Universitat Autònoma de Barcelona

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PhD Thesis

Enzyme-Benzalkonium chloride combined strategies to remove *Listeria monocytogenes* mixed-species biofilms

by

PEDRO RODRÍGUEZ LÓPEZ

A dissertation submitted in the **Department of Genetics and Microbiology**

of the **Autonomous University of Barcelona** in partial fulfilment

of the requirements for the degree of **Doctor of Philosophy in Microbiology**

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If I set out to prove something I am no real scientist

– I have to learn to follow where the *facts* lead me –

I have to learn to whip my prejudices…

Lazzaro Spallanzani (Scandiano 1729 – Pavia 1799)

Biologist and Catholic Priest

A scientist in his laboratory is not a mere technician:

he is also a child confronting natural phenomena

that impress him as though they were fairy tales.

Maria Sklodowska (Warsaw 1867 – Sallanches 1934)

Physicist and Chemist

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List of abbreviations

Amp	Ampicillin
AVC	Adhered viable cultivable cells
BAC	Benzalkonium chloride
BHI	Brain-heart infusion
BPW	Buffered peptone water
CA	Covered area
CEL	Cellulase
CFU	Colony forming unit
Cm	Chloramphenicol
CV	Coefficient of variation
DI	Discrimination index
DNA	Deoxyribonucleic acid
eDNA	Extracellular deoxyribonucleic acid
EFSA	European food safety authority
EO	Essential oil
EPS	Exopolymeric substances
EW	Electrolysed water
FISH	Fluorescence in situ hybridisation
GAPs	Good agricultural practices
GHPs	Good hygienic practices
GLPs	Good laboratory practices
GMPs	Good manufacturing practices
HACCP	Hazard analysis and critical control points
LAB	Lactic acid bacteria
LD90	Lethal dose 90
MBEC	Minimum biofilm eradication concentration
MDD	Maximum diffusion distance
mTSB	Modified trypticase soy broth
OA	Occupied area
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PFU	Plaque forming unit
PNA	Peptide nucleic acid
POA	Percentage of occupied area
PRN	Pronase

QAC	Quaternary ammonium compound
QS	Quorum sensing
RAPD	Random amplified polymorphic DNA
RTE	Ready-to-eat
RVC	Released viable culturable cells
Sm	Streptomycin
SS	Stainless steel
TD	Tolerance development
TSA	Trypticase soy agar
TSB	Trypticase soy broth
VBNC	Viable but not cultivable cells

Summary

Listeria monocytogenes is considered one of the major foodborne pathogenic bacteria in Europe. In nature, it is found forming part of multispecies biofilms, resistance structures constituted by an extracellular matrix acting as a protective barrier against external agents, hindering their action and generating sublethal concentrations inside the biofilm.

In industrial environments, biofilms are usually exposed to sublethal concentrations of biocides, due to the fact that the biofilm is located in inaccessible locations or because of inefficient application. This can favour the appearance of resistant and persistent bacteria in industrial plants, which lead to an excessive biocide deployment with a subsequent higher environmental impact.

Is hence necessary, to propose more effective and efficient cleaning and disinfection systems, able to ensure pathogen control, generate less resistance while maintaining the main environmental impact standards.

In the present dissertation, the design of a specific cleaning and disinfection system based on the combined application of enzymes and benzalkonium chloride (BAC) against *L. monocytogenes* mixed biofilms present in the food industry is proposed.

The initial hypothesis is that the application of enzymes might produce the disruption of the biofilm matrix that acts as a barrier to antimicrobials, facilitating the subsequent effect of the disinfectant. The specificity is achieved based on the previous characterisation of the *L. monocytogenes*-carrying biofilms present in industry that permits the enzyme selection, the dose adjustment and the study of the possible tolerance development.

The experimental work was development in the following stages:

- **Characterisation of the *L. monocytogenes*-carrying communities present in fish, meat and dairy industry.** This allowed detecting the presence and subtypes of *L. monocytogenes*, to characterise the accompanying microbiota and to study the adhesion dynamics of *L. monocytogenes* isolates on stainless steel (SS) as well as the association capacity and biofilm formation in mixed culture with the accompanying species.

- **Effectiveness of the enzyme-BAC combination to remove early-stage *L. monocytogenes*-carrying biofilms.** The effects of different enzymes alone and combined with BAC against early-stage *L. monocytogenes* mixed biofilms grown on SS was assessed. Results obtained demonstrated the efficacy of the enzyme-BAC combined application to remove *L. monocytogenes* mixed biofilms and highlighted

that this efficacy varies with the composition and age of the biofilm, pointing out the importance of designing strain-specific cleaning and disinfection strategies.

- **Quantification of the effects of pronase-BAC combined application against *L. monocytogenes-E. coli* late-stage dual-species biofilms.** The individual and combined effects on the occupied surface, and the number of viable adhered and released cells after the application of pronase and BAC against late-stage *L. monocytogenes-E. coli* dual-species biofilms were assessed. Results demonstrated a synergistic effect of pronase-BAC application against *L. monocytogenes-E. coli* dual-species biofilms, a higher efficacy against *L. monocytogenes*, and the need to use high BAC doses to ensure the absence of adhered and released viable cells.

- **Tolerance development to pronase-BAC combined treatments in *L. monocytogenes-E. coli* mixed biofilms.** The effects of the frequency and duration of consecutive sublethal exposures to pronase-BAC on the development of tolerance in *L. monocytogenes-E. coli* mixed biofilms was assessed. Results showed that only when sublethal exposures are alternated with recovery periods, a tolerance development to the application of pronase-BAC combined treatments takes place.

Resumen

Listeria monocytogenes está considerada una de las bacterias patógenas transmitidas por alimentos de mayor relevancia en Europa. En la naturaleza, se encuentra formando biopelículas multiespecie, estructuras de resistencia constituidas por una matriz extracelular que actúa de barrera protectora frente a agentes externos, dificulta su acción y genera concentraciones subletales en el interior de la biopelícula.

En el ámbito industrial, es habitual que las biopelículas estén expuestas a concentraciones subletales de biocidas, bien debido a que estas se encuentran en ubicaciones de difícil acceso, bien como consecuencia de una aplicación ineficiente. Ello favorece la aparición de bacterias resistentes y persistentes en plantas industriales, lo que ha llevado a un exceso en el uso de biocidas y al consecuente incremento del impacto ambiental.

Es necesario, pues, proponer sistemas de limpieza y desinfección más efectivos y eficientes, que aseguren el control de patógenos, generen menor resistencia y mantengan los cánones de impacto ambiental.

En la presente tesis se propone el diseño de un sistema de limpieza y desinfección específico frente a biopelículas mixtas de *L. monocytogenes* presentes en la industria alimentaria basado en la aplicación combinada de enzimas y cloruro de benzalconio (CB).

La hipótesis de partida se basa en que la aplicación de las enzimas podría suponer la disrupción de la matriz de la biopelícula que actúa como barrera frente a antimicrobianos facilitando la acción posterior del desinfectante. La especificidad se consigue a partir de la caracterización previa de las biopelículas portadoras de *L. monocytogenes* presentes en la industria, que permite la selección de las enzimas, el ajuste de las dosis y el estudio del posible desarrollo de tolerancia.

El trabajo se desarrolló en las siguientes etapas:

- **Caracterización de las comunidades portadoras de *L. monocytogenes* presentes en superficies de industrias pesquera, cárnica y láctica.** Esto permitió detectar la presencia y subtipos de *L. monocytogenes,* caracterizar la microbiota acompañante y estudiar las dinámicas de adhesión de los aislados de *L. monocytogenes* sobre acero inoxidable (AI) así como la capacidad de su asociación y formación de biopelículas en cultivo mixto con las especies acompañantes.

- **Efectividad de la combinación de enzimas-CB sobre la eliminación de biopelículas tempranas portadoras de *L. monocytogenes*.** Se estudiaron los efectos de diferentes enzimas solas y combinadas con CB sobre biopelículas

tempranas mixtas de *L. monocytogenes* formadas en AI. Los resultados obtenidos demostraron la efectividad de la aplicación combinada enzima-CB sobre la eliminación de biopelículas mixtas portadoras de *L. monocytogenes* y pusieron de manifiesto que dicha efectividad varía con la composición y edad de la biopelícula, señalando la importancia de diseñar sistemas específicos de limpieza y desinfección.

- **Cuantificación de los efectos de la aplicación combinada de pronasa-CB sobre la eliminación de biopelículas tardías de *L. monocytogenes-E. coli*.** Se cuantificaron los efectos individuales y combinados de la aplicación de pronasa y CB sobre la superficie ocupada por las biopelículas tardías mixtas y el número de células viables adheridas y desprendidas después de la aplicación de los tratamientos. Los resultados demostraron un efecto sinérgico de pronasa-CB sobre la eliminación de biopelículas de *L. monocytogenes-E. coli*, una mayor efectividad frente a *L. monocytogenes* y la necesidad de dosificar elevadas concentraciones de BAC para asegurar la ausencia de células viables adheridas y liberadas.

