N**
-1	-1	5.313	5.72	3.00	6.51	**-1.18 CN**
0	0	4.760	4.25	Average value =	4.65	
0	0	5.100	4.25	Expected average value =	4.25	
0	0	4.250	4.25	Var (Ee) =	0.1324	
0	0	4.500	4.25	t(<0,05; υ=3) =	3.182	

	SS	υ	MS		
				MSM / MSE = 15.470	VLM_e (α=0,05) = 6.590
Model	19.908	3	6.636	MSMLF / MSM = 0.800	VLM_e (α=0,05) = 9.120
Error	1.716	4	0.429	MSE / MSEe = 3.241	VLM_e (α=0,05) = 9.120
Experim. error	0.397	3	0.132	MSLF / MSEe = 9.964	VLM_e (α=0,05) = 10.13
Lack of fitting	1.319	1	1.319	r^2 =	0.921
Total	21.624	7	3.089	corrected r^2 =	0.861

VLM (in log CFU/g); VLMe: expected response; NS: not significant coefficients; SS: sum of squares; υ: degrees of freedom; MSE, MSEe and MSLF: mean squares for total error experimental error and lack of fitting, respectively.

Table related to equation 3.5.2**:** Effects of CO_2 (C) and nisin (N) on the Viable Cells of *L. monocytogenes* (VLM) CECT 5873 after 11 days of storage after 2.5 ºC.

C	N	VLM	VLM_e	Coefficients	t	Model
1	1	4.48	4.52	9.00	52.83	**5.25**
1	-1	5.04	5.08	3.00	3.22	**-0.45 C**
-1	1	4.46	4.50	-2.00	5.27	**-0.74 N**
-1	-1	6.87	6.91	3.00	3.28	**0.46 CN**
0	0	4.95	5.25	Average value =	5.30	
0	0	5.36	5.25	Expected average value =	5.25	
0	0	5.63	5.25	Var (Ee) =	0.0792	
0	0	5.25	5.25	t(<0,05; υ=3) =	3.182	

	SS	υ	MS		
				MSM / MSE = 20.454	VLM_e (α=0,05) = 6.590
Model	3.867	3	1.289	MSMLF / MSM = 0.753	VLM_e (α=0,05) = 9.12
Error	0.252	4	0.063	MSE / MSEe = 0.796	VLM_e (α=0,05) = 9.12
Experim. error	0.237	3	0.079	MSLF / MSEe = 0.185	VLM_e (α=0,05) = 10.13
Lack of fitting	0.015	1	0.015	r^2 =	0.939
Total	4.119	7	0.588	corrected r^2 =	0.893

Notations in previous table.

Table related to equation 3.5.3: Effects of CO_2 (C) and nisin (N) on the Viable Cells of *L. monocytogenes* (VLM) CECT 5873 after 20 days of storage after 2.5 ºC.

C	N	VLM	VLM_e	Coefficients	t	Model
1	1	3.76	3.43	9.00	130.74	**5.72**
1	-1	5.35	5.15	3.00	23.15	**-1.43C**
-1	1	6.50	6.29	-2.00	13.90	**-0.86N**
-1	-1	8.34	8.02	3.00	1.03	**0CN**
0	0	5.55	5.72	Average value =	5.46	
0	0	5.56	5.72	Expected average value =	5.72	
0	0	5.30	5.72	Var (Ee) =	0.0153	
0	0	5.43	5.72	t(<0,05; υ=3) =	3.182	

	SS	υ	MS		
				MSM / MSE = 44.749	VLM_e (α=0,05) = 6.590
Model	11.171	2	5.585	MSMLF / MSM = 0.526	VLM_e (α=0,05) = 9.120
Error	0.624	5	0.125	MSE / MSEe = 8.145	VLM_e (α=0,05) = 9.010
Experim. error	0.046	3	0.015	MSLF / MSEe = 9.550	VLM_e (α=0,05) = 10.13
Lack of fitting	0.578	2	0.289	r^2 =	0.947
Total	11.795	7	1.685	corrected r^2 =	0.926

Notations in previous table.

Table related to equation 3.5.4**:** Effects of CO_2 (C) and nisin (N) on the Viable Cells of *L. monocytogenes* (VLM) CECT 5873 BAC-adapted after 7 days of storage after 2.5 ºC.

C	N	VLM	VLM_e	Coefficients	t	Model
1	1	2.04	2.21	9.00	36.69	**3.65**
1	-1	3.65	3.94	3.00	4.05	**-0.57 C**
-1	1	3.04	3.35	-2.00	6.15	**-0.87 N**
-1	-1	4.91	5.08	3.00	0.50	**0 CN**
0	0	4.16	3.65	Average value =	3.89	
0	0	3.90	3.65	Expected average value =	3.65	
0	0	4.00	3.65	Var (Ee) =	0.0791	
0	0	3.50	3.65	t(<0,05; υ=3) =	3.182	

	SS	υ	MS		
				MSM / MSE = 14.756	VLM_e α=0,05) = 6.590
Model	4.293	2	2.146	MSMLF / MSM = 0.557	VLM_e (α=0,05) = 9.120
Error	0.727	5	0.145	MSE / MSEe = 1.840	VLM_e (α=0,05) = 9.010
Experim. error	0.237	3	0.079	MSLF / MSEe = 3.099	VLM_e (α=0,05) = 9.550
Lack of fitting	0.490	2	0.245	r^2 =	0.855
Total	5.020	7	0.717	corrected r^2 =	0.797

Notations in previous table.

Table related to equation 3.5.5**:** Effects of CO_2 (C) and nisin (N) on the Viable Cells of *L. monocytogenes* (VLM) CECT 5873 BAC-adapted after 11 days of storage after 2.5 ºC.

C	N	VLM	VLM_e	Coefficients	t	Model
1	1	3.78	3.85	9.00	105.19	**5.24**
1	-1	5.54	5.73	3.00	6.32	**-0.45 C**
-1	1	4.55	5.74	-2.00	13.35	**-0.94 N**
-1	-1	6.55	6.62	3.00	0.85	**0.0CN**
0	0	5.44	5.24	Average value =	5.37	
0	0	5.26	5.24	Expected average value =	5.24	
0	0	5.24	5.24	Var (Ee) =	0.0198	
0	0	5.53	5.24	t(<0,05; υ=3) =	3.182	

	SS	υ	MS	MSM / MSE = 51.095	VLM_e (α=0,05) = 5.790
Model	4.327	2	2.163	MSMLF / MSM = 0.518	VLM_e (α=0,05) = 19.250
Error	0.212	5	0.042	MSE / MSEe = 2.136	VLM_e (α=0,05) = 9.010
Experim. error	0.059	3	0.020	MSLF / MSEe = 3.839	VLM_e (α=0,05) = 9.550
Lack of fitting	0.152	2	0.076	r^2 =	0.953
Total	4.538	7	0.648	corrected r^2 =	0.935

Notations in previous table.

Table related to equation 3.5.6**:** Effects of CO_2 (C) and nisin (N) on the Viable Cells of *L. monocytogenes* (VLM) CECT 5873 BAC-adapted after 20 days of storage after 2.5 ºC..

C	N	VLM	VLM_e	Coefficients	t	Model
1	1	4.90	4.85	9.00	76.06	**5.47**
1	-1	5.40	5.35	3.00	3.66	**-0.37 C**
-1	1	4.57	4.51	-2.00	7.79	**-0.79 N**
-1	-1	7.23	7.18	3.00	5.31	**0.54 CN**
0	0	5.63	5.47	Average value =	5.42	
0	0	5.14	5.47	Expected average value =	5.47	
0	0	5.44	5.47	Var (Ee) =	0.0414	
0	0	5.45	5.47	t(<0,05; υ=3) =	3.182	

	SS	υ	MS		
				MSM / MSE = 38.310	VLM_e (α=0,05) =6.590
Model	4.233	3	1.411	MSMLF / MSM = 0.754	VLM_e (α=0,05) = 9.120
Error	0.147	4	0.037	MSE / MSEe = 0.890	VLM_e (α=0,05) = 9.120
Experim. error	0.124	3	0.041	MSLF / MSEe = 0.560	VLM_e (α=0,05) = 10.13
Lack of fitting	0.023	1	0.023	r^2 =	0.966
Total	4.380	7	0.626	corrected r^2 =	0.941

Notations in previous table.

7. Perspectives

PERSPECTIVES

The results achieved throughout this Ph.D. thesis and the consequent conclusions drawn from those results allows a number of future research lines to be enunciated:

1. Search for genes and proteins associated with biofilm formation in *L. monocytogenes*, and quantitative analysis of such genes and proteins during biofilm formation in different strains. A high variability was observed when initial adhesion and biofilm formation of several strains were compared, and this seems to indicate that there might be biological determinants associated with the type of biofilm formed.

2. Examine the potential of the method newly proposed for attaining benzalkonium chloride-adapted *L. monocytogenes*. This should comprise a screening of a high number of strains, firstly. Subsequently it should be studied if it can be extended to other biocides and even to further bacterial species with the aim of establishing this method as a general protocol in microbial physiology.

3. Design of disinfection strategies aimed at removing mature *L. monocytogenes* biofilms. This also comprises the search of agents (of chemical, physical or biological origin) that can diffuse into and disturb such biofilms and have or enhance bactericidal effects. This search needs, in turn, to identify the chemical composition of the extracellular matrix, which is unknown, and determine the microstructure of those biofilms. Furthermore, bacterial species commonly associated with *L. monocytogenes* in the food environment must be identified and physiological characteristics of mixed biofilms formed by these consortia must be determined in order to design disinfection strategies correctly. Also, the study of the role of each microorganism could give new interesting possibilities (based in microbial ecology) to control high persistant biofilms.

4. Identify cross-responses to environmental and technological factors resulting from the adaptation of *L. monocytogenes* to industrial biocides. For instance, it would be highly interesting to find out if the cross-adaptive response between benzalkonium chloride and CO_2 shown by *L. monocytogenes* CECT 5873 is also developed by other strains. Additionally, the mechanisms of adaptation should be clarified by using molecular biology tools. These studies

would permit the potential risk associated with the routine use of each disinfectant in each food processing plant to be better known.

Resumen

RESUMEN

Introducción

Características biológicas: L. monocytogenes es un bacilo Gram positivo, anaerobio facultativo, móvil a temperaturas inferiores a 25 ºC (Seeliger and Jones, 1986) y altamente resistente en condiciones de estrés: pHs ácidos, baja aw, bajas concentraciones de O_2 y baja temperatura (Ross et al., 2000, Kathariou, 2002). Todo ello contribuye a su ubicuidad (Cox et al. 1989, Ivanek et al. 2006) y a su condición de bacteria patógena, causante de listeriosis.

Patogeneidad: está asociada a un grupo de riesgo constituido por mujeres embarazadas, individuos de avanzada edad e inmunodeprimidos. A pesar de su ubicuidad, la incidencia anual de listeriosis es de 0.3 casos al año por cada 100.000 habitantes, baja si la comparamos con otras infecciones trasmitidas por alimentos (EFSA 2006). Lo que contrasta con su elevada tasa de mortandad (20-30%), haciéndola especialmente relevante. Existen 13 serotipos: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 7 (Seeliger and Höhne, 1979; Seeliger and Jones, 1986), siendo 1/2a, 1/2b, 1/2c and 4b los responsables del 95% de los casos de listeriosis en humanos (Farber and Peterkin, 1991; Doumith et al., 2004; Swaminathan and Gerner-Smidt, 2007).

Incidencia en alimentos: Según los datos epidemiológicos, el 99% de los casos de listeriosis en humanos se deben al consumo de productos alimenticios contaminados. En Europa rara vez se detectan productos contaminados por encima del límite de seguridad (contaminación <10 UFC/g) pero los productos listos para el consumo en los que ha sido detectada son productos de la pesca, cárnicos y quesos. De entre alimentos anteriores, las últimas inspecciones realizadas en Europa mostraron una mayor incidencia en productos de la pesca listos para el consumo, en los cuales se detectaron una mayor proporción de muestras contaminadas por encima de 100 UFC/g (2.4%) (EFSA, 2009), sobre todo durante y después del procesado (Cox et al., 1989; Hu et al., 2006; Samelis and Metaxopoulos, 1999, Autio et al., 1999, Miettinen et al., 1999; Norton et al., 2001; Rørvik et al., 1995; Vogel et al., 2001a, Wulff et al., 2006).).