- **Desarrollo de tolerancia a tratamientos combinados de pronasa-CB en biopelículas mixtas de *L. monocytogenes-E. coli*.** Se evaluó el efecto de la frecuencia y duración de exposiciones subletales consecutivas de pronasa-CB sobre el desarrollo de tolerancia en biopelículas mixtas de *L. monocytogenes-E. coli*. Los resultados demostraron que únicamente cuando las exposiciones subletales se acompañan de un periodo de recuperación se produce el desarrollo de tolerancia a la aplicación de los tratamientos combinados pronasa-CB.

1

Introduction and objectives

Listeria monocytogenes: from environment to human disease

Historical facts, taxonomy and general characteristics

The genus *Listeria* is classified along with the genus *Brochotrhix* within the family *Listeriaceae*, based on phylogenetic analyses and 16S rDNA sequences (Figure 1.1). Cells into this family are low G+C, Gram-positive, short rods that may form filaments. Hitherto, the genus *Listeria* includes seventeen species: *L. monocytogenes*, *L. aquatic*a, *L. booriae*, *L. cornellensis, L. fleishmannii*, *L. floridensis*, *L. grandensis L. grayi*, *L. innocua*, *L. ivanovii*, *L. marthii*, *L. newyorkensis, L. riparia, L. rocourtiae*, *L. seeligeri*, *L. weihenstephanensis* and *L. welshimeri* [1–4].

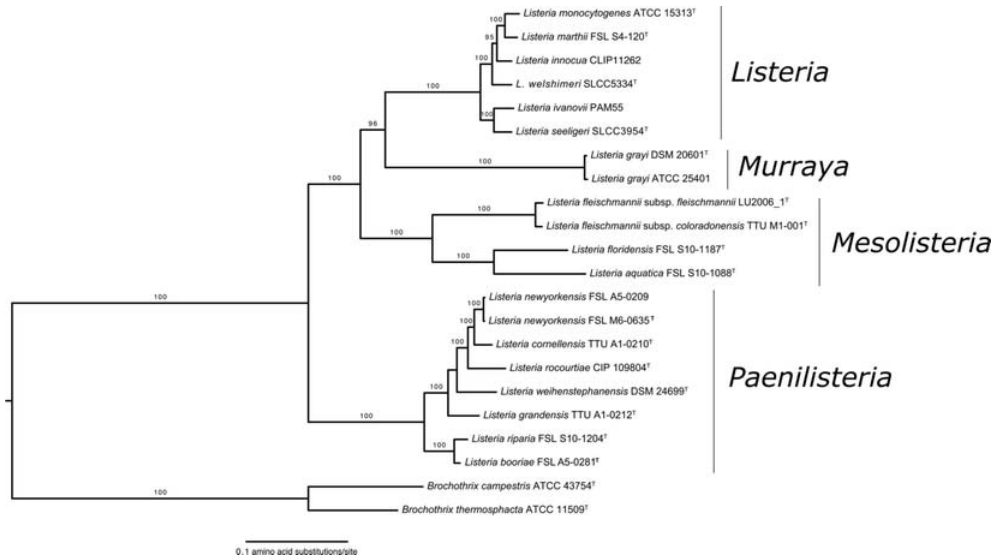

Figure 1.1: Maximum likelihood phylogeny of the genus *Listeria* based on concatenated amino acid sequences and new genera names proposed for every monophyletic group. Values on branches: bootstrap values (>70 %) based on 250 replicates. [3]

L. monocytogenes was firstly isolated in 1926 by E.G.D. Murray from the blood of rabbits and guinea pigs [5] but it was not until 1940 that J. Pirie gave its current name for these Gram-positive, catalase-positive bacteria [6]. Typical selective media for *L. monocytogenes* isolation include PALCAM and Oxford agar where they grow as small, round colonies (Figure 1.2). Microscopically, they are short rods, 1-2 μm by 0.4-0.5 μm with parallel sides and round

ends that grow as single or short chains [1]. They can be motile due to the presence of four to seven peritrichous flagella when cultured below 30 ºC and present a facultative anaerobic metabolism [1,7].

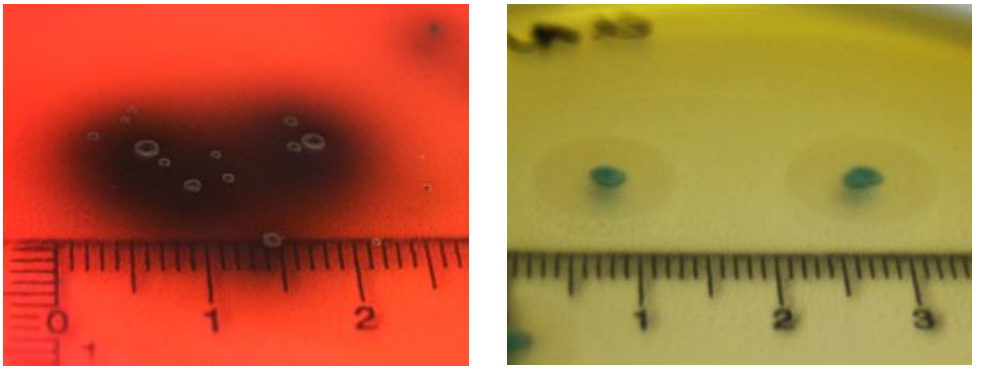

Figure 1.2: Typical aspect of *L. monocytogenes* colonies grown on PALCAM (left) and Oxford (right) selective agars.

To date, *L. monocytogenes* stains are subtyped following two main criteria:

i). *Antigenic structure*. The serotypes of *L. monocytogenes* were described by Paterson [8] and later redefined by Seeliger and Höhne [9] depending on the typology of its somatic (O) and flagellar (H) antigens. This approach classifies the species into 13 different variants: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7. Despite all serotypes are potentially pathogenic for humans, it has been reported that serotypes 1/2a, 1/2b and , especially, 4b are responsible for about 98% of the reported human listerioses [10–12], whilst 4a and 4c are not normally outbreak-associated serotypes [12,13].

ii). *Genotypic analyses*. According to the presence of genes related to virulence factors, *L. monocytogenes* can be divided into three major lineages [14]: lineage I, that includes the serotypes 1/2b, 3b, 3c and 4b; lineage II, that includes the serotypes 1/2a, 1/2c and 3a and lineage III, that includes the serotypes 4a and 4c [15]. *L. monocytogenes* invasive illness is mainly caused by lineage I strains, whilst strains belonging to lineage II are frequently isolated from food samples. Comparatively, lineage I is more significant, being lineages II and II rarely associated with foodborne listeriosis.

The ubiquity of *L. monocytogenes* can be attributed to the outstanding ability to cope with different environmental conditions. In fact, this pathogen is considered one of the most robust non-spore forming organism. It can proliferate under a broad range of temperatures, from about 0 to 45 ºC, tolerate salt concentrations up to 12 % (w/v) and pH values from 4.3 to 9.2 [16]. This wide variety of environmental conditions under which *L. monocytogenes* can grow and survive, make it a difficult pathogen to eliminate in the food industry and food-related areas.

Ecological aspects of *L. monocytogenes*

Because of its adaptive characteristics *Listeria monocytogenes* is considered a ubiquitous organism that can be found in soils rich of decay plant matter as well as in faecal samples, water environments or attached to food-related premises [17–19] (Figure 1.3). A relatively high incidence of *L. monocytogenes*, around 8 and 44 %, has been reported in soil samples [20]. On the other hand, the concentration is relatively low with magnitudes of 1 to 100 CFU/g in positive samples [21]. However, all these survival values can vary significantly depending on the physicochemical characteristics of the soil in which *L. monocytogenes* is present [22].

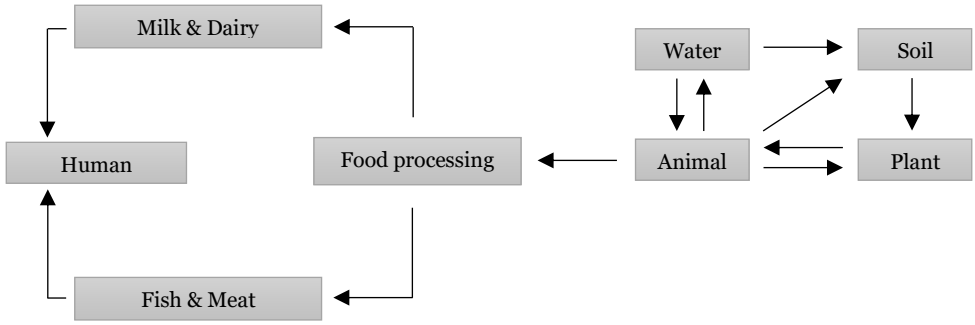

Figure 1.3: Diagram schematising the transmission cycle and the main ecological niches of *L. monocytogenes*.

Animals are also natural reservoirs of *L. monocytogenes* carrying this pathogen without developing symptoms of listeriosis, being frequently isolated from livestock although with a higher incidence in cattle [23,24]. Derived products like milk can be direct vector of

contamination to humans, but essentially the transmission of the pathogen is due to the routes involving food processing environments. In fact, ready-to-eat (RTE) products, soft cheese, fish, shellfish and deli products [25,26] are some of the most common foodstuffs through which *L. monocytogenes* infection takes place. Gombas et al. [27] demonstrated that in the USA the prevalence of *L. monocytogenes* is generally associated with seafood salads (4.7 %) and smoked seafood (4.3 %), whereas in the EU, non-compliance among RTE products was significantly lower. In addition to RTE, raw products also harbour *L. monocytogenes* as demonstrated in a study performed by Pagadala et al. [28] reporting a *L. monocytogenes* incidence of 4.5 % in blue crab processing plants. Other authors have reported presence of *L. monocytogenes* in raw meat of chicken [29,30] of pork [30,31]. Although in a lower proportion compared to other products [32], vegetables and fruits and related processing environments are also associated with *L. monocytogenes* incidence [33,34].

In food related environments, this pathogen can be a difficult pathogen to control and become persistent [35,36] being usually associated with other microorganisms in complex multi-species communities [37]. Remark that *L. monocytogenes* is among the major agents causing death due to foodborne illnesses in the United States [38] and in Europe [39], which justifies the importance to study and understand the different aspects regarding the life cycle of *L. monocytogenes* to develop effective strategies to control this bacterium especially in food processing facilities.

L. monocytogenes as a foodborne pathogen

Inside the genus, *L. monocytogenes* is the only species considered as pathogen for humans causing mainly foodborne infections [36]. Human listeriosis typically courses as a two-phase illness with an initial phase of mild symptoms including sub-febrile episodes that can last from 3 to 10 days and may be concomitant with headache, ataxia, general physical discomfort and nausea followed by a subsequent phase with severe signs of central nervous system affection [40]. These meningeal forms usually provoke consciousness alteration, motor disorders or even partial nervous paralysis [40].

L. monocytogenes is considered an important paradigm due to its particular replication cycle. They are intracellular pathogens that undertake cell-to-cell spreading and therefore they remain invisible for host defences [41]. They can proliferate within macrophages, once the pathogen is engulfed, carrying out an early scape from the phagocytic vacuole followed by a multiplication in the cytosol of endothelial and epithelial cells and in hepatocytes. This

leads to an eventual intracytosolic mobilisation via actin filaments and a final protrusion and invasion of the neighbouring cells where all the invasive cycle reinitiates [41].

According to the European Food Safety Authority (EFSA), *L. monocytogenes* appears to be a microorganism of a great concern because even though its incidence among population is relatively low, it is maintained throughout time with high morbidity and mortality rates among the major risk groups: newborns, elderly people, people with weakened immune system, and pregnant women [42,43]. In fact, the latest report of the European Food Safety Authority shows that the incidence of confirmed European *L. monocytogenes* infections has increased by 30 % regarding previous data [39]. Large food-borne listeriosis outbreaks with relatively high mortality rates are still being reported [36,44]. As a matter of example, some of the most resounded *L. monocytogenes*-associated outbreaks in the last decade are listed in Table 1.1.

Contry/Region	**Year**	**Foodstuff**	**Cases**	**Deaths**	**Serovar**	Ref.
Switzerland	2005	Tomme cheese	10	3	1/2a	[45]
Czech Republic	2006	Mature cheese and mixed salad	75	12	1/2b	[46]
Germany	2006-2007	Cheese (acid curd)	189	26	4b	[47]
United States	2007	Pasteurised milk	5	3	N.D.	[48]
Norway	2007	Camembert cheese	17	3	Serogroup I	[49]
Canada	2008	RTE products	57	22	1/2a	[50]
Austria & Germany	2009	*Quargel* cheese	14	4	1/2a	[51]
Portugal	2009-2012	Cheese	30	11	Serogroup IVb	[52]
United States	2011	Cantaloupe melons	147	33	1/2a, 1/2b	[53,54]
United States	2012	Ricotta cheese	22	4	N.D.	[54]
Germany	2012-present	N.D.	66	6	1/2a	[55]
Spain	2013-2014	N.D.	35	6	1/2a, 1/2b, 4b	[56]
United States	2014	Caramel apples	35	7	N.D.	[54]
Denmark	2014	RTE meat	41	17	N.D.	[57]
United States	2015	Soft cheeses	30	10	N.D.	[54]
United States	2016	Packaged salads	19	1	N.D.	[54]

N.D.: Not determined

Table 1.1: Outbreaks of *L. monocytogenes* reported in Europe and the United States over the last decade.

Regarding *L. monocytogenes*-associated foodborne epidemiology, serotype 4b appears to be the most frequent serotype causing large outbreaks and invasive illness [58]. This serotype has been associated with the consumption of contaminated foodstuffs such as paté, cheese and coleslaw [59,60]. On the other hand, serotype 1/2b is the most frequent in non-invasive listerioses and it has been isolated among outbreaks involving contaminated dairies and rice salad [61,62].

Several reasons have been postulated to explain the apparent deficient control of this pathogen in food industry: lack of sensitivity among methods leading to an inadequate *L. monocytogenes* detection due to the existence of viable non cultivable cells [63,64], inefficient procedures for cleaning and disinfection [65] and principally biofilm formation by *L. monocytogenes* and subsequent increase of its capability to resist sanitizers [66–68].

Biofilm formation in *Listeria monocytogenes*

Bacterial biofilms: sessile but not stuck communities

Even though biofilms may be considered as a modern concept, the reality is that the very first observations of these structures were carried out in 1684 by Antoni van Leeuwenhoek in dental plaque samples. He reported those results to the Royal Society of London, referring to his observations of the vast quantity of microorganisms present stating that: "*the number of these animicules in the scurf of a man's teeth are so many that I believe they exceed the number of men in a kingdom"*. However, it was not until 1975 that the word "biofilm" was not used in a scientific publication [69].

The currently accepted definition of a biofilm was coined in 2002 by Donlan and Costerton who elegantly described them as microbially derived sessile communities characterised by cells that are irreversively attached to a surface, an interface or to each other, are embedded in a matrix of extracellular polymeric substances (EPS) and have an altered phenotype regarding its growth rate and genic expression [70]. Therefore, biofilms are considered the main structure in which bacteria can be found ubiquitously in sanitary, environmental and industrial settings [71–74]. The capability to grown as a biofilm demonstrates somehow the social component of bacteria even though the formation rates and the physicochemical features of the final structure are highly variable and depend on the strain (or strains) composing the actual biofilm and the abiotic factors involved [75,76]. Despite its sessile nature, biofilms cannot be considered as halted structures. They are made up in a large part of water canalicules which constitute a metabolically active and effective oxygen and

nutrients distribution network [77]. The establishment of microscale chemical gradients inside the biofilm, leads to the presence of local phenotypical and genotypical cellular variations among the resident population [76] and, therefore, cells present are in a broad range of physiological states [76–78].

Steps in biofilm formation

The development of surface-adhered bacterial biofilms can be divided into three fundamental steps schematised in Figure 1.4: (i) attachment; (ii) maturation and growth; and (iii) detachment and/or dispersion [77,79]. All these phases are deeply regulated by chemical stimuli that act as modulators modifying the communal behaviour in a concentration-dependent manner. This mechanism of signalling and molecule recognition known as *quorum sensing* (QS) still remains partially unknown to microbiology due to its complexity [80,81]. In *L. monocytogenes* the main regulation pathways are dependent of the so-called *auto-inducer 2* (AI-2) [82], the *agr* (accessory gene regulator) system [83] and the transcriptional regulator of stress response *sigB* [84]. Other factors that influence biofilm development include medium composition and presence of antimicrobials, temperature, bacterial concentration in the bulk phase and shear forces [70].

Step I: Attachment

Among the steps involved in the development of a biofilm, the phenomenon of initial adhesion is the phase in which bacteria shift from a free-living (planktonic) cell to a sessile state. This initial stage is strongly influenced by the environment and bacteria involved undertake several physiological changes.

It is important to remark, that primary contact generally occurs between bacteria and a conditioned surface. This conditioning is an accumulation in the solid-liquid interface of different inorganic and organic molecules that are present in the bulk phase. This accumulation leads to a local higher concentration of nutrients that alters the physicochemical properties of the surface [85]. Following the formation of this conditioning film, bacteria are deposited onto the surface either passively via Brownian motion, sedimentation or convective transport [86] although it has been reported that active transport via flagella and chemical sensing also plays an essential role [87].