Control: Dada la importancia del este patógeno en el ámbito alimentario, se han desarrollado diferentes estrategias para el control de *Listeria monocytogenes* a diferentes niveles, desde la consideración de medidas para evitar su aparición mediante la implementación del sistema de prerrequisitos y análisis de riesgo de los puntos críticos de control (HACCP), hasta el diseño de estrategias de conservación que aseguren el control de *L. monocytogenes*, pasando además por los esfuerzos realizados en la aplicación de protocolos de limpieza y desinfección efectivos.

Justificación y objetivos

Con el objetivo final de incrementar la seguridad alimentaria respecto a *L. monocytogenes*, tres razones justifican la realización del presente trabajo:

1. Que *L. monocytogenes* puede persistir en plantas de procesado formando biopelículas, definidas como comunidades microbianas sésiles caracterizadas por células irreversiblemente adheridas al sustrato o interfase y embebidas en una matriz de sustancias poliméricas extracelulares (Donlan and Costerton 2002). En el desarrollo de estas biopelículas desde el estado planctónico se pueden diferenciar tres fases: 1) Fase de adherencia: reversible y dependiente de los nutrientes del medio (Kim and Frank 1994; Mai and Corner 2007; Stepanovic et al., 2004; Hood et al., 1997), del movimiento flagelar (Lemon et al. 2007; Vatanyoopaisarn et al. 2000; Tresse et al., 2006 and Geeriri et al., 2008), del tipo de superficie (Balckman and Frank, 1996; Di Bonaventura et al., 2008; Krysinski and Brown 1992; Meylheuc et al., 2001; Rodríguez et al. 2008; Saá et al., 2009; Safyan et al. 2006; Sinde and Carballo, 2000; Somers and Wong, 2004; Smoot and Pierson, 1998; Teixeira et al., 2008) y de variables de estado como pH (Herald and Zottola 1988; Tresse et al., 2006; Poimenidou et al., 2009) 2) Maduración: durante la que las células bacterianas se adhieren ya de manera irreversible para formar estructuras tipo microcolonias que segregan una matriz polimérica (EPSs) (Høiby et. al. 2010) y forman biopelículas que van incrementando su densidad a lo largo del tiempo (Takhistov and George 2004, Marsh and Wang, 2003) dependiendo del medio externo (Folsom et al 2006; Rodrigues et al. 2009) y 3) Desprendimiento: en la que el biofilm se

desprende del sustrato por diversas razones tales como falta de nutrientes (Marshall, 1988) o restricciones en la transferencia de gases (Applegate and Bryers, 1991) pudiendo ser fuente de contaminaciones cruzadas. En la actualidad se conocen dos sistemas de comunicación célula-célula que regulan la formación de biopelículas en *L. monocytogenes*: los sistemas LuxS y Agr (Belval et al. 2006; Garmyn et al., 2009).

Pero la importancia de la formación de biofilms en bacterias en general y en *L. monocytogenes* en particular es todavía mayor si tenemos en cuenta que las biopelículas de una sola especie raramente se encuentran en el ambiente y que, al menos en algunos casos, la asociación entre diferentes especies para formar una biopelícula mixta incrementa su resistencia a antimicrobianos (Simões et al., 2007a).

2: Que la persistencia de *L. monocytogenes* en plantas de procesado pudiera relacionarse con su resistencia a aquellos biocidas frecuentemente utilizados en los procesos de limpieza y desinfección (Gilbert et al. 2002; Lundén et al. 2000, 2003a). Y de hecho, en numerosos estudios se ha demostrado que los biofilms de *Listeria monocytogenes* son más resistentes que las células plantónicas a los desinfectantes comúnmente empleados en la industria (Aarnisalo et al., 2000; Aarnisalo et al., 2007; Amalaradjou et al., 2009; Ammor et al., 2004; Blackmann et al., 1996, Bremer et al., 2002, Chavant et al., 2003; Gram et al., 2007, Kastberg 2009; Holah et al., 2002, Leriche et al., 1999; Minei et al., 2008, Frank et al., 2003; 48 h: Pan et al., 2006, Yang et al., 2009) tales como cloruro de benzalconio (perteneciente a la familia de QACs) y ácido peracético.

3: Que las biopelículas de *L. monocytogenes* que persisten en las plantas de procesado (con resistencias adquiridas) pueden transferirse fácilmente por contacto al alimento, causando problemas graves de contaminación cruzada.

De acuerdo con lo expuesto, los objetivos del presente trabajo fueron:

1. Comparar las cinéticas de adhesión de diferentes cepas de *L. monocytogenes* (CECT 5873, CECT 936, CECT 911 y CECT 4032) en polipropileno (PP) y acero inoxidable (SS) con y sin superficie de acondicionamiento, simulando residuos presentes en plantas de procesado de mejillón. A partir de los resultados obtenidos en este primer objetivo se

seleccionaron aquellos escenarios de mayor riesgo, que constituyeron los casos experimentales para el desarrollo de los objetivos subsiguientes.

2. Desarrollar un procedimiento eficiente para la obtención de células de *L. monocytogenes* adaptadas al cloruro de benzalconio (BAC). Para ello, se estudiaron, se discutieron y se formalizaron los efectos del tamaño de inóculo y concentración de BAC durante la exposición sobre el nivel final de adaptación. Además, se compararon los perfiles proteicos de células adaptadas y no adaptadas al BAC.

3. Estudiar la formación de biopelículas maduras por tres cepas de *L. monocytogenes* (CECT 4032, CECT 911, CECT 5873 y CECT 5873 adaptada al BAC) en aquellos escenarios seleccionados en 1. como de mayor riesgo mediante el estudio del aumento de resistencia a biocidas (BAC, ácido peracético y nisina) y el análisis microscópico de la estructura biopelicular formada en diferentes etapas de la maduración a 25 ºC. Ello permitió además comparar la efectividad de los biocidas e identificar algunas respuestas cruzadas derivadas de la adaptación al cloruro de benzalconio.

4. Estudiar la influencia de la asociación entre *Pseudomonas putida* y *L. monocytogenes* sobre la resistencia al BAC y la estructura microscópica de la biopelícula mixta formada en diferentes escenarios respecto a la correspondiente biopelícula monoespecie de *L. monocytogenes*.

5. Estudiar el efecto de la aplicación de atmósferas ricas CO_2 y O_2 sobre la viabilidad de células procedentes de biopelículas de *L. monocytogenes* no adaptadas y adaptadas al cloruro de benzalconio una vez transferidas a mejillón cocido y vivo, respectivamente. En el caso del mejillón cocido, se estudió además la aplicación combinada de CO_2 y nisina mediante un diseño factorial de primer orden.

Resultados y Discusión

1. Efectos de la presencia de residuos de plantas de procesado de mejillón sobre la adherencia de *Listeria monocytogenes* en polipropileno y acero inoxidable. *J. Food Protect.* 72(9):1885-1890 (2009).

1.1. Introducción

L. monocytogenes puede adherirse a las superficies en las superficies en la industria alimentaria (Kim and Frank 1995) y desarrollar un biofilm, lo cual puede contribuir a su persistencia (Frank and Koffi 1990; Pan et al., 2006). La adherencia bacteriana a superficies es la fase inicial del desarrollo de biofilms. Los estudios recogidos en bibliografía hasta el momento presentaban controversias entre sí (Harvey et al., 2007). Por tanto, el estudio llevado a cabo, trató de esclarecerlas determinando la adherencia no solo durante las primeras horas, y evitandom la utilización de medios comerciales puesto que no reproducen las condiciones reales de las plantas de procesado.

Para ello, se compararon las cinéticas de adherencia de *L. monocytogenes* (cepas CECT 5873, CECT 936, CECT 911 y CECT 4032) en polipropileno (PP) y acero inoxidable (INOX). Además, se simularon dos situaciones que reproducen a escala experimental dos casuísticas diferentes respecto a la acumulación de residuos resultado de una limpieza deficitaria en una planta de procesado:

- Contaminación con *L. monocytogenes* de superficies con residuos secos, pegados al material, formando una *película de acondicionamiento*: PA.
- Contaminación con *L. monocytogenes* de superficies limpias con residuos de mejillón en disolución.

1.2. Materiales y Métodos

Cepas: las cepas se obtuvieron en la Colección Española de Cultivos Tipo (CECT)

Medios residuales: en estos experimentos se utilizaron el agua de cocción de mejillón (ACM) y el agua intervalvar (AIV). La primera, obtenida a partir de la cocción de los mejillones durante 1 min., resultó tener la siguiente composición: 9,19 g/l de carbohidratos totales y 1698,7 mg/l de Nitrógeno; pH=7.74. El agua intervalvar, obtenida a partir del escurrido de mejillón vivo, resultó tener la siguiente composición: 2,7g/l de carbohidratos totales, 185 mg/l de Nitrógeno; pH=8.80.

El inóculo se preparó ajustando el cultivo activado a una absorbancia de 0.1 a 700 nm correspondiente a una densidad celular de 10^8 CFU/ml de acuerdo con calibraciones previas. Una vez se centrifuga a 6000 rpm, 25 ºC durante 10 min, las células

recolectadas se resuspenden en el mismo volumen del medio residual objeto de estudio en cada caso (tampón fosfato 0.05M diluido en agua destilada (1:1) (v:v) para aquellos casos con película de acondicionamiento, ACM o bien AIV). En cada cupón se dispensaron alícuotas de 250 µl de inóculo dentro del área circular (15 mm de diámetro) previamente delimitada en los cupones (20 mm x 20 mm) mediante *typpex*. A continuación se incubaron a 25 ºC y el número de células adheridas se cuantificó a diferentes tiempos durante un período en torno a 60-80 h mediante el método puesto a punto previamente en nuestro laboratorio (Herrera et al., 2007).

1.3. Resultados y discusión

Los valores de adherencia obtenidos en las diferentes condiciones ensayadas se describieron satisfactoriamente mediante un modelo logístico modificado de acuerdo con Cabo et al. (1999).

$$NCA = \left(\frac{a_{ad}}{1 + e^{r_{ad}(m_{ad} - t)}} - \frac{1}{1 + e^{r_{ad} m_{ad}}} \right) \qquad [\mathbf{1.1}]$$

No obstante, en 3 de los 8 casos objeto de estudio en presencia de película de acondicionamiento, se produce un descenso pronunciado del número de células adheridas después de aproximadamente 24 h (CECT 911-PP y en INOX y la CECT 936-PP (**Fig.1.1**)), que resultó describible mediante un modelo empírico aditivo representado por 2 términos logísticos:

$$NCA = \left(\frac{a_{ad}}{1 + e^{r_{ad}(m_{ad} - t)}} - \frac{1}{1 + e^{r_{ad} m_{ad}}} \right) - \left(\frac{a_{D}}{1 + e^{r_{D}(m_{D} - t)}} - \frac{1}{1 + e^{r_{D} m_{D}}} \right) \qquad [\mathbf{1.2}]$$

- NCA: número de células adheridas (UFC/mm^2).
- a_{ad}: máximo número de células adheridas (asíntota)
- r_{ad}: coeficiente de adherencia específico (t^{-1}).
- m_{ad}: tiempo al cual el número de células adheridas es la mitad del máximo.
- a_D: número total de células adheridas que mueren o bien se desprenden (UFC/mm^2).

- r_D: coeficiente de desprendimiento/muerte (t^{-1}).
- m_D: tiempo al cual el número de células desprendidas/muertas es la mitad del máximo.