After that, initial attachment of bacteria takes place in which van der Waals forces, electrostatic forces and hydrophobic interactions contribute to stabilise the cell-surface

interaction [86]. The nature of this primary adhesion is weak and cells can be effortless removed by shear forces (e.g. rinsing). This reversible feature allows bacterial cells to move along the surface to find an appropriate place to adhere. The duration of this initial phase tends to be short and cells rapidly carry out transition from reversible to irreversible attached cells in which the production of specific ligands, such as pili and fimbriae, and also secretion of exopolymeric substances (EPS) makes bacteria to be strongly adhered to the surface and therefore much more difficult to remove both by physical (e.g. scraping) and chemical (e.g. cleaners) methods [88]. In *L. monocytogenes*, Schwab et al. [89] observed that this phenotypical shift is produced in approximately 5 min after initial adhesion.

Besides the formation of the conditioning film, environmental conditions also modulate the adhesion phenomena. Major factors affecting biofilm adhesion are:

i). *pH*. Many authors have studied the effects of the pH in culture medium on the initial steps of *L. monocytogenes* biofilm formation. Nevertheless, results depict contradictory results and are highly influenced by the rest of the conditions in each assay and, therefore, the actual effects still remain obscure. As a matter of example of this divergence, Herald and Zottola [90] and lately Poimenidou et al. [91], reported that *L. monocytogenes* initial adhesion was hampered at acidic pH whereas Briandet et al. [92] observed that adherences was increased at low pHs due to a higher hydrophobicity of the cell wall in *L. monocytogenes* Scott A.

ii). *Temperature*. Even though *L. monocytogenes* is able to grow and adhere to food-related surfaces in a broad range of temperatures (0 – 45 ºC), this and other processes like flagella synthesis [93], are influenced by temperature. Briandet et al. [92] demonstrated that *L. monocytogenes* Scott A adhered significantly better in Trypticase soy-yeast extract broth (TSYE) at 37 ºC compared to lower incubation temperatures. Despite this, subsequent studies demonstrated that this temperature dependent favouring is produced until certain extent [94].

iii). *Nutrient availability*. It has been observed that the nutrients of the medium stimulate or not the adherence of *L. monocytogenes* depending on the strain. Thus, in some cases nutrient starvation promotes the initial adherence [95]. Kim and Frank [96] reported a higher adherence in biofilms grown in chemically defined medium compared to those grown in trypicase soy broth (TSB) while Mai and Conner [94] observed that rich media promoted *L. monocytogenes* adhesion. Glucose availability also alters adhesion in *L. monocytogenes*. With this regard, Guilbaud et al. [97] used glucose supplements to enhance biofilm formation, whereas other studies report that rich media with high glucose concentrations give

rise to biofilms with fewer adhered cells and a block of the *Listeria* adhesion protein (LAP) expression [98] despite a higher EPS production [99].

iv). *Characteristics of the surface*. Several studies have demonstrated that *L. monocytogenes* is able to adhere and undertake biofilm formation on a wide range of surfaces routinely used in food-related environments [17,73,100–103]. Among them, stainless steel (SS) is the most common material used for food contact purposes in the food industry because it is easy to produce, durable and straightforwardly cleaned and disinfected [86]. However, the under scanning electron microscopy (SEM) the surface of SS reveals cracks and crevices, susceptible to provide bacteria a greater surface to adhere [101] and a shelter for antimicrobials [86]. With this regard, Mosquera-Fernández et al. [101] demonstrated that *L. monocytogenes* is able to adhere better to AISI 304 SS (rough) than to polished AISI 316 SS (smooth). Due to its relevance in the food industry, SS will be the only surface used in the present thesis to grow both mono and multi-species biofilms.

v). *Flagella and cellular motility*. Contrarily to many bacterial species, flagella synthesis in *L. monocytogenes* is temperature-dependent [93]. Incubation temperatures higher than 37 ºC impede the flagellin polymerisation, and subsequent motility failure, due to MogR repression of flagellar gene transcription. At temperatures of 30 ºC and below, MogR is inhibited by GmaR antirepressor, restoring flagella biosynthesis and cellular motility [104]. Studies such as those carried out by Guerrieri et al. [105], Lemon et al. [104], Tresse et al. [106] and Vatanyoopaisarn et al. [107], among others, demonstrated that flagella are critical for *L. monocytogenes* biofilm formation during the first stages. However, in subsequent stages of biofilm formation flagella presence seems not to have any deleterious effects [108].

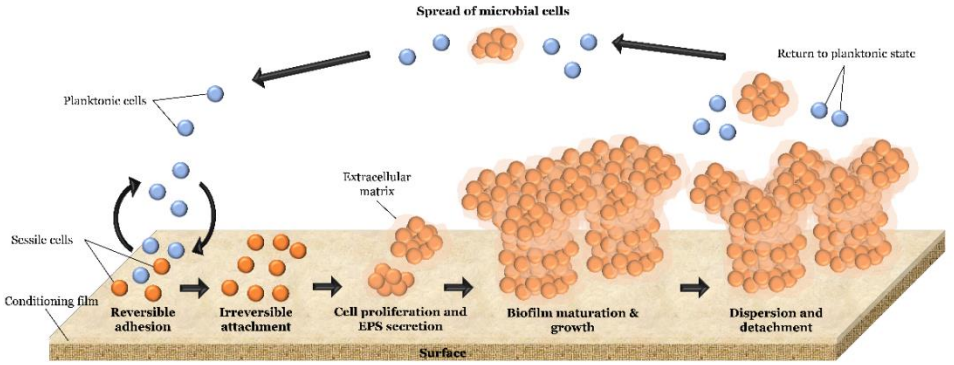

Figure 1.4: General processes involved during the development of a biofilm.

Step II: Maturation and growth

After bacteria become irreversively attached to the surface the biofilm starts to growth and maturate. The potential growth of *L. monocytogenes* and, generally, of any bacterial biofilm reaches its limit depending on the surrounding environment. In this stage, the overall density and thus the complexity of the biofilm increases as attached organisms start to actively multiply (and die) and to produce and secrete various extrapolymeric substances (EPS) [109], that may interact with other organic and inorganic compounds in the immediate environment to construct the biofilm matrix [88,110].

As happened in the adhesion phase, maturation and growth of a biofilm are processes influenced by gases diffusion rate, the mechanisms for waste disposal, nutrient availability in the bulk phase, and the penetration of these nutrients into the structure [88,111]. In the particular case of *L. monocytogenes*, a preference to media with relatively high nutrient concentrations for biofilm formation is observed, unlike other species do [109,112].

In addition to nutrients, cell embedding into EPS is another determinant factor for biofilm formation. Up-regulation of EPS biosynthesis in biofilms generally occurs shortly after irreversible adhesion [113] and it has been demonstrated that in *L. monocytogenes* is regulated by c-di-GMP [114,115]. EPS composition present in the *L. monocytogenes* biofilm matrix is mainly constituted by proteins [116,117] and extracellular DNA (eDNA) [116–118] and influenced depending on the strain and culture conditions [116]. This significantly differs from other species' matrixes such as *Staphylococcus* sp. or *Pseudomonas* sp., generally rich in polysaccharides. Although in a lower proportion, *L. monocytogenes* biofilm

matrix contains polysaccharides such as teichoic acids equals to the ones found in the bacterial membrane [119].

EPS secretion and, subsequently, matrix formation plays an important role in biofilms providing protection against environmental aggressions impeding e.g. antimicrobial molecules to reach the cells due to the reduced diffusion or by direct neutralisation of these molecules with matrix components. In addition, the matrix also confers the biofilm a physical stability that influences the final tridimensional conformation [110,120], favours the genetic exchange between cells [110] and acts as a reserve of carbon, nitrogen and phosphorus [121].

Step III: Dispersion and detachment

These two phenomena can take place separately of simultaneously in a given biofilm. The two processes are similar, because both refer to a certain amount of cells are physically separated from the biofilm and returned to the bulk phase, but different, since dispersion is a process related with active genetic and metabolic processes undertaken inside the cell whereas detachment is more of a passive phenomenon related to biofilm sloughing and erosion produced by shear forces [122,123].

In spite of the advances in the field, dispersion phenomena in biofilms still remain as a controversial issue. Several reasons have been attributed to this. It has been demonstrated that can be regulated by QS [124], the production of glycolipids [125], the production of endogenous enzymes [126] or due to nutrient depletion [109]. In these last two cases, eventual matrix decay may take place and therefore the extrusion of parts of the biofilm would be facilitated.

The most immediate consequence of mobilisation of parts of the biofilm in the context of the food industry is the creation of new contamination foci [127] that could finally affect final product safety and quality via cross contamination. Because of this, in the present PhD thesis, the pool of live viable cells released from the biofilms after the application of cleaning and disinfection strategies will be addressed.

L. monocytogenes mixed-species biofilms

Although considered as a relatively poor biofilm former compared to other species [128], *L. monocytogenes* can easily associate with other bacterial species forming part of complex microbial communities with both Gram-positive and Gram-negative species [37,129–132]

and the interaction among species forming these consortia varies depending of the genera implicated and the environmental conditions [132]. Various studies involving *L. monocytogenes* multispecies biofilms have highlighted the complexity of such interactions and the different effects that associated bacteria could have in terms of the number of adhered cells [129,131,133] and the EPS composition of the biofilm matrix [134]. In these polymicrobial communities, *L. monocytogenes* can act as primary coloniser or as later biofilm partner establishing interactions with other microorganisms present [135], therefore increasing the complexity of bacterial ecological niches [136].

Considering this, it seems to be clear that increasing the knowledge regarding multispecies biofilms could provide key information to develop new cleaning and disinfection strategies against a given target [137,138]. In the particular context of the food industry, this would reduce the number of bacterial foci thus reducing subsequent cross-contaminations of food products. Despite this, the number of studies regarding mixed-species biofilms in food industry-related environments dealing with a characterisation of the whole microbiota in a particular surface is relatively low compared to other ambits such as oral biofilms [139]. In this line, various authors have remarked the need to characterise the bacterial interactions among *L. monocytogenes*-multispecies biofilms present in real scenarios. These would include, those influencing the biofilm formation patterns as well as other phenotypical characteristics [140–142], especially when designing new disinfection strategies. However, most of the studies dealing with polymicrobial biofilms, use model structures based on the literature rather than using bacteria isolated previously from relevant environments, since they can present unique phenotypical features [143].

Regarding interactions within *L. monocytogenes* mixed-species biofilms, Carpentier and Chassaing [129] analysed 29 different *L. monocytogenes* dual-species biofilms and observed how the number of adhered *L. monocytogenes* was increased, decreased o unaltered depending on the accompanying bacterium. Other studies demonstrated how certain bacteria such as *Pseudomonas fluorescens* clearly contributes to *L. monocytogenes* adhesion in mixed biofilms [144,145]. In a posterior study, it was discussed how the effects of such interactions affected the level of *L. monocytogenes* persistence in a processing plant [35]. Almeida et al. [146] characterised *L. monocytogenes-Salmonella enterica-E. coli* mixed biofilms using peptide nucleic acid fluorescence *in situ* hybridisation (PNA-FISH) on SS coupons describing a defined structural pattern in which *S. enterica* and *L. monocytogenes* were in the bottom parts of the biofilm while *E. coli* was located on the top layer. These results were consistent with those of Puga et al. [144] who observed the disposition in the bottom layers of *L. monocytogenes* in mixed biofilms with *P. fluorescens* grown on glass. A posterior work of this group, demonstrated that *L. monocytogenes-P. fluorescens* biofilms

became more compact with the age especially in those grown at 4 ºC, despite cell viability remained unaltered [147], showing higher resistance to chitosan [148].

The present thesis, contributes to the knowledge of the ecological aspects of *L. monocytogenes*-carrying biofilms present on surfaces of the food industry characterising the composition and distribution of *L. monocytogenes* polymicrobial communities in industrial premises, gaining further insight into the accompanying species' distribution depending on the environmental factors [37]. In addition to these determinations, evidence on how the accompanying species clearly affects the final morphology of the *L. monocytogenes* mixed biofilm and the susceptibility to enzyme-based combined antimicrobial treatments is also provided [145].

Methods for biofilm quantification and structural studies

The structural studies in biofilms have been highly conditioned by the technological advances. First studies performed in the 70s and the 80s were mostly based in the quantification of adhered cells via agar plating despite its limitations [149] and the lack of information provided other that the number of viable-and-cultivable cells.

Light field and electron microscopy allowed the very first studies in this field. However, the lack of resolution in the first case and the need for dehydrate the sample in the second, limited the accuracy of such observations [150]. Therefore, biofilms were initially considered as flat and homogeneous instead of complex and heterogeneous structures. The various structural models currently accepted were not observed and described until the fabrication and utilisation of higher resolution microscopy techniques. In this line, it is usual to incorporate microscopic assays, mainly fluorescence and confocal laser scanning microscopy (CLSM). In many studies regarding structure and spatio-temporal distribution of cells into biofilms [101,151–153] CLSM is preferred since it permits to gather 3D data from hydrated biofilms *in vivo*. However, CLSM microscopes are expensive and not available in all research centres, which undoubtedly represents a major drawback at the time to incorporate this technique in the habitual laboratory technique [154].

Although numerous image analysis software (COMSTAT, ImageJ, ISA, Imaris, MATLAB…), and structural 2D and 3D parameters have been used in the literature for biofilm quantification [101,151,152,155,156], the main issue of concern still is remains when selecting the most appropriate parameters to give an accurate description of the structures. Theoretically, reliable parameters should be easily related to biological processes, however,

it is a consensus that the high complexity of biofilms makes difficult to correlate structural changes within a specific biological process associated with a biofilm forming cycle.

Looking for solutions, some authors recommended to use image analysis only as auxiliary information [157] whereas others came to the conclusion that an *ad hoc* selection of the structural parameters for biofilm characterisation and quantification is required [153] and the number should be as few as possible [158]. In biofilm quantification areal parameters such as areal porosity, were described by Lewandowski et al. [159] have been considered as good biofilm descriptors with an intuitive approach, used in many biofilm studies [152,153,157,160]. When it comes to the study of the effects of antimicrobial substances on biofilms Beyenal et al. [153] demonstrated how areal parameters allow to easily gather information from microscopic data.

An analogue parameter to areal porosity, occupied area, is proposed in this PhD thesis to, along with plate count, describe and quantify biofilm formation and antimicrobial effectiveness of enzyme-benzalkonium chloride treatments against *L. monocytogenes* mixed-species biofilms. This parameter was chosen since it was considered as the 2D structural parameter with the most biologically meaningful, easy-to-interpret outcome. Besides, it can be calculated with most of the commercial and home-made software.

Listeria monocytogenes in the food industry

Incidence of *L. monocytogenes*. A major concern in food processing plants

In Europe, it is estimated that the food industry annually invests about five trillion euros in the implementation and application of cleaning and disinfections systems. Nonetheless, bacterial contamination of foodstuffs is still a major problem with a remarkable increasing incidence of *L. monocytogenes* over the last decade [39]. Numerous studies have demonstrated that *L. monocytogenes* can be present in food processing facilities and how some strains are able to persist in these premises for various months or even years [30,161–165] mostly found associated with other bacteria forming mixed-species biofilms [130]. Molecular methods have been used in various surveys to point out equipment, floors and drains as important contamination sources in food processing lines. In many cases harbouring the same *L. monocytogenes* subtype in different locations therefore demonstrating that clonal expansion due to cross-contamination occurs [37,164,166,167].

A high prevalence of *L. monocytogenes* has been detected in food-related facilities in North America and Europe representing a serious concern in dairies, smoked fish houses and RTE

meat processing plants [168]. In the light of these facts, various authors have suggested to establish routing programs for of environmental sampling for *L. monocytogenes* detection and control in an effort to ensure microbiological safety of the foodstuffs intended for human consumption especially those belonging to the risk groups [162,169–171].

Strategies to control *L. monocytogenes*

Hazard analysis and critical control points (HACCP)

HACCP is an effective management system that ensures safety in all relevant points of the production, storage, distribution and consumption of food products, by anticipation and control of associated health hazards. Pre-requisite programs provide the basics in environmental and operating conditions needed for the production of safe, wholesome foodstuffs. The combination with Good Agricultural Practices (GAPs), Good Manufacturing Practices (GMPs), Good Hygienic Practices (GHPs) and Good Laboratory Practices (GLPs) is the best strategy to control potential hazards [172].

Some of these basic conditions to avoid product contamination by foodborne pathogens are listed below [168]:

i). Good quality of the raw material.
ii). Effective training of employees in food hygiene.
iii). Appropriate design of the processing environment, including equipment, to ensure proper cleaning and disinfection of all the in-contact surfaces.

These should be appropriately documented and regularly audited, and established and managed separately from the HACCP plan. In *L. monocytogenes*, early studies reported that *L. monocytogenes* is able to get access to the processing plants via operators' shoes, clothing and transport equipment, raw material and, probably, asymptomatic human carriers [173]. Despite great advances have been made for in HACCP, the widespread and survival capacity of this pathogen still makes *L. monocytogenes* to be a difficult microorganism to control [174].