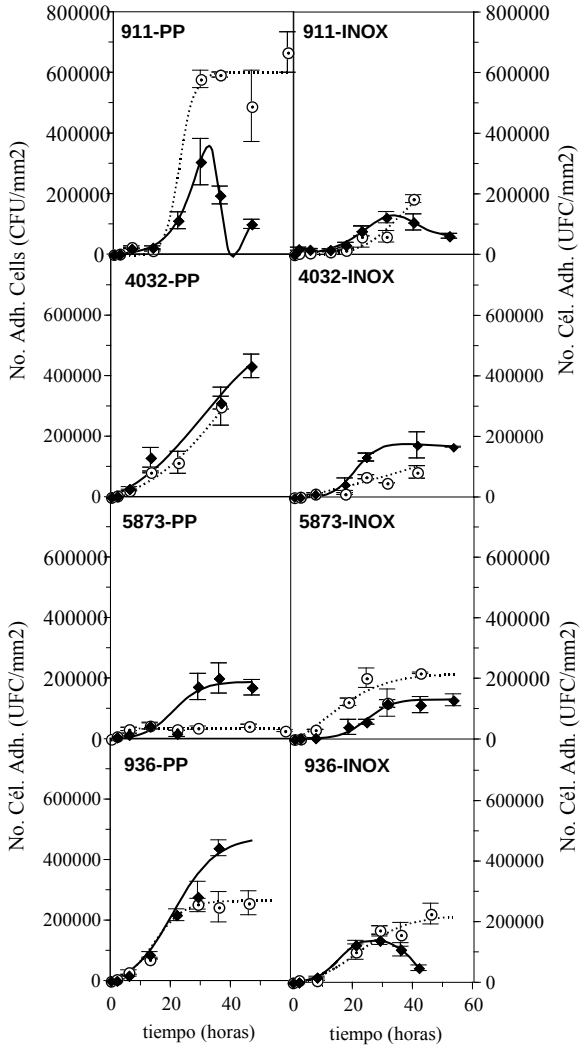

Figura 1.1 Cinéticas de adherencia de diferentes cepas de *Listeria monocytogenes* en cupones de polipropileno (PP) y en acero inoxidable (INOX) en presencia de agua de cocción de mejillón (ACM) sin (**O**) y con (**F**) película de acondicionamiento. Las líneas representan las estimaciones obtenidas empleando las ecuaciones [**1.1**] y [**1.2**].

En la **Figura 1.1.** se representaron los ajustes de los resultados experimentales obtenidos a las ecuaciones [**1.1**] y [**1.2**]. Tal y como se observa, los resultados mostraron una mayor adherencia en polipropileno que en acero inoxidable en todas las cepas de *L. monocytogenes* excepto para la CECT 5873. Se demostró además un efecto positivo de la presencia de película de acondicionamiento sobre la adherencia inicial de *L. monocytogenes*, aunque en la fase más tardía del estudio se produce desprendimiento celular (**Figura 1.1**).

Además, a partir de estos primeros resultados se seleccionaron aquellos casos experimentales (escenarios) que podrían dar lugar a unos mayores niveles de adherencia: CECT 911-PP-SPA, 4032-PP-PA, 5873-INOX-SPA, y 4032-INOX-PA. Precisamente en estos casos concretos, se comparó la adherencia con la obtenida en el supuesto de una contaminación producida en plantas de procesado de mejillón vivo, para lo que se utilizó agua intervalvar (AIV) de mejillón como medio de cultivo (**Figura 1.2**).

Los resultados demostraron claramente mayores niveles de adherencia en presencia de agua intervalvar, especialmente en los dos casos experimentales sin película de acondicionamiento. Aunque para conocer la razón exacta de estos resultados sería necesario recurrir a experimentación adicional, se postulan como posibilidades el que la nueva composición del medio implique condiciones físico-químicas que incrementen la adherencia celular o que dejen mayor espacio disponible.

Para los casos seleccionados a partir de los experimentos realizados como más representativos de los resultados obtenidos se estudió la formación de biopelículas maduras después de 4 y 11 días de incubación a 25 ºC (**subapartado 3** de resultados y discusión). Asimismo, con la finalidad de simular la formación de biopelículas adaptadas al BAC en condiciones reales y de comparar la capacidad adaptativa a este desinfectante de las tres cepas objeto de estudio, a continuación se desarrolló el segundo de los objetivos expuestos, que permitió proponer un procedimiento para la obtención de células de *L. monocytogenes* adaptadas al cloruro de benzalconio y mejorar notablemente el método clásico basado en la exposición sucesiva de células en fase estacionaria a concentraciones subletales de BAC.

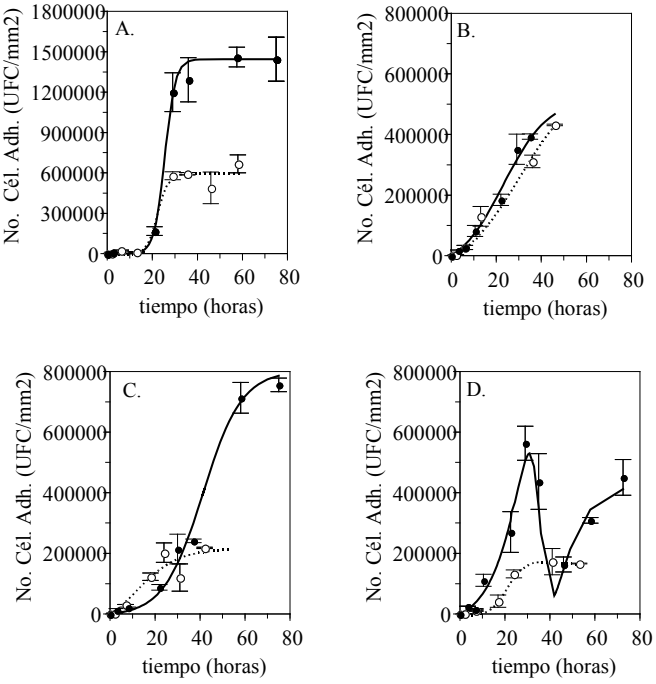

Figura 1.2 Comparación de las cinéticas de adherencia de *Listeria monocytogenes* bajo las condiciones de mayor riesgo en presencia de agua de cocción de mejillón (○) y de agua intervalvar (●) en diferentes casos experimentales. **A.** CECT 911 en polipropileno en ausencia de película de acondicionamiento (PP-SPA). **B.** CECT 4032 en polipropileno en presencia de película de acondicionamiento (PP-PA). **C.** CECT 5873 en acero inoxidable en ausencia de película de acondicionamiento (INOX-SPA). **D.** CECT 4032 en acero inoxidable en presencia de película de acondicionamiento (INOX-PA). Las líneas representan las estimaciones obtenidas empleando las ec. **.[1.1]** o **[1.2]**

2. Desarrollo de un procedimiento eficiente para la obtención de células de *Listeria monocytogenes* CECT 5873 adaptadas al cloruro de benzalconio.

2.1. Introducción

Numerosos estudios han demostrado que las cepas persistentes de *L. monocytogenes* son resistentes a los compuestos de amonio cuaternario (QACs) (Aase et al. 2000;

Mullapudi et al., 2008), entre ellos el cloruro de benzalconio (BAC), biocida perteneciente a la familia QACs que se emplea comúnmente en la industria alimentaria. Sin embargo, no existen trabajos previos centrados en el estudio de la adaptación de *L. monocytogenes* al BAC.

En general, la obtención de bacterias resistentes adaptadas a biocidas se lleva a cabo experimentalmente mediante subcultivos sucesivos de células en fase estacionaria en medio de cultivo con concentraciones crecientes de BAC hasta la ausencia de crecimiento, considerándose éste como el punto máximo de adaptación. Sin embargo, este método clásico resulta tedioso, con fases experimentales demasiado largas y no asegura alcanzar el máximo nivel de adaptación (To et al. 2002, Aarnisalo et al. 2007, Aase et al. 2000). En este sentido, y partiendo de que las células en fase exponencial son metabólicamente más activas (Whistler et al., 1998; Zotta et al., 2009), una alternativa posible podría ser diseñar un procedimiento experimental basado en la exposición de células en fase exponencial.

De acuerdo con ello, en el presente trabajo se planteó en dos etapas: 1) comparación entre el nivel de adaptación alcanzado por 3 cepas de *L. monocytogenes* (CECT 4032, CECT 911 Y CECT 5873) una vez aplicado el protocolo experimental clásico y selección de aquella cepa con mayor capacidad de adaptación y 2) estudio del efecto del tamaño del inóculo y de la concentración de BAC sobre el nivel de adaptación alcanzado tras una sola exposición de células de *L. monocytogenes* CECT 5873 en fase exponencial. Para ello, se utilizó un diseño factorial de primer orden que permitió describir adecuadamente los resultados en el ámbito experimental ensayado. A partir de los resultados obtenidos, se propuso un método que mejora notablemente el actualmente utilizado, permitiendo alcanzar mayores niveles de adaptación en menos tiempo. Además, se realizaron estudios proteómicos preeliminares para identificar cambios en los perfiles proteicos consecuencia de la adaptación al BAC y se identificaron posibles genes (así como sus proteínas asociadas) relacionados con dicha adaptación.

2.2. Materiales y Métodos

Cepas: Las cepas utilizadas se obtuvieron en la CECT.

Procedimientos experimentales:

i. *Cultivos sucesivos en fase estacionaria (método clásico)*: consistente en siembras sucesivas de 1 ml de inóculo en fase estacionaria en 49 ml de TSB (Caldo de Soja y Triptona, Cultimed, S.L.) con concentraciones crecientes de BAC (Sigma Chemical Co) hasta la ausencia de crecimiento. Estos primeros experimentos se realizaron con las cepas CECT 911, CECT 4032 y CECT 5873.

ii. *Cultivos en fase exponencial*: consistente una sola exposición de combinaciones [inóculo de *L. monocytogenes* CECT 5873 (I): BAC (B)] de acuerdo a un plan factorial ortogonal de primer orden (**Tabla 2.1**).

Recolección de células: después de cada exposición, las células se recolectaron mediante centrifugación (2000 g durante 5 minutos a 25 ºC) y se almacenaron congeladas en glicerol al 50% (v/v) hasta los ensayos para determinar su resistencia.

Determinación de la resistencia de las células de L. monocytogenes CECT 5873 recolectadas al cloruro de benzalconio. Mediante el cálculo de la dosis letal 50 (DL_{50}) a partir de un ensayo tipo dosis-respuesta, de acuerdo con lo descrito por Cabo et al. 1999.

Tabla 2.1: Valores naturales y codificados de las variables en el diseño factorial

	Valores naturales	
Valores codificados	**BAC (mg/l)**	**Inóculo (ml)**
1;1	9	25
1;-1	9	5
-1;1	3	25
-1;-1	3	5
0;0	6	15

Ensayos de proteómica: la obtención y comparación de los perfiles proteicos de las células adaptadas y no adaptadas al BAC se realizó de acuerdo a los procedimientos de Sánchez et al. 2010.

2.3. Resultados y discusión

Adaptación de células de L. monocytogenes CECT 5873, 911 y 4032 al BAC aplicando el método clásico.

Los resultados obtenidos mostraron que en las cepas 911 y 4032 el nivel de adaptación máxima se alcanzó después de tan solo una exposición (desde DL_{50} de 3.80 mg/l en la salvaje hasta 5.06 mg/l en el caso de la 911 y desde 6.96 mg/l hasta DL_{50} 9.19 mg/l en el caso de la 4032), lo cual demuestra la falta de optimización del método clásico e indica que el control de la adaptación de la cepa objeto de estudio después de cada exposición al BAC permitiría, al menos en algunos casos, reducir el tiempo de experimentación empleado en estudios previos (Aase et al. 2000; To et al. 2002; Romanova et al. 2006).

Por el contrario, la cepa 5873 no alcanzó su nivel máximo de adaptación ni después de 5 exposiciones sucesivas al BAC, indicando una mayor capacidad adaptativa de esta cepa respecto a las anteriores. Dicha capacidad adaptativa se refleja en un mayor incremento entre la resistencia de la cepa 5873 salvaje (DL_{50}=2.71 mg/l) y la cepa 5873 adaptada después de 5 exposiciones (DL_{50}=7.1 mg/l). Capacidad que justifica la selección de esta cepa para los estudios subsiguientes, en los que se ensayaron los efectos del tamaño del inóculo y de la concentración de BAC sobre el nivel de adaptación tras una sola exposición de células de *L. monocytogenes* 5873 en fase exponencial.

Una estrategia alternativa para la obtención de células adaptadas de L. monocytogenes CECT 5873 al cloruro de benzalconio.