Non-chemical agents for L. monocytogenes control

i). *Enzymes*. A promising strategy, especially for biofilm control, is the use of molecules that can interfere in biofilm formation processes or even degrade specific components of the extracellular matrix, some of them listed in Table 1.3. Following this line of research, in the last few years the use of different enzymes has

increasingly become a method used for biofilm control. These environmentally friendly compounds have been shown to both prevent the initial adhesion and remove formed structures [175–178] because of their dispersive effect on the sessile structures acting on target molecules present in the biofilm matrix [141,179,180]. However, enzymes do not necessarily have bactericidal activity which makes them unsuitable to be used as a strategy for disinfection [117]. To overcome this, a feasible strategy to obtain both biofilm disinfection and removal would be the combination of enzymes with chemical biocidals [181]. With this regard, in the present PhD thesis the feasibility of combining enzymes with chemically-based disinfectants will be addressed not only demonstrating that such combination is possible [145], but also how the synergic action of these two components, effectively remove *L. monocytogenes* mixed-species biofilms.

ii). *Essential oils (EOs)*. These comprise a broad family of approximately 3000 different aromatic and volatile liquid preparations extracted from plant material, such as roots, fruits, herbs, flowers, etc. [182,183] with different antioxidant and antimicrobial properties [184,185]. EOs cause changes in cell morphology, physicochemical properties of membranes, as well as several intracellular phenomena by interfering in the metabolic pathways, including cell division, and/or altering the molecular interaction [186]. Despite the practical application of EOs has been limited due to their alteration of organoleptic properties of foodstuffs, poor solubility and partial volatility [184].

iii). *Bacteriocins*. They form part of a heterogeneous group of small, bacterially produced, ribosomally synthesised peptides with antimicrobial properties classified according to the post-translational modifications that they undergo once synthesised [187]. Recent studies have shown the effectiveness of bacteriocins on *L. monocytogenes* biofilms [188–190] and they have been proposed as an environmentally friendly alternative to the currently used strategies. In addition to bacteriocin direct use, the co-culture of *L. monocytogenes* with bacteriocin-producing bacteria, have also demonstrated to be effective for biofilm control.

iv). *Bacteriophages*. The application of viruses infecting bacteria and, therefore, inducing the lysis of the host is considered nowadays as a versatile biofilm control tool, highly active and specific, without deleterious effects to mammalian cells and relatively low cost [191–193]. In *L. monocytogenes* phage-therapy has been reported to be effective both in medical and industrial environments [194]. To date, approximately 500 *Listeria* phages have been identified [195] all belonging to the *Caudovirales* family [196]. Among them, bacteriophage P100 is one of the best characterised being effective against *L. mononcytogenes* biofilms [196]. As a matter

of example, Soni and Nannapaneni [197] observed a reduction of 3.4 to 5.4 CFU/cm² in 168 h *L. monocytogenes* biofilms belonging to different serotypes after 24 h treatment with 1 ml of 10^9 PFU of phage P100. Additionally, Montañez-Izquierdo et al. [198] found that a significant disaggregation of 72 h *L. monocytogenes* biofilms grown on stainless steel was achieved after application of 100 µl of a 7-8 PFU/ml solution of phage P100 together with a decreasing in the number of viable cells up to undetectable levels after 48 h treatment. Despite this proven efficacy and theoretical innocuousness for human beings, the EFSA proposed a series of recommendations on the virulence, host-range, mutants, persistence, etc. of the bacteriophages intended to be used in the food industry concluding that information in the existing literature is still not enough to determine whether bacteriophages are able or unable to protect against recontamination of food with bacterial pathogens [199].

Mode(s) of action	**Enzyme(s)**	**Target(s)**
Anti QS	Lactonase	*Pseudomonas aeruginosa*
	Acylase	Multi-species biofilms in reverse osmosis membranes
	Lactonase	*P. aeruginosa, Escherichia coli*
Oxidative	DNase	*Enterococcus faecalis*, *L. monocytogenes*
Polysaccharide-degrading	DispersinB	*Staphylococcus epidermidis*
	α-amilase	*S. epidermidis, Staphylococcus aureus*
Proteolytic	Resinase	*P. aeruginosa*
	Spezyme	*P. aeruginosa*
	Pronase	*Pseudomonas fluorescens*
Anti QS + proteolytic	Acylase I + Proteinase K	Multi-species biofilms in reverse osmosis membranes
Oxidative + polysaccharide-degrading	Glucose oxydase + Lactoperoxidase	*S. aureus, S. epidermidis, P. aeruginosa, P. fluorescens*
Proteolytic + polysaccharide-degrading	Cellulase + Pronase	*P. fluorescens*

Table 1.3: Examples of anti-biofilm enzymes and their target microorganism described in the literature [181].

Chemically-based agents for L. monocytogenes control

i). *Classical disinfectants*. The choice of a chemical disinfectant depends on the efficacy, safety, toxicity, among other prerequisites [200]. Generally, disinfectants must have a broad spectrum of targets i.e. bacteria, fungi and viruses, although their mechanism of action is rarely fully understood [201] (Figure 1.6). A wide range of chemical disinfectants are available for the food-industry the mostly used are listed in Table 1.4. Among them, quaternary ammonium compounds (QACs), are one of the most widely used disinfectants proved to be effective against algae, fungi, viruses, spores, and mycobacteria even at low concentrations [202]. Besides, they are non-corrosive, low-aggressive, odourless agents with high stability which makes them especially suitable to be used in food industry [202]. More specifically, benzalkonium chloride (BAC) is usually preferred due to its bactericidal effects affecting permeability of the cell wall and membrane and finally inducing irreversible cell damage due to intracellular content leakage and cell lysis [201,203]. Nevertheless, it has been extensively demonstrated that biofilms exhibit higher tolerance to BAC compared to planktonic cells both in Gram-positives such as *L. monocytogenes* [67,204] and in Gram-negatives such as *Escherichia coli* [205] or *Pseudomonas sp.* [134]. Moreover, previous authors have demonstrated the development of adaptive resistance to BAC by *E. coli-P. aeruginosa* mixed biofilms [206]. To overcome this, a feasible strategy to obtain both biofilm disinfection and removal would be the combination of an enzyme prior to BAC application [141,181,207]. This enzymatic breakdown of the matrix would allow BAC to penetrate easier into the biofilm at effective concentrations. Although enzyme-based cleaners and detergents have been proved to be effective for biofilm removal [208–210], previous to the studies carried out in this PhD thesis, only the work performed by Kaplan [211] had reported the effectiveness of combining enzymes and BAC against 24 h *Staphylococcus aureus* biofilms. In the present work, the efficacy of different enzymes alone and combined with BAC against young and late-stage *L. monocytogenes*-carrying biofilms will be undertaken in order to test not only the efficacy of such combinations, but also to prove how the effect of these strategies varies depending on the age and the composition of the biofilm.

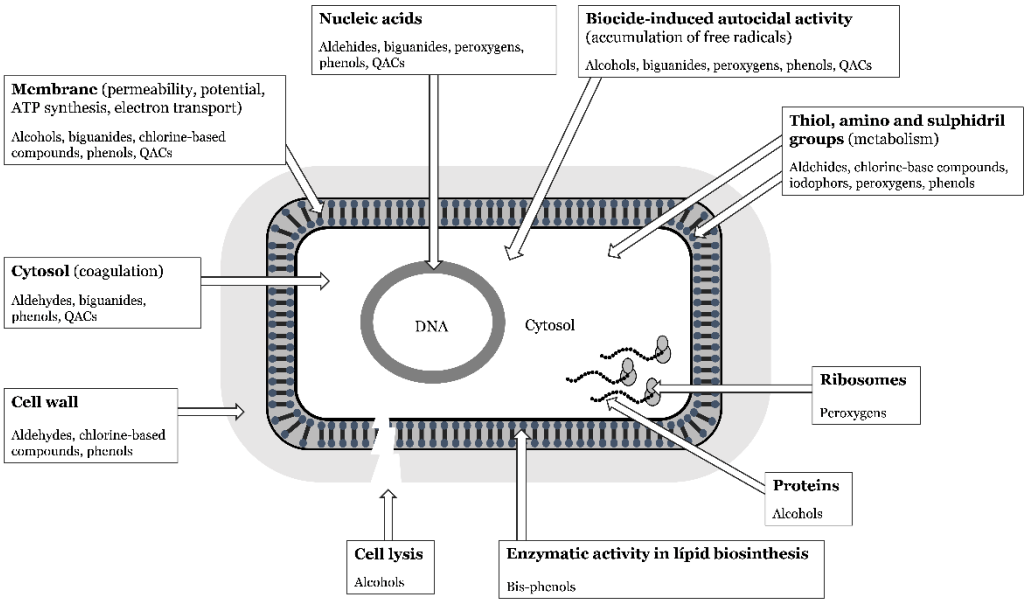

Figure 1.6: Cellular targets and effects of disinfectants commonly used in the food industry (based on Denyer and Stewart [212] and Maillard [213]).

ii). *Electrolysed water (EW)*. Straightforwardly produced with salt and water, several advantages have been identified in EW over other traditional disinfecting agents: higher effectiveness, easy-to-use, relatively inexpensive, and environmentally friendly [214]. The bactericidal activity of EW derives from the combined action of pH, redox potential, and available chlorine concentrations. Thus, EW damages the bacterial protective barriers, increases membrane permeability leading to intracellular content leakage, and causes an activity decrease on critical enzymatic pathways [200,214]. The efficacy of EW against *L. monocytogenes* has been demonstrated to dwindle the bacterial load in food-contact surfaces [215]. Besides, combinations of EWs with other antibacterial systems, has been proved to have synergic effects reducing the *L. monocytogenes* load in different food products [216,217].