Los resultados experimentales resultaron describibles mediante la siguiente ecuación empírica (poner el valor de r=0.925), cuya representación se observa en la **Figura 2.1**:

$$DL_{50}=7,82-0,99B+1,42I+2,24BI \qquad [\mathbf{2.1}]$$

A partir de la ecuacion **[2.1]** se deduce claramente la importancia del tamaño del inóculo durante las exposiciones en la resistencia final adquirida así como una

interacción positiva entre aquél y la concentración de BAC. Sin embargo, el efecto individual negativo del cloruro de benzalconio refleja la necesidad de mantener la relación [nºde células: BAC] en un valor tal que se encuentre por encima de los límites de crecimiento de la cepa. En definitiva, como consecuencia de la aplicación de este protocolo, y dentro del ámbito experimental, se consiguió incrementar la resistencia al BAC aproximadamente por un factor de 3.69 (DL_{50}=10 mg/l) con respecto a la cepa salvaje (células no adaptadas DL_{50}=2.71 mg/l) después de 33 horas de exposición. Ello supuso una mejora considerable respecto al método tradicional, con el que la resistencia al BAC de *L. monocytogenes* CECT 5873 se incrementó por un factor de factor: 2.61 después de después de 5 días de exposiciones sucesivas a concentraciones subletales de BAC.

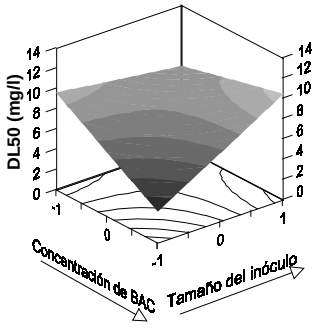

Figura 2.1. Efectos del tamaño de inóculo y de la concentración de BAC durante la exposición sobre la resistencia (DL_{50}) de células de *L. monocytogenes* CECT 5873. Las variables independientes se expresan en valores codificados (**Tabla 2.1**).

Además, un análisis comparativo inicial entre los perfiles proteicos de células de *L. monocytogenes* CECT 5873 no adaptada y adaptada al BAC demostró que la adaptación al biocida parece acompañarse de una represión proteica.

Por último, con el objetivo final de identificar aquellos genes responsables de la resistencia al BAC en *L. monocytogenes*, se llevó a cabo un estudio de sensibilidad al BAC de un banco de mutantes (alrededor de 10000 para ambas cepas) de *Listeria monocytogenes* EGDe y LO28, identificándose los genes y proteínas asociadas con la resistencia al biocida en aquellos casos que obtuvimos valores de MIC superiores a la cepa salvaje. La implicación exacta de la proteína codificada *lmo2277* en la resistencia

de *L. monocytogenes* EDGe al BAC se está llevando a cabo mediante mutagénesis y estrategias de clonación.

3. Resistencia al cloruro de benzalconio, ácido peracético y nisina durante la formación de biofilms maduros de *Listeria monocytogenes.* *Food Microbiology*. *doi:10.1016/j.fm.2010.09.014.*

3.1. Introducción

Una de las consecuencias de la formación de biofilms que puede provocar serios problemas económicos y de salud es la adquisición de resistencia (adaptativa) a biocidas (Aarnisalo et al., 2007; Gram et al., 2007; Leriche et al., 1999; Minei et al., 2008). De hecho, la eficacia de diferentes biocidas para erradicar biofilms de *L. monocytogenes* ha sido evaluada en varios estudios (Aarnisalo et al., 2000; Ammor et al., 2004; Chavant et al., 2003; Tyh-JenK and Frank, 2003). La mayoría se han centrado en desinfectantes industriales como compuestos de cloruro cuaternario, alcoholes, compuestos clorados y otros agentes oxidantes tales como el ácido peracético, ozono y derivados de peróxidos (Aarnisalo et al., 2007; Frank et al., 2003; Pan et al., 2006; Krysinski and Brown, 1992; González-Fandos et al., 2005). Por el contrario, existen pocos estudios de antimicrobianos naturales y sólo algunos de aceites esenciales (Chorianopoulus et al., 2008; Sandasi et al., 2008; Sandasi et al., 2009) y ácido láctico y bacteriocinas producidos por biofilms de bacterias lácticas (Leriche et al., 1999; Minei et al., 2008; Guerrieri et al., 2009).

La mayoría de los trabajos existentes en bibliografía emplean *biofilms* inmaduros (72 h: Aarnisalo et al., 2007; 24 h: Bonaventura et al., 2008; 40 h: Borucki et al., 2003; 28 h: Frank et al., 2003; 48 h: Pan et al., 2006). La excepción es el grupo de Gram et al., (2007, 2008), en cuyos estudios simulan condiciones reales con biopelículas de más de 7 días. Sin embargo, de acuerdo con resultados previos obtenidos en esta tesis, 60 horas de incubación a 25ºC resultaba insuficiente para la formación de una biopelícula madura resistente. Por ello, en el presente capítulo se estudió la formación de las biopelículas maduras durante 11 días a 25 ºC en aquellos casos seleccionados como de mayor riesgo a partir del desarrollo del objetivo 1. y de la CECT 5873 adaptada al BAC obtenida tras

el desarrollo del objetivo 2. Para la evaluación del estado de maduración de las biopelículas se utilizaron dos criterios complementarios: la adquisición de resistencia a tres biocidas (cloruro de benzalconio (BAC), ácido peracético (PA) y nisina (NIS) y el análisis microscópico de las estructuras biopeliculares formadas. Ello supone una mejora importante del planteamiento experimental habitual en la bibliografía publicada, en donde la formación de biopelículas es evaluada frecuentemente cuantificando simplemente biomasa (Di Bonaventura, 2008; Djordjevic et al., 2002; Harvey et al., 2006; Folsom et al., 2006). Y sólo en algunos casos se realizaron análisis microscópicos *in situ* (Borucky et al., 2003; Chae and Scraft, 2000; Chavant et al., 2003; Kalmokoff et al., 2001; Rodríguez et al., 2008; Moltz and Martin, 2005).

3.2. Materiales y Métodos

Casos experimentales estudiados:

- 911-PP-AIV (Biopelículas maduras de *L. monocytogenes* CECT 911 formadas sobre polipropileno y con agua intervalvar de mejillón)
- 4032-PP-ACM (Biopelículas maduras de *L. monocytogenes* CECT 4032 formadas sobre polipropileno (PP) y con agua de cocción de mejillón)
- 5873-INOX-ACM (Biopelículas maduras de *L. monocytogenes* CECT 5873 formadas sobre acero inoxidable y con agua de cocción de mejillón).
- 5873A-INOX-ACM (Biopelículas maduras de *L. monocytogenes* CECT 5873 adaptada al BAC (LD_{50}: 2.71 mg/l; Cepa adaptada al BAC: 7.2 mg/l) formadas sobre acero inoxidable (INOX) y con agua de cocción de mejillón. Esta cepa se obtuvo en experimentos previos mediante 2 exposiciones sucesivas a concentraciones crecientes de inóculo y subletales de BAC de la cepa salvaje correspondiente.

Medios residuales y biocidas: el agua de cocción de mejillón e intervalvar se preparó tal y como se describe en el primer apartado (Saá et al., 2009). Posteriormente el pH se ajustó a 7 y se autoclavó a 121 ºC durante 15 minutos para su esterilización.

Biopelículas: la formación de las biopelículas se llevó a cabo en cupones (20 mm x 20 mm) de acero inoxidable o polipropileno previamente esterilizados y situados individualmente en mini placas Petri. En todos los casos, se añadieron 9 ml de inóculo para el desarrollo del biofilm. Y a cada tiempo de ensayo (4 y 11 días), los cupones se lavan mediante inmersión en PBS durante 10 s para eliminar las células no adheridas.

Ensayos dosis-respuesta y determinación del nº de células viables

Se añaden 0.5 ml de cada concentración de biocida (BAC (mg/l): 2.5, 5,10, 17, 25, 50, 100, 250, 500, 1000; PA (mg/l): 1, 2.5, 5, 10, 17, 25, 50, 100, 250, 500; Nisina (UI/ml): 5, 10, 15, 20, 50, 75, 100, 125, 150, 165, 185, 200, 400, 1500) sobre toda la superficie del cupón previamente lavado. Se espera el tiempo de exposición predeterminado (10 minutos) y se neutraliza añadiendo 10 ml de una disolución neutralizante (34 g/l KH_2PO_4 ajustado a pH 7.2 con NaOH, 3 g/l soybean lecithin, 30 ml/l Tween 80, 5 g/l $Na_2S_2O_3$ y 1 g/l L-histidine). El número de células viables se determina siguiendo el método ya descrito. En los ensayos realizados en células plantónicas se utilizó el mismo procedimiento, pero en tubos y utilizando TSB como medio de ensayo. Experimentos preeliminares permitieron conseguir aproximadamente la misma concentración de inóculo en ambos sistemas. Todos los experimentos dosis-respuesta se realizaron por triplicado

3.3. Resultados

Resistencia al cloruro de benzalconio de células plantónicas y biopelículas de L. monocytogenes.

Se compararon los valores de resistencia (LD_{90}) al BAC obtenidos en células plantónicas con los obtenidos en el caso de biopelículas maduras (tras 4 y 11 días)**.** Además, y con el objetivo de investigar si la adaptación fisiológica después de la exposición al BAC desencadenaba resistencias cruzadas en las biopelículas maduras que pudieran formar, la cepa CECT 5873 adaptada al BAC (LD_{50}= 7.22) se incluyó en este y en los siguientes estudios.

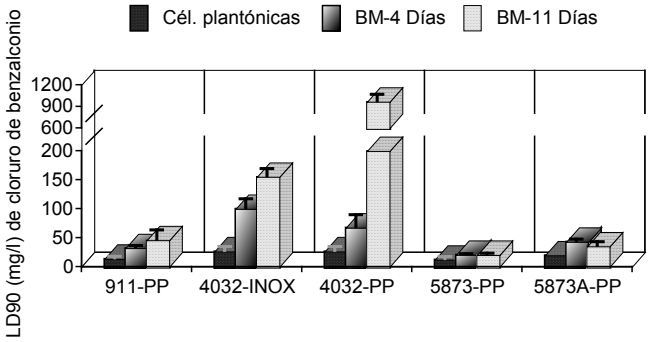

Figura 3.1: Valores de DL_{90} (mg/l) obtenidos después de la exposición de células plantónicas y biopelículas maduras de *L. monocytogenes* al cloruro de benzalconio (BAC).

Los resultados obtenidos (**Figura 3.1**) muestran un incremento significativo ($p<0.05$) en los valores de LD_{90} (es decir resistencia) entre las células plantónicas y los *biofilms* al igual que para la nisina. Sin embargo, mientras que en el caso de la CECT 5873 no se produce un aumento significativo de la resistencia de los *biofilms* formados en polipropileno (PP) después de 4 y 11 días, la resistencia de las biopelículas formadas por la cepa 911 y la 4032 aumenta significativamente con el tiempo de maduración y fue significativamente mayor para la 4032 ($p<0.05$) tanto en INOX como en PP, siendo superior en PP donde la resistencia aumenta por un factor de 36 después de 11 días con respecto al sistema plantónico. Los valores de los parámetros obtenidos tras los ajustes de las curvas dosis-respuesta obtenidas tras la aplicación de los tres biocidas a la ecuación logística se muestran en la **tabla 3.1**.

Resistencia a la nisina de células plantónicas y biopelículas de L. monocytogenes.

En este caso, a diferencia del BAC, no se observaron diferencias significativas entre los valores de LD_{90} obtenidos para los *biofilms* de 4 días. Sin embargo, después de 11 días, los *biofilms* formados por la cepa 4032 en INOX fueron significativamente más resistentes que los formados por la misma cepa en polipropileno y por la cepa 911 (**Figura 3.2**).

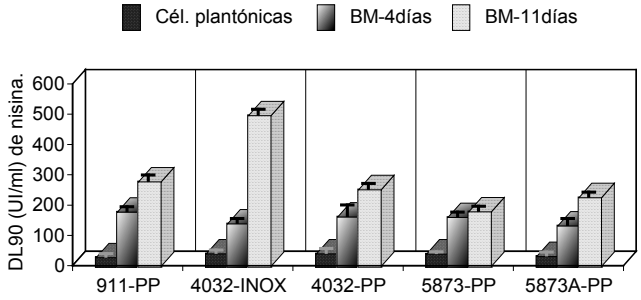

Figura 3.2: Valores de DL_{90} (mg/l) obtenidos después de la exposición de células plantónicas y biopelículas maduras de *L. monocytogenes* a nisina.

De nuevo, los *biofilms* formados por la cepa 5873 resultaron ser los más sensibles, no observándose diferencias significativas entre los valores de su resistencia obtenidos durante la maduración. Sin embargo, se produjo un incremento significativo en la resistencia ($p < 0.05$) al BAC de la cepa adaptada con respecto a los *biofilms* formados por la no adaptada después de 11 días de incubación.

Table 3.1: Valores de los parámetros (k y r) obtenidos del ajuste de los datos experimentales a un modelo logístico (Cabo et al., 1999; Cabo et al., 2009)

	911-PP-IWM		**4032-SS-MCJ**		**4032-PP-MCJ**		**5873-PP-MCJ**		**5873A-PP-MCJ**	
	BM. 4D	BM.11D	BM. 4D	BM.11D	BM. 4D	BM.11D	BM. 4D	BM.11D	BM. 4D	BM.11D
K_{BAC}	97.363	95.506	99.65	99.450	99.060	93.188	99.477	99.097	99.748	91.275
r_{BAC}	0.308	0.1746	0.053	0.033	0.235	0.007	0.256	0.231	0.138	0.404
K_{nisin}	99.595	99.056	86.413	91.913	84.872	87.189	69.412	83.274	76.843	89.048
r_{nisin}	0.013	0.007	0.215	0.006	0.255	0.023	0.028	0.327	0.0730	0.029
K_{PA}	99.342	99.810	99.704	99.736	99.998	97.457	98.976	98.560	94.353	99.883
r_{PA}	0.330	0.419	0.169	0.069	0.480	0.097	0.439	0.263	2.816	2.255

Por último, en el caso de la nisina, y tal y como se muestra en la **tabla 3.1**., se obtienen menores valores de k y de r que en el caso del BAC y el ácido peracético

Resistencia a ácido peracético (AP) de células plantónicas y biopelículas de L. monocytogenes.

En el caso del ácido peracético, y a diferencia de los anteriores, se obtuvieron valores de LD_{90} significativamente más altos para las células plantónicas cuando se utilizaba TSB como medio de cultivo, que para las biopelículas maduras después de 4 y 11 días de incubación (Figura 3.3). Sin embargo, cuando en experimentos posteriores (*data not shown*) se sustituyó el TSB por tampón fosfato (PBS) se obtenían los mismos valores de resistencia que los obtenidos en los biofilms de 4 días de maduración, indicando un efecto de la materia orgánica presente en el medio (data not shown).

Únicamente en el caso de la cepa 4032 se observó un incremento significativo en la resistencia ($p < 0.05$) entre 4 y 11 días de maduración en ambos materiales. Finalmente, en el caso de la cepa 5873 adaptada al BAC, los valores de resistencia de las biopelículas maduras formadas tras 4 y 11 días de incubación fueron significativamente menores que en el caso de las formadas por la cepa salvaje correspondiente.

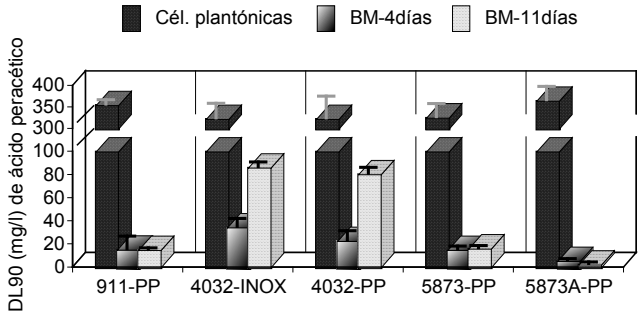

Figura 3.3: Valores de DL_{90} (mg/l) obtenidos después de la exposición de células plantónicas y biopelículas maduras de *L. monocytogenes* al ácido peracético (AP)

Análisis microscópico de biopelículas de L. monocytogenes formadas en los escenarios ensayados.

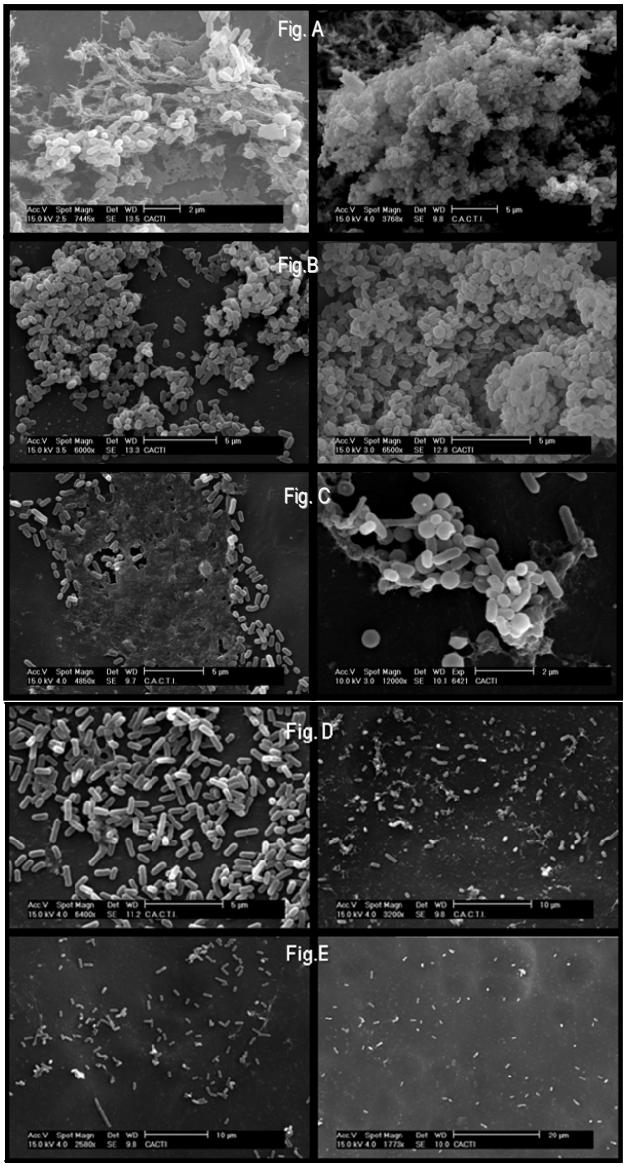

Figura 3.4: Imágenes de microscopio electrónico (SEM) de la cepa 4032 (4b) en acero inoxidable (INOX) (**A**) y polipropileno (PP) (**B**), CECT 911 (1/2 c) en PP (**C**) y la CECT 5873 (**D**) y 5873A (**E**) en PP después de 4 (derecha) y 11 días (izquierda).

Las imágenes obtenidas después de 4 y 11 días de maduración mostradas en la **Figura 3.4** permitieron diferenciar tres niveles de densidad celular, siendo claramente la CECT 4032 la que muestra una estructura tridimensional más compleja.

Estudio de la relación de las cinéticas de adherencia y la formación de biopelículas de L. monocytogenes.

Las cinéticas de adherencia obtenidas (**Figura 3.5)** muestran la ausencia de diferencias significativas entre el número máximo de células adheridas de *Listeria* en los diferentes escenarios.

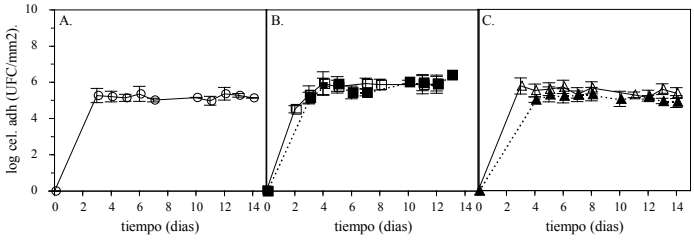

Figura 3.5: Cinéticas de adherencia de *Listeria monocytogenes* en diferentes casos experimentales. CECT 911 en polipropileno (E) en presencia de agua intervalvar (AI). CECT 4032 en acero inoxidable (**V**) en presencia de agua de cocción de mejillón (ACM) y en polipropileno (B**)** en ACM **C:** CECT 5873 (**C**) y la CECT 5873A (adaptada al BAC) (**H**) en polipropileno en ACM.

3.4. Discusión

Los resultados obtenidos demostraron que la cepa 4032 es la que forma biopelículas maduras más resistentes después de 11 días de incubación a 25 ºC (**Figuras 3.1, 3.2, 3.3**) con respecto a los 4 días y al estado plantónico. Además, las imágenes obtenidas tras el análisis microscópico mostraron que es también esta cepa la que forma estructuras tridimensionales más complejas (**Figura 3.4**). Los resultados demostraron, por tanto, una relación entre la estructura tridimensional de las biopelículas maduras de *L. monocytogenes* y su resistencia a los biocidas ensayados. Esta aparente relación entre estructura-resistencia apoya la teoría de que el aumento de resistencia observado en biopelículas bacterianas tiene más que ver con la disposición de las células que con

cambios fisiológicos o metabólicos a nivel celular (Folsom et al., 2006; Kalmokoff et al., 2001) y explica aquellos casos en los que no se observan diferencias significativas con respecto a las células planctónicas (Kastbjerg and Gram, 2009; Stopforth et al., 2002). Sin embargo, dicho incremento de resistencia a biocidas y complejidad parece no depender de la adherencia inicial (**Figura 3.5**), lo que indica la necesidad de considerar biopelículas y no células plantónicas cuando se trata de optimizar protocolos de desinfección. De hecho, parece que la capacidad de formación de biopelículas maduras es inherente a cada cepa de *L. monocytogenes* y que no depende estrictamente de la capacidad inicial de las células de adherirse. Prueba de ello es que los mayores valores de adherencia inicial se observaron en los casos 911-PP-AI y 4032-PP-ACM, siendo mucho menores en aquellos casos donde se utilizó como superficie experimental el acero inoxidable. Estos resultados evidencian además la necesidad de conocer la composición de la matriz extracelular para mejorar las estrategias actuales de desinfección actuales.

El ácido peracético parece ser el más efectivo de los tres desinfectantes empleados en biopelículas (**Figura 3.3**). Su elevada capacidad oxidante junto con su bajo peso molecular podría facilitar su penetración dentro del biofilm. Sin embargo, tiene una aplicabilidad reducida en superficies sucias ya que su elevada reactividad implica la disminución de su acción en presencia de materia orgánica disuelta, mucho más abundante en cultivos líquidos que en las células adheridas lavadas previamente a la exposición al desinfectante. En el extremo opuesto se encuentra la nisina, para la que el aumento de complejidad de la biopelícula proporciona valores de inhibición máxima por debajo del 90 % (tabla 3.1) debido probablemente a que la formación de la matriz de exopolisacáridos dificulta más la efectividad de este antimicrobiano, lo cual pudiera relacionarse con su enorme capacidad de adsorción en superficies.

Si comparamos la cepa 5873 salvaje con la adapatada (5873A) al BAC, se identificaron varias respuestas cruzadas: una de signo positivo, entre el BAC y la nisina, y otra de signo negativo, entre el BAC y el ácido peracético. Estos resultados ponen de manifiesto la importancia del estudio de respuestas cruzadas en biofilms para preveer posibles situaciones de riesgo derivadas de la adaptación a desinfectantes de amplio uso industrial. En este sentido, sólo hemos podido encontrar un estudio (Stopforth et al., 2002) en el cual los autores no encontraron respuestas cruzadas entre la adaptación de

biofilms de *L. monocytogenes* al ácido y la exposición subsiguiente al AP, al BAC y al hipoclorito sódico.

Finalmente, los resultados obtenidos muestran que la utilización de ingredientes puros activos mejora los empleados habitualmente, productos comerciales mezclas de varios compuestos, ya que evitan posibles respuestas de resistencia múltiples (Frank et al., 2003; González-Fandos et al., 2005; Jacquet and Reynaud, 1994) Paula, yo este párrafo lo sacaría, realmente no hacemos ninguna comparación entre los desinfectantes puros y los comerciales.

La determinación de la resistencia del biofilm (DL_{90}) a partir de principios básicos de la cinética microbiana permite su adecuada cuantificación y posibilita la comparación objetiva entre los distintos casos estudiados. Ello supone además una alternativa útil a la utilizada en la mayoría de los trabajos previos, en donde sólo se estudian 2 o tres concentraciones de biocidas en los ensayos dosis-respuesta (Aarnisalo et al., 2000; Thy-Jenq et al., 1993), se dosifican mezclas de desinfectantes (en algunos casos de composición desconocida) y se cuantifica la resistencia del biofilm mediante métodos semicuantitativos (MIC) y durante un periodo demasiado corto de incubación.