Disinfectant	Pros	Cons
Alcohols (e.g. ethanol)	Cheap, fast-acting biocides of broad microbial spectrum, non-toxic, easy-to-use, colourless, harmless on skin, soluble in water and volatile	Biostatics. Lack of effectiveness against spores.
Chlorine-based compounds (e.g. sodium hypochlorite)	Cheap, fast-acting oxidisers of broad microbial spectrum. Easy-to-use and unaffected by hard water. Effective against planktonic cells and spores, even at low temperatures. Non-film forming without residues	Toxic, irritating, unstable, potentially explosive and corrosive. Inactivated by organic matter. pH sensitive. Discoloration of products. Resistance development.
Glutaraldehyde	Cheap biocide of broan microbial spectrum, non-corrosive	Biostatic. Non-biodegradable. Low penetration in biofilms.
Idophors	Sanitisers of broad activity spectrum, non-corrosive, non-irritating and easy-to-use. Low toxicity and stable at a very low pH. Little affected by organic matter	Alters flavour and odour of foodstuffs Stain plastics and porous materials. Highly foaming, unsuitable for cleaning-in-place (CIP) systems. Reduced efficacy at high pH and temperatures >50 ºC. Expensive.
Peroxygens	Strong fast acting oxidisers of broad microbial spectrum, relatively non-toxic and easy-to-use. Low foaming, suitable for CIP. Effective against bacterial biofilms and spores, even at low temperatures. Non-corrosive to stainless steel.	Loss of effectiveness in the presence of organic material and some metals contained in water. May corrode some metals. Low efficacy against yeasts and moulds. Relatively expensive.
QACs (e.g. BAC)	Stable, surface-active agents. Non-toxic, non-irritating, non-corrosive, odour and flavourless. Little affective by organic materials. Support microbial detachment	Limited effectiveness, which is affected by hard water, low temperatures and low pH. Incompatible with most detergents. Highly foaming. Unsuitable for CIP. Residual antimicrobial film forming. Resistance development. Relatively expensive.

Table 1.4: Pros and cons of some disinfectants widely used in the food industry (based on Wirtanen and Salo [218] and Marriott and Gravani [200]).

L. monocytogenes resistance to disinfection

The increased resistance to disinfectants, especially to QACs, has been a topic of concern in the context of the food industry. It has been demonstrated that resistance to BAC can be attributed to two main factors: the expression of membrane active efflux pumps [219–222] or the modification in the membrane fatty acid composition [223]. The effects of such resistance have been recently investigated by Møretrø et al. [224] concluding that resistance to BAC thanks to the presence of *qacH* and *bcrABC* genes, may contribute to an increased growth of *L. monocytogenes* in food-related premises.

In biofilms, several mechanisms leading to a resistance to biocides can take place. According to the observations of Costerton et al. [225] and Donlan and Costerton [70], some of them may be the following:

i). Lack of penetration and further diffusion of the antimicrobial agent due to the biofilm matrix [68,113,226]. Additionally, some authors have also pointed out that the abiotic part of the biofilm may have a neutralising effect on many compounds [110,120,227].
ii). Altered growth rate of cells into the biofilms [66,228].
iii). Other physiological changes due to the biofilm mode of growth supposing a coexistence of different cell phenotypes can be present within the biofilm [228,229].
iv). Formation of multispecies biofilms [226,230].

Regarding this last point, the association of *L. monocytogenes* with other microorganisms can increase the resistance to sanitisation treatments, despite results vary depending on the study. Van der Veen and Abee [132] observed that in *L. monocytogenes-Lactobacillus plantarum* mixed biofilms in polystyrene microtiter plates, application of 100 μg/ml BAC caused about 2.5 log CFU/well less compared with monocultures. In a similar way, Saá Ibusquiza et al. [230] observed denser biofilm formation and a five-fold increase in the lethal dose 90 (LD_{90}) value to BAC of *L. monocytogenes* CECT 4032 in 96 h mixed biofilms with *Pseudomonas putida* CECT 845 grown on SS. Contrarily, Kostaki et al. [231] did not found any difference in the level of resistance to BAC, NaClO and peracetic acid in *L. monocytogenes-S. enterica* biofilms compared with monocultures. In addition, a recent study demonstrated that *P. putida* resistance to BAC is increased in co-culture with *L. monocytogenes* while the resistance of the latter remains the same [134]. These contradictory findings highlight the necessity to continue exploring the mechanisms underneath bacterial associations in biofilms and the relationship with antimicrobial resistance.

Effects of antimicrobial sublethal exposure

It has been reported that continuous misuse of biocides e.g. using sublethal concentrations unable to kill bacteria within a biofilm, can have long-term deleterious effects, contributing to the selection of multi-drug resistant variants. Development of these resistances could be detected as changes in the susceptibility to these biocides such as increased minimum biofilm eradication concentration (MBEC) values, being especially relevant in the food industry where chemical biocidals (quaternary ammonium compounds (QACs), chlorine, etc.) are constantly deployed [113]. Besides, this incorrect use can alter the biofilm phenotypic heterogeneity and the intra-specific relationships that take place. Therefore, the late effects of these treatments will be the selection of the most resistant phenotypic variant of the biofilm. Some authors have related this fact with the presence of persistent *L. monocytogenes* through time, although this issue still arouses controversial discussion [35].

Motivation and general objectives

Listeria monocytogenes is considered one of the main foodborne pathogens causing human listeriosis, a rare but serious illness that, in susceptible individuals, can end into fatality. Recent reports have highlighted an increasing incidence over the last years which turns *L. monocytogenes* into a major concern in food safety.

Listeria monocytogenes, like all bacteria, lives in natural environments as bacterial multispecies biofilms, where resident microorganisms interact with each other different manners. Despite this, most of the studies found in the literature use collection strains, being the number of studies regarding mixed-species biofilms in food industry-related environments scarce. In the present PhD, the experimental design used was developed with the aim to reproduce as much as possible the natural consortia found in real industrial settings.

Cleaning and disinfection protocols used in food industry are non-specifically designed being frequent that their application implies sublethal expositions to biocides, thus generating tolerance and resistance or persistence among the pathogenic bacterial associations present. Additionally, a high number of studies have reported cross-resistance between different antimicrobials e.g. disinfectants and antibiotics. In this situation, the tendency is to increase the concentration of biocides applied causing, ultimately, a major problem of environmental pollution. Then, it is necessary to purpose specific and more efficient cleaning and disinfection strategies that could generates lower levels of resistance and lower pollution whereas assuring the control of the pathogenic bacteria.

Enzymes were chosen as good candidates to specifically damage and degrade the matrix components of the *L. monocytogenes* mixed biofilms, in order to facilitate penetration of the disinfectant in a synergistic manner. Additionally, if this occurs, the concentration of the disinfectant and the subsequent generation of resistance and environmental impact could be decreased.

In summary, the studies detailed within this PhD thesis were mainly motivated by the need to develop specific and efficient cleaning and disinfection strategies to remove *L. monocytogenes*-carrying biofilms present in food-related industrial environments. With this global aim, the main objectives of the present thesis were:

i). Detect and characterise the composition of *L. monocytogenes*-carrying consortia present in surfaces of fish and seafood, meat, and dairy industries. This characterisation included molecular subtyping to establish ecological distributions and further demonstration of how *L. monocytogenes* can associate with the

different bacteria present to form dual-species biofilms. Besides, the dynamics of *L. monocytogenes* monospecies biofilm on SS was determined using fluorescence microscopy and image analysis. The isolates obtained were used in the subsequent studies, and in those environments where no isolates were detected model biofilms formed by strains also isolated from environmental samples were used.

ii). Determine the biofilm-removal effects of different enzymes aiming to degrade specific components of the biofilm matrix in young *L. monocytogenes* dual-species biofilms. The enzymes showing the highest efficacy were combined with benzalkonium chloride to determine its biofilm removal effects in two different *L. monocytogenes* dual-species biofilms also taking into consideration the amount of viable cells released after the treatment.

iii). Ascertain the synergic effects of a pronase-benzalkonium chloride combined treatment against late-stage *L. monocytogenes-E. coli* biofilms. Determination of synergy was performed following a first order factorial design using images of fluorescence microscopy and further determination of areal parameters as well as the effects of viable adhered and released cells after the application of combined treatments. In addition to this, a previous statistical study to determine the feasibility of this method was also carried out.

iv). Determine the capacity of *L. monocytogenes-E. coli* biofilms to develop tolerance after application of sublethal concentrations of pronase-benzalkonium chloride combined treatments using different exposure approaches.

2

Listeria monocytogenes-carrying consortia present in the food industry

Introduction

The ability of *L. monocytogenes* to survive and persist for long periods in food-related industrial environments has been extensively documented in the last decades [30,162,164,232]. Besides, its ecological flexibility permits this pathogen to associate with both Gram-positive and Gram-negative species [130,131] forming multispecies biofilms [132]. Since the composition and the interaction heterogeneity in these structures determines a series of characteristics such as the number of adhered *L. monocytogenes* [129,131,133] or the capability to endure sanitation treatments [68,132,134,230,231], it seems obvious that gaining further insight into the actual composition of multispecies biofilms present in industrial environments, would provide valuable information for development of novel strategies for pathogen control.