4. Cinéticas de adherencia, resistencia al cloruro de benzalconio y análisis microscópico de biopelículas mixtas formadas por *Listeria monocytogenes* y *Pseudomonas putida.*

4.1. Introducción

Una de las vías frecuentes de contaminación alimentaria es mediante contaminación cruzada a través de superficies de plantas de procesado de alimentos (Ammor et al., 2004; Norwood and Gilmour, 1999; Porsby et al., 2008) donde *L. monocytogenes* puede adherirse y formar biofilms (Aase et al., 2000; To et al., 2002; Takahashi et al., 2009). Esta resistencia se relaciona con la estructura tridimensional de las células que constituyen el biofilm, la cual es el resultado de interacciones entre las especies bacterianas presentes (Wuertz et al., 2004) y determina el grado de acceso de los biocidas a las células (Bourion and Cerf, 1996, Qu et al., 2010). Además, se ha demostrado que las células de los biofilms son fenotípica y genotípicamente diferentes a

las células plantónicas (Nadell et al., 2008). Esta situación se complica aún más debido a que en situaciones reales más de dos especies bacterianas coexisten formando biofilms y se ha demostrado que esto podría incrementar su resistencia a los biocidas (Sharma and Anand, 2002; Kastbjerg and Gram, 2009).

4.2. Matreriales y métodos

En el presente trabajo se estudió el efecto de la presencia de *P. putida* CECT 845 sobre la resistencia al cloruro de benzalconio de biopelículas maduras formadas por *L. monocytogenes* en los 5 escenarios considerados en el apartado 3. Se utilizó el mismo diseño experimental que el descrito en dicho apartado, salvo la inoculación simultánea de *P. putida* y *L. monocytogenes* a la misma concentración (10^8 UFC/ml en ambos casos).

4.3. Resultados y discusión

Comparación entre la adherencia de L. monocytogenes en monocultivo y en presencia de Pseudomonas putida CECT 845

Se compararon las cinéticas de adherencia de 3 cepas de *L. monocytogenes* (CECT 911, 4032 and 5873) en monocultivo y co-cultivo con *P. putida* en diferentes escenarios seleccionados de acuerdo a los estudios previos (Saá et al. 2009; Saá Ibusquiza et al. 2010).

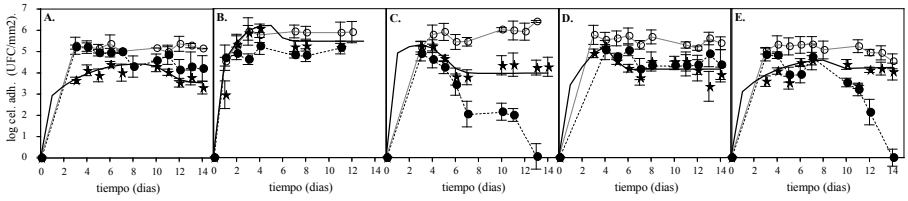

Figura 4.1: Cinéticas de adherencia de *Listeria monocytogenes* en monocultivo (**O**) y en presencia de *Pseudomonas putida* 845 (●)**. A:** CECT 911 en polipropileno y agua intervalvar (AIV); **B:** CECT 4032 en acero inoxidable y agua de cocción de mejillón (ACM); **C:** CECT 4032 en polipropileno en ACM; **D:** CECT 5873 en polipropileno en ACM and **E**: CECT 5873A (adaptada al BAC) en polipropileno en ACM. Además se incluyen en esta gráfica las cinéticas de adherencia observadas de *P. putida* (∗) y esperadas (línea continua) en las biopelículas mixtas.

Tal y como muestra la **figura 4.1,** el resultado más significativo es el descenso pronunciado del número de células adheridas en presencia de *P. putida* observado en el caso de la cepa 4032 y la 5873 adaptada al BAC en polipropileno (**Figura 4.1.B**. y **4.1.E.**).

Aunque el modelado de los datos experimentales facilita su comparación, en el caso de *L. monocytogenes* la ausencia de puntos antes en los 4 primeros días imposibilitó el ajuste matemático. No así en el caso de *P. putida*, donde los resultados de adherencia resultaron describibles (r^2=0.995) mediante un modelo aditivo de los ecuaciones logísticas que describen adecuadamente el incremento y descenso de la población de la biopelícula (**ecuación 1.2)** (Herrera et al. 2007; Saá et al. 2009). Y este ajuste de los datos experimentales permitió identificar 2 patrones de adherencia de este microorganismo en presencia de *L. monocytogenes*, que, como veremos, van a dar lugar a dos patrones de asociación del inóculo mixto:

- Patrón tipo A: que tiene lugar cuando *Pseudomonas* se asocia con las cepas de *Listeria* 4032, especialmente en INOX, y con la 5873 salvaje (**Fig. 4.1.B. C. y D.** respectivamente). Se caracteriza por mayores niveles de adherencia máxima (veánse valores de parámetro a_{ad} y r_{ad} en la **tabla 4.1**) que los obtenidos en el patrón B.

Tabla 4.1.: Valores de los parámetros obtenidos tras ajuste de los resultados experimentales de adherencia de *Ps. putida* en presencia de diferentes cepas de *L. monocytogenes* a la ecuación [1.2]

	911-PP-IWM	**4032-SS-MCJ**	**4032-PP-MCJ**	**5873-PP-MCJ**	**5873-PP-MCJ**
a_{ad}	30499	1800000	370723	199237	45000
r_{ad}	5,014	2,974	1,101	3,204	5,014
m_{ad}	0,700	1,843	0,998	1,881	0,700
a_D	26244	1497876	268936	184311	26244
r_D	10,425	5,500	4,173	4,965	8,500
m_D	1,676	4,500	1,820	51,632	8,000

- Patrón tipo B: que tiene lugar cuando *Pseudomonas* se asocia con las cepas de *Listeria* 911 y la 5873 adaptada al BAC **(Fig. 4.1.A. y E.**

respectivamente). En este caso, los valores máximos de adherencia de *P. putida* descienden 1-log con respecto al patrón de tipo A.

Estos dos patrones identificados van a dar lugar a biopelículas maduras de 4 días de maduración con diferente resistencia al cloruro de benzalconio.

Comparación entre la resistencia al BAC de biopelículas monoespecie de L. monocytogenes y biopelículas mixtas formadas tras la asociación de L. monocytogenes y P. putida

Los resultados obtenidos mostraron que *P. putida* tiene un papel determinante en la formación de las estructuras biopeliculares mixtas, observándose que su asociación con *L. monocytogenes* modifica significativamente ($p<0.05$) la resistencia al BAC después de 4 y 11 días de incubación a 25 ºC respecto a los *biofilms* monoespecie (**Figura 4.2**). No obstante, los efectos de la presencia de *P. putida* fueron diferentes tras 4 y 11 días de incubación.

Después de 4 días de incubación, los resultados obtenidos demostraron que la presencia de *P. putida* en la biopelícula supone un incremento significativo de la resistencia al cloruro de benzalconio en todos los casos experimentales ensayados (**Figura 4.2**.). Estos resultados están de acuerdo con los obtenidos previamente por otros autores (Ammor et al., 2004; Bourion and Cerf 1996). Sin embargo, en todos los escenarios ensayados los niveles de adherencia de *L. monocytogenes* disminuyen significativamente en presencia de *Pseudomonas putida* respecto a los observados en monocultivo (**Figura 4.1**).

A este tiempo de maduración, y tal y como se anunció previamente, los resultados de resistencia al BAC permitieron diferenciar los casos experimentales en dos grupos de acuerdo con las cinéticas de adherencia de *P. putida* desde el inóculo mixto: i) patrón favorable para la formación de biopelículas, que ocurre en 3 de los 5 escenarios estudiados (cepas 4032 y 5873 salvaje, **Fig. 4.1.B. C. y D.** respectivamente) y se caracteriza por una mayor velocidad de adherencia de *P. putida* respecto a *L. monocytogenes*. Y que después de 4 días de incubación da lugar a las biopelículas más resistentes al BAC, llegándose a multiplicar los valores de la resistencia (medida en términos de la DL_{90}) por un factor de 6 y ii) patrón desfavorable para la formación de

biopelículas, que ocurre en los escenarios de las cepas 911 y 5873 adaptada al BAC, (**Fig. 4.1.A. y E.** respectivamente) en los que la velocidad de adherencia de *P. putida* es menor que la de *L. monocytogenes.* En estos casos la resistencia aumenta por un factor de 2. Especialmente significativo es el aumento de resistencia observado en la CECT 5873, cuya asociación con *L. monocytogenes* le permite formar biopelículas resistentes.

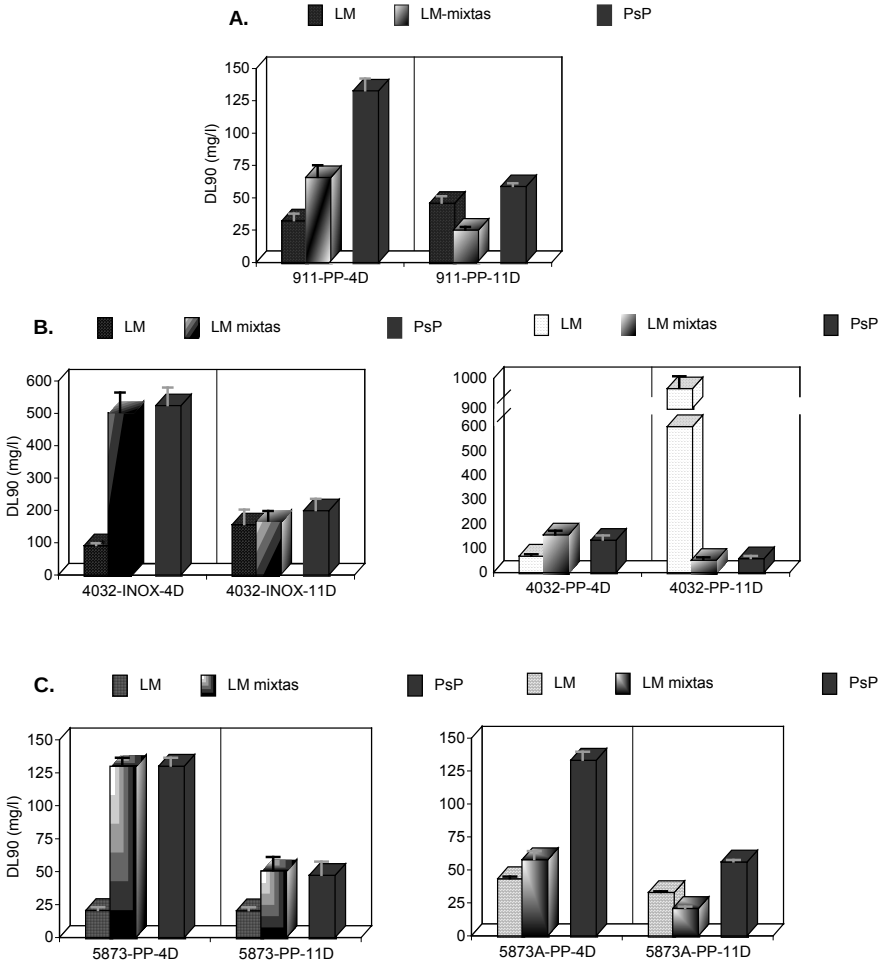

Figura 4.2: Comparación entre la DL_{90} de BAC (mg/l) frente a biofilms monoespecie y biofilms mixtos de *L. monocytogenes* en presencia de *P. putida* empleando diferentes cepas de *L. monocytogenes*. **A.:** con la CECT 911-PP-AI; **B.:** CECT 4032-INOX-ACM, 4032-P-ACM; **C.:** CECT 5873-PP-ACM y CECT 5873A (adaptada al BAC)-PP-ACM

Por el contrario, después de 11 días de incubación se observa una disminución significativa ($p<0.05$) de la resistencia al cloruro de benzalconio de la biopelícula constituida por *L. monocytogenes* y *P. putida* respecto a los valores obtenidos a los 4 días de incubación que refleja una desestructuración de la biopelícula mixta ocurrida en todos los casos experimentales ensayados, estando acompañada además en 2 de los 5 casos ensayados (4032 y 5873A en polipropileno) por un descenso pronunciado del número de células adheridas (**Figura 4.1.C.** y **E.**). Mientras que el desprendimiento de células de biofilms de monoespecie ha sido descrito previamente por varios autores (*S.aureus*: Herrera et al., 2007, *P. aeruginosa*: Boles et al., 2005, *B. cereus*: Wijman et al., 2007), las razones por las cuales se produce son todavía inciertas (Marshall, 1988, Takhistov and George, 2004, Rodríguez et al., 2007, Sauer et al., 2004; Liu et al., 2007; Boles et al., 2005). Además, los resultados obtenidos demuestran que la adaptación de la cepa 5873 al BAC disminuye su capacidad de asociación con *P. putida*, proporcionando biopelículas mixtas significativamente menos resistentes ($p<0.05$) al BAC y con una mayor tendencia a desestabilizarse que la cepa salvaje.

Análisis de microscopio

El análisis microscópico de las estructuras formadas demostraron una mayor complejidad tridimensional para el *biofilm* formado por la cepa CECT 4032-INOX (**Figuras 4.3 A y B**) tanto en presencia como en ausencia de *P. putida* tras 11 días de incubación. En el extremo opuesto se encuentra la CECT 5873A, cepa cuya asociación al BAC proporciona biopelículas de menor resistencia y con el mayor nivel de desestructuración de todos los casos ensayados (**Figura 3.3.C** y **D**)

Además, la distribución de las cepas de acuerdo a su resistencia al BAC se mantiene en presencia de *P. putida* respecto a la obtenida en las correspondientes biopelículas maduras monoespecie, siendo la 4032 (serotipo 4b) la más resistente y la cepa 911 la que presenta valores de DLs iguales en ambos casos a 4 y 11 días de incubación. La excepción es la cepa 5873 salvaje, que en presencia de *P. putida* forma una biopelícula mucho más resistente al BAC que en el monocultivo, donde no se produce un aumento de la resistencia con el tiempo de incubación. En cualquier caso, y salvo este caso concreto, los resultados obtenidos apoyan la teoría de que la capacidad de *L.*

monocytogenes de formar biofilms y el tipo de arquitectura del biofilm formado no es un proceso estocástico, sino regulado a nivel celular y poblacional (*quorum sensing*) de manera específica en cada cepa, tal y como ha sido demostrado en otros géneros bacterianos.

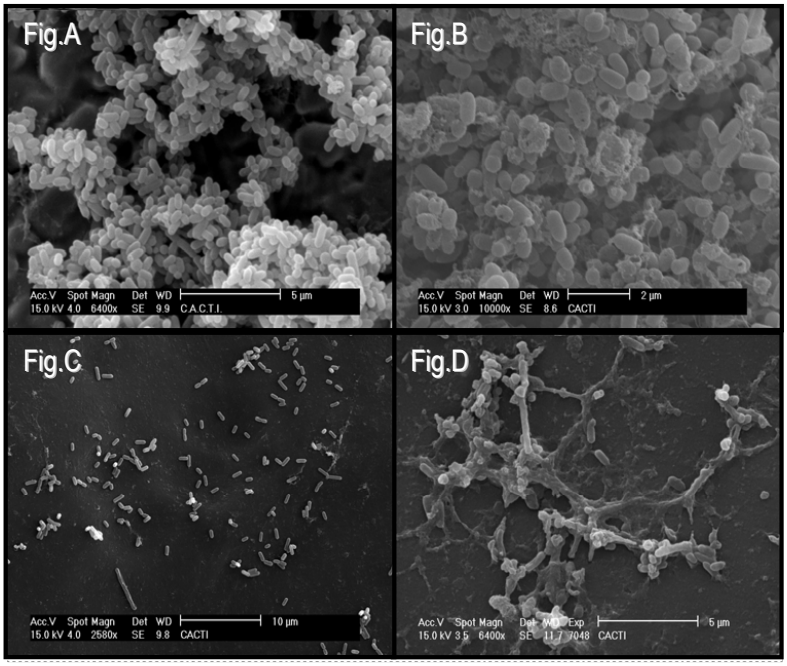

Figura 4.3: Imágenes de microscopía electrónica (SEM) de biofilms maduros de 11 días formados por las cepas CECT 4032-INOX (**3A**) y la CECT 5873A (**3C**)-PP monoespecie y asociados con *P. putida* CECT 845 (**3B y 3D**).

Los resultados obtenidos mostraron además un claro efecto del material sobre la asociación entre *P. putida* y *L. monocytogenes* CECT 4032, tal y como se refleja en la **Figura 4.4**., en donde se observa cómo la desestructuración de la biopelícula mixta después de 11 días de maduración es significativamente mayor en polipropileno (4.4. B) que en acero inoxidable.

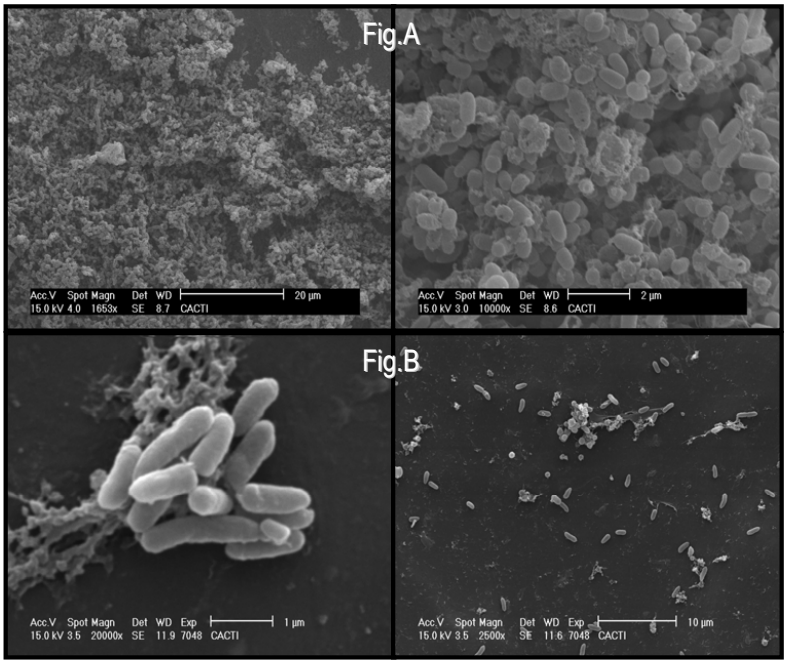

Figura 4.4: Imágenes de microscopía electrónica (SEM) de biofilms maduros de 11 días formados por la cepa CECT 4032 en INOX (4A) y en PP (4B) con *P. putida.*

En conjunto, estos resultados reflejan que la consideración de las asociaciones bacterianas que ocurren en la naturaleza en el diseño de protocolos de desinfección redundaría en una mejora de los sistemas de control suponiendo un incremento de la seguridad alimentaria. Pero además, la aplicación de biocidas debe de asegurar la eliminación de materia orgánica residual, que puede servir de anclaje para recontaminaciones y formación de nuevas biopelículas. De hecho, el análisis microscópico realizado demuestra la presencia de materia orgánica residual después de la desinfección con cloruro de benzalconio de 100 mg/l (**Figura 4.5**). Por tanto, se hace necesario el diseño de protocolos de desinfección que aseguren la eliminación completa de la matriz después de la desinfección.

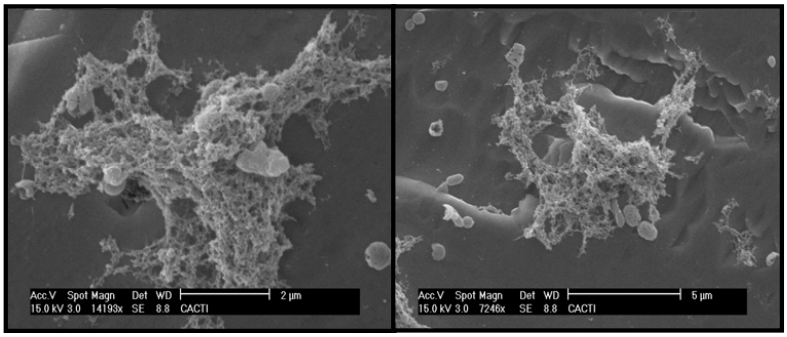

Figura 4.5: Imágenes de microscopía electrónica (SEM) de los residuos de los biofilms formados por la cepa CECT 4032 en INOX y *P. putida* tras la acción del BAC empleando una concentración de 100 mg/l.

Aunque tanto los resultados obtenidos en el capítulo previo como en este dejan claro que la mayor o menor capacidad de formar biopelículas es inherente a cada cepa bacteriana, también ponen en evidencia que la presencia de *P. putida* en el biofilm no es irrelevante. Dos hechos principales observados apoyan esta afirmación:

1) Que la cepa salvaje 5873 en presencia de *Ps. putida* forma *biofilms* con una mayor resistencia al BAC que los correspondientes *biofilms* monoespecie (**Figura 4.2 C**).

2) Que las diferencias entre los *biofilms* formados por la cepa 4032 en polipropileno y en acero inoxidable se deben a una mayor capacidad de adherencia de *P. putida* esta última, con máximos de adherencia incrementados en más de 1-log. Como consecuencia, se forma un *biofilm* con una mayor resistencia al BAC en INOX, después de 4 y 11 días de incubación (**Figura 4.2. B**). Sin embargo, otra explicación para estas diferencias podría deberse a un descenso en la actividad metabólica derivada de la formación de un *biofilm* más denso y complejo sobre acero inoxidable (Rodrigues et al. 2009), lo cual implica una mayor estabilidad y probablemente un retraso en el comienzo de la fase de desestructuración del *biofilm*. Además, la presencia de conexiones intercelulares y la proximidad entre las células de diferentes especies en el *biofilm* podría facilitar la transferencia de material genético, incluídos los genes asociados a la resistencia a estímulos externos (Ammor et al., 2004, Wuertz et al., 2004, Nadell et al., 2009).

El desarrollo de los objetivos anteriores, permitió concluir que la cepa 4032 (4b), sola o asociada con *P. putida*, es la que forma estructuras biopeliculares más resistentes a desinfectantes y que el incremento de dicha resistencia se relaciona sin duda con su capacidad de formar estructuras tridimensionales complejas. Sin embargo, para los estudios de adaptación al BAC se eligió la cepa CECT 5873 por ser la que mostraba la mayor capacidad adaptativa al cloruro de benzalconio, demostrándose *a posteriori* su condición de mala formadora de biopelículas y como consecuencia, su mayor facilidad para su transferencia a alimentos.

5. Efectos combinado de CO_2 y nisina sobre células de *Listeria monocytogenes* transferidas a mejillón desde biopelículas adaptadas y no adaptadas al cloruro de benzalconio. Accepted in Journal of Food Protection

5.1. Introducción

De acuerdo con las últimas inspecciones realizadas por la EFSA (2009, 2010), uno de los tipos de alimentos con mayor incidencia en *L. monocytogenes* son los productos de la pesca listos para el consumo. Tal y como se ha descrito, su presencia en alimentos se asocia a su transferencia por contacto desde las plantas de procesado, donde *L. monocytogenes* persiste formando biopelículas (Porsby et al. 2008, Rodríguez and McLandsborough 2007 dicho en el apartado anterior). Y también, en algunos casos se ha demostrado que las cepas de *L. monocytogenes* persistentes son resistentes a QACs como el cloruro de benzalconio (BAC) (Aase et al 2000). Por otro lado, el envasado en atmósferas modificadas (EAM) es una técnica de conservación aplicada con éxito en alimentos de origen marino (Pastoriza et al. 1996a, 1996b, 1998, 2002, 2004; Cabo et al. 2003), pudiendo, sin embargo, favorecer el crecimiento de microorganismos anaerobios facultativos como *L. monocytogenes*. En estas situaciones, la aplicación de bacteriocinas constituye una solución útil para incrementar la calidad y seguridad (Cabo et al. 2001, 2005, 2009). De este modo, en una planta de procesado de mejillón puede existir el riesgo de que células de *Listeria monocytogenes* adaptadas al BAC persistan formando biopelículas y se transfieran por contacto al mejillón antes del envasado. Sin embargo, no existen estudios previos acerca de los efectos de la adaptación de *L.*

monocytogenes a desinfectantes de amplio uso industrial sobre su resistencia a la aplicación de atmósferas modificadas.

El principal objetivo de este trabajo es comparar la viabilidad de células procedentes de una biopelícula adaptada y no adaptada al BAC en atmósferas ricas en CO_2 y O_2 una vez han sido transferidas por contacto a mejillón cocido y vivo, respectivamente, y almacenadas a 2.5ºC. Además, en el caso concreto del mejillón cocido, se recurrió a un diseño factorial de primer orden (Box & Hunter, 1988) para el estudio adicional del efecto combinado del CO_2 y la nisina.

5.2.Materiales y métodos

C*epas*: *Listeria monocytogenes* CECT 5873 adaptada al BAC (DL_{50}=7.2 mg/l) se obtuvo en experimentos previos a partir de dos cultivos sucesivos de la cepa salvaje (DL_{50}=2.7 mg/l) en concentraciones subletales de BAC.

Medios residuales, inóculo y formación e biopelículas: La preparación de los medios residuales y el procedimiento para la formación de biopelículas se realizó como se describe en los apartados previos. Se utilizaron *biofilms* de *L. monocytogenes* CECT 5873 adaptada y no adaptada al BAC formados en cupones de INOX (10 mm x 10 mm) después de 7 días de incubación a 25 ºC. Tras el lavado con PBS durante 30 segundo, el número

Ensayos de transferencia: la transferencia de las células adheridas a los mejillones se realizó mediante el contacto del cupón y la vianda (mejillón cocido) o concha (mejillón vivo) durante 2 minutos. El tiempo de contacto (10 min) se determinó a partir de estudios preliminares, alcanzándose 5.6 log UFC de *L. monocytogenes* por de mejillón cocido y 7.2 log UFC por gramo de mejillón vivo.

Aplicación de la nisina: se dosificó en disolución acuosa (en concentraciones de 210, 115, 21 UI/ml) mediante pipeteo sobre la vianda. Dicha dosificación se realizó después de la transferencia de *L. monocytogenes* y antes del envasado.

Envasado: Las muestras así preparadas se envasaron en bolsas tipo barrera (Cryovac, SL) y se insuflaron a continuación las mezclas de gases (Carburos Metálicos, Barcelona) correspondientes, almacenándose posteriormente en cámara de refrigeración

a 2.5ºC hasta la toma de muestra. A cada tiempo prefijado, se determinó del número de células viables de *L. monocytogenes* mediante los métodos tradicionales de siembra en placa y utilizando agar Palcam (Liofilchem, S.L.R., Italia) a pH=9 como medio de cultivo. El pH del agar se ajustó a 9 para asegurar la inactivación de la nisina.

5.3. Resultados y discusión

En mejillón cocido

El número de células viables de *L. monocytogenes* (LMV) obtenidos resultaron describibles mediante las siguientes ecuaciones empíricas significativas después de 7, 11 y 20 días de almacenamiento, representadas en las **Figuras 5.1** y **5.2**:

- *L. monocytogenes* CECT 5873 no adaptada (DL_{50}=2.7 mg/l):

Despúes de 7 días:	LMV=4.25-1.18C-1.48N-1.18CN	[5.1]
Despúes de 11 días:	LMV=5.25-0.45C-0.74N+0.46CN	[5.2]
Despúes de 20 días a:	LMV=5.72-1.43C-0.86N	[5.3]

- Cepas 5873 adaptada (DL_{50}=7.2 mg/l):

Después de 7 días:	LMV=3.65-0.57C-0.87N	[5.4]
Después de 11 días:	LMV=5.24-0.45C-0.94N	[5.5]
Después de 20 días:	VLM=5.47-0.37C-0.79N+0.54CN	[5.6]

Las ecuaciones obtenidas mostraron el conocido efecto inhibitorio del CO_2 (C) y de la nisina (N) frente a *L. monocytogenes* (efecto del CO_2: Sheridan et al 1995, Olarte et al 2002; efecto de la nisina: Nilson et al. 1997, López- Mendoza et al. 2007). Sin embargo, no fue posible encontrar estudios previos sobre la efectividad del CO_2 en biofilms y solo uno en el caso de la nisina (Minei et al., 2008). Por otro lado, la interacción de signo positivo puso de manifiesto la incompatibilidad de ambas variables a concentraciones altas. Esto último contrasta con estudios previos en donde se demostró un efecto sinérgico entre CO_2 y nisina sobre la supervivencia de células planctónicas de *L. monocytogenes* (López-Mendoza et al. 2007, Nilsson et al. 1997, Nilsson et al 1999,

Szabo et al. 1998). Esta diferencia entre el efecto conjunto de ambas variables en sistemas plantónicos y sésiles podría reflejar un aumento de la incompatibilidad entre antimicrobianos con acción a nivel de membrana derivada de la presencia de exopolisacáridos en las biopelículas transferidas al mejillón cocido. De hecho, Fang and Lin (1994) demuestran la inefectividad de la nisina en presencia de CO_2 frente a *P. fragi* productora de exopolisacáridos.

Los resultados obtenidos demostraron que las biopelículas formadas por células adaptadas de BAC podían ser más resistentes a la aplicación de atmósferas modificadas ricas en CO_2 y nisina una vez transferidas al mejillón cocido por contacto (simulando contaminación cruzada) tal y como reflejan las ecuaciones obtenidas tras 7 y 20 días de almacenamiento siendo los coeficientes obtenidos tanto para el BAC como para la nisina significativamente mayores ($p<0.05$). Después de 7 días, el modelo predice una reducción logarítmica (RL) de 3.39 log y de 5.12 log en *L. monocytogenes* adaptada y no adaptada, respectivamente, una vez transferidas a mejillón cocido y envasado bajo condiciones de 90% de CO_2 y 210 UI/ml de nisina (**ec. 5.1** y **5.4**). Esto supone un incremento en el riesgo asociado a la presencia de este género en las plantas de procesado. Aunque no se encontraron estudios previos sobre resistencias cruzadas derivadas de la resistencia a desinfectantes, sí se ha demostrado una respuesta cruzada entre la tolerancia a ácido de *L. monocytogenes* y su mayor resistencia al CO_2 (Francis et al. 2007). Además, estos resultados concuerdan con el estudio previo desarrollado en el apartado 3 (Saá Ibusquiza et al., 2010) en el que se obtenía que los *biofilms* maduros adaptados al BAC eran más resistentes a la nisina que los no adaptados al BAC.

Sin embargo, después de 11 días de incubación las diferencias entre ambas células biopeliculares de *L. monocytogenes* (adaptadas y no adaptadas) decrecen y se obtienen ecuaciones polinómicas (**ec. 5.2** y **5.5**) y superficies de respuesta (**Fig 5.2** y **5.3**) similares, lo que sin duda responde a la variación de los efectos de las variables resultado de la condición cinética de los sistemas microbianos en alimentos.

Por último, el hecho de que los efectos de las variables decrezcan con el tiempo de almacenamiento demuestra que los biofilms de *L. monocytogenes* resisten los tratamientos empleados en mejillón cocido a esta temperatura.

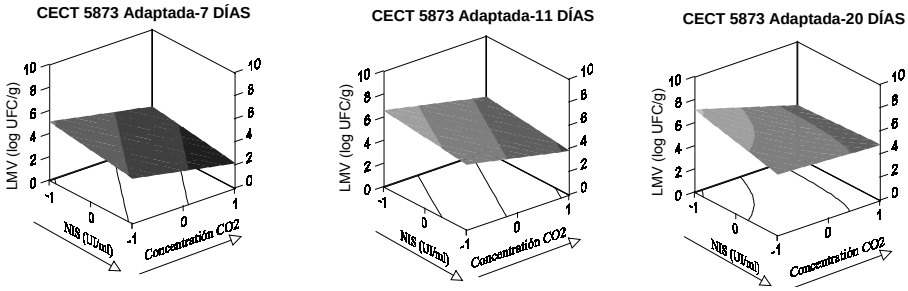

Figura 5.2: Superficies de respuesta correspondientes al efecto combinado del CO_2 y de la nisina frente a las células viables de *L. monocytogenes* CECT 5873 procedentes de una biopelícula adaptada al BAC (LMV en log UFC/g) después de 7, 11 y 20 días de almacenamiento a 2.5 ºC. Las variables se expresan en valores codificados.

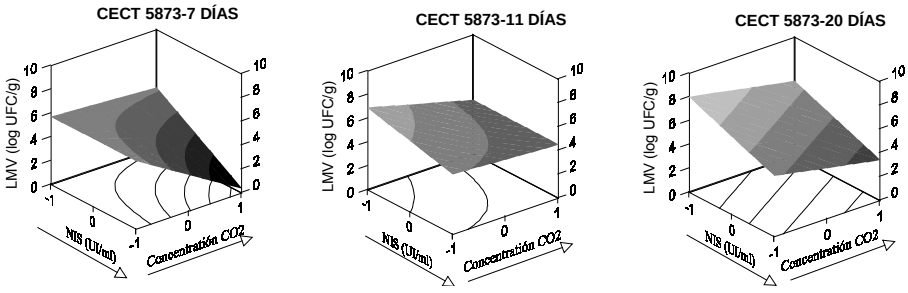

Figura 5.3: Superficies de respuesta correspondientes al efecto combinado del CO_2 y de la nisina frente a las células viables de *L. monocytogenes* procedentes de una biopelícula CECT 5873 no adaptada al BAC (LMV en Log UFC/g) después de 7, 11 y 20 días de almacenamiento a 2.5 ºC. Las variables se expresan como valores codificados.

A pesar de que la explicación de los resultados obtenidos necesitaría de experimentos adicionales, una revisión bibliográfica permitió encontrar algunas razones posibles, que abrirían varias posibilidades para futuras investigaciones:

- Que el BAC, la nisina y el CO_2 actúan sobre la membrana celular (CO_2: Jydegaard-Axelson et al. 2004, Lungu et al., 2009, Tassou et al., 2004, Nilsson et al.,

2000; nisin: (Christ et al., 2007, Dalmau et al., 2002, Wiedemann et al., 2007; BAC: Ceragioli et al., 2010, Soumet et al., 2005, Walton et al., 2008)

- Que cepas de *L. monocytogenes* tolerantes al ácido son también más resistentes al CO_2 (Francis et al., 2007, Jydegaard-Axelsen et al., 2004) y a la nisina (Badaoui Najjar et al., 2009; Begley et al., 2010; Bonnet and Montville 2005).
- Que la resistencia al BAC y a la nisina podría estar relacionada con las bombas de protones (nisin: Bruno et al., 1992, BAC: Romanova et al., 2006) lo que podría disminuir el efecto del CO_2 (García-González et al. 2007)

En mejillón vivo

Dado que en un ensayo previo se observó que hasta el día 13 de almacenamiento no había diferencias significativas entre los valores de viabilidad del mejillón vivo almacenado atmósferas con concentración creciente de O_2, la toma de muestra se fijo tras 14 días de almacenamiento. A este tiempo, se observa una clara relación entre el porciento de viabilidad y la concentración de oxígeno en la atmósfera, tal y como se muestra en la **Figura 5.3**.

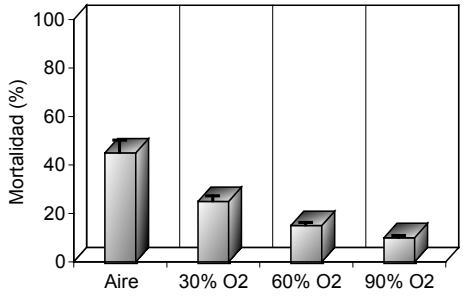

Figura 5.3 Mortalidad de mejillones vivos envasados bajo diferentes atmósferas ricas en O_2 después de 14 días de almacenamiento a 2.5 ºC.

Los resultados de viabilidad obtenidos (**Figura 5.4**) demuestran que *L. monocytogenes* puede persistir después de una contaminación cruzada durante el procesado de mejillón vivo por ello es necesario incrementar las condiciones de seguridad durante el empaquetado en atmósferas ricas en O_2.

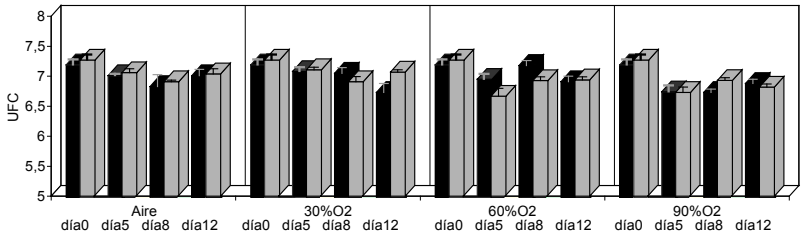

Figura 5.4 Viabilidad de mejillones vivos envasados bajo atmósferas ricas en O_2 después de 5, 8 y 13 días de almacenamiento a 2.5 ºC.