However, most of the research carried out involving *L. monocytogenes*-carrying biofilms, generally uses the most representative species of the expected accompanying microbiota instead of bacteria isolated from real food processing premises. For this reason, as a starting point in this thesis, the main aim of this work was to detect and characterise the bacterial communities carrying *L. monocytogenes* present in surfaces of fish, meat and dairy industries. This included sampling, isolation, identification and subtyping of *L. monocytogenes* isolates and related microbiota and a study of *L. monocytogenes* biofilm formation dynamics on stainless steel.

Methods

Sample collection

Sampling was carried out between September 2010 and July 2011 in eight different surveys in Northwest Spain (Galicia and Asturias) in different food-related premises obtaining total of 270 samples from fish, meat and cheese production industries (Table 2.1). In each survey all samples were collected the same day. A detailed list of all samples obtained for food industry-related consortia is stated in Supplementary Table 1 (available online). Surveys in fish industry were carried out by personnel from the Microbiology and Technology of Marine Products personnel whereas an external company was needed to perform meat and cheese industry samplings since they did not grant us access due to their legal and privacy policy.

Samples corresponding to 200 cm^2 from every selected surface were aseptically collected by thoroughly rubbing with a sterile sponge moistened with 10 ml of sterile LPT Neutralizing

broth (composition per litre: 0.7 g soy lecithin, 5 g NaCl, 1 g $Na_2S_2O_3$, 2.5 g $NaHSO_3$, 1 g $HSCH_2COONa$, 5 g Yeast Extract, 1 g L-histidine, 5 ml Tween 80, pH 7.6 ± 0.2). Sponges were introduced individually in auto-sealable bags, kept refrigerated at 4ºC and processed within the 24 hours following the sample collection.

Isolation of *Listeria monocytogenes* and accompanying microbiota

Sponges were mixed with 50 ml of sterile buffered peptone water (BPW; Cultimed, Barcelona, Spain) and digested with a stomacher masticator (IUL Instruments, Barcelona, Spain) during 1 minute. An aliquot of 100 µl of the resultant suspension was directly spread onto TSA plates (Cultimed, Barcelona, Spain) and incubated at 25 ºC for subsequent isolation of accompanying microbiota in case of *L. monocytogenes*-positive sample, where morphologically different colonies were picked and subcultured twice in TSA to obtain pure cultures (isolates). These isolates were finally cultured in TSB (Cultimed, Barcelona, Spain) at 25 ºC for DNA extraction.

To detect *L. monocytogenes* 1 ml was directly transferred to a flask containing 25 ml of sterile Half Fraser broth (Oxoid, Hampshire, England) and incubated at 30 ºC during 24 hours. 100 µl of positive samples (changing medium from green to black) was transferred to 10 ml of sterile Fraser broth (Oxoid, Hampshire, England) and incubated at 37 ºC for 24 hours. Finally, 100 µl of positive samples was plated in Chromogenic (ISO) Listeria Agar (Oxoid, Hampshire, England) and incubated for further 24 hours at 37 ºC. Presumptive *L. monocytogenes* appeared blue presenting a clear halo around them. These were recovered and subcultured twice in TSA to ensure purity of cultures and finally cultured in TSB at 37 ºC for DNA extraction.

Stock cultures of every sample were made and kept at -80 ºC in BHI (Biolife, Italy) containing 50 % glycerol 1:1 (v/v) mixed. Work cultures were maintained at -20 ºC in TSB (Cultimed, Barcelona, Spain) containing 50 % glycerol 1:1 (v/v) mixed.

Survey	n. factories surveyed	Sort of factory	Sampling date	n. samples	*L. monocytogenes* positive samples	Isolate ID	Serotype	*Apa*I, *Asc*I pulsotype
A	1	Fish and seafood processing	Sept. 2010	106	3	A1	1/2a – 3a	I
						A2	1/2a – 3a	I
						A3	1/2a – 3a	I
D	1	Mussel processing	March 2001	17	3	D1	4b -4d – 4e	II
						D2	4b -4d – 4e	II
						D3	4b -4d – 4e	II
E	7	Meat processing, slaughterhouse and quartering	April 2011			E1	1/2a – 3a	III
						E2	1/2a – 3a	IV
F	8	Meat processing and	April 2011			F1	1/2a – 3a	V
						F2	½b – 3b – 7	VI
						F3	1/2a – 3a	VII
						F4	½b – 3b – 7	VI
G	1	Cheese producer	June 2011	24	0			
H	1	Cheese producer	June 2011	12	0			
I	1	Cheese producer	June 2011	12	0			
J	1	Cheese producer	July 2011	24	0			
Total				270				

Table 2.1: Origin, dates, number of samples and subtyping of *L. monocytogenes* isolates obtained in this study.

Bacterial identification

Genomic DNA was extracted from liquid cultures as described previously (Vázquez-Sánchez et al., 2012). 16S rRNA gene amplification was performed using primers 27FYM and 1492R' (Table 2.2) as previously described [233] using a MyCycler™ Thermal Cycler (Bio-Rad, Hercules, CA) and PCR amplicon size was checked using a 50 - 200 bp molecular marker (Hyperladder 50 bp, Bioline) in a 1.5 % agarose gel stained with ethidium bromide.

Purification of PCR products was performed using a GenElute™ PCR Clean Up Kit (Sigma-Aldrich) and sequencing was carried out at Secugen, S.L. (Madrid, Spain) using an ABI Prism gene sequencer (Applied Biosystems, Foster City, CA). Chromatograms were processed and strain identification was undertaken using the Nucleotide-BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Assay	Primer	Sequence (5'→3')	Ref.
16S r DNA gene	27FYM	AGAGTTTGATCCTGGCTCAG	[233]
	1492R'	GGTTACCTTGTTACGACTT	
***L. monocytogenes* serotyping**	*lmo0737*	For: AGGGCTTCAAGGACTTACCC	[11]
		Rev: ACGATTTCTGCTTGCCATTC	
	lmo1118	For: AGGGGTCTTAAATCCTGGAA	
		Rev: CGGCTTGTTCGGCATACTTA	
	ORF2819	For: AGCAAAATGCCAAAACTCGT	
		Rev: CATCACTAAAGCCTCCCATTG	
	ORF2110	For: AGTGGACAATTGATTGGTGAA	
		Rev: CATCCATCCCTTACTTTGGAC	
	prs	For: GCTGAAGAGATTGCGAAAGAAG	
		Rev: CAAAGAAACCTTGGATTTGCGG	
Accompanying microbiota RAPD-PCR	S	TCACGATGCA	[234,235]
	AP7	GTGGATGCGA	
	ERIC-2	AAGTAAGTGACTGGGGTGAGCG	

[a]For, forward; Rev, Reverse

Table 2.2: Sequences of primers used in this work.

PFGE subtyping

For those confirmed *L. monocytogenes* samples, pulsed field gel electrophoresis (PFGE) assays were performed in Complexo Hospitalario Universitario Xeral - Cíes (Vigo, Spain) in a CHEF-DR®III Electrophoresis Apparatus (Bio-Rad Laboratories, Hercules, CA) using the re-evaluation of PulseNet protocol for *L. monocytogenes* [236]. Agarose plugs were digested with *Asc*I and *Apa*I restriction endonucleases (NewEngland Biolabs) and Lambda Ladder PFG Marker (NewEngland Biolabs) was used in all experiments. After electrophoresis, gels were stained with ethidium bromide and visualised under UV light.

Similarity factors based on Dice coefficient, cluster analysis by UPGMA system and strain dendograms (Tolerance 1%, Optimisation 0.5%) were obtained using GelComparII software (Applied Maths NV, Belgium).

Listeria monocytogenes serotyping

So as to differentiate the major serovars (1/2a, 1/2b, 1/2c, and 4b) among the obtained L. monocytogenes isolates, a multiplex PCR was used following a modified protocol of that described previously [11]. Briefly, 5 µl of confirmed L. monocytogenes DNA sample was mixed in a 50 µl PCR reaction mixture containing 0.2 mM of each dNTP (Bio-Rad, Hercules, CA), 5 µl 10X Advanced Taq buffer without Mg2+ (5 Prime), 2 mM MgCl2, 1 µM for primers lmo0737, ORF2819 and ORF2110, 1.5 µM for primer lmo1118 and 0.2 µM for primer prs (Table 2.2) and 1 U Taq polymerase (5 Prime). Conditions consisted of an initial denaturing step at 95 ºC (3 min), followed by 35 cycles of 94 ºC (1 min), 53 ºC (1:15 min) and 72 ºC (1:15 min), with a final extension of 7 min at 72 ºC. Amplicons were resolved in a 1.5 % agarose gel stained with ethidium bromide and bands were visualised using a GelDoc 2000 Apparatus equipped with Quantity One software (Bio-Rad, Hercules, CA) using Hyperladder 50 bp (Bioline) as a molecular marker.

RAPD-PCR for accompanying microbiota

Sequences of oligomers used in Random Amplified Polymorphic DNA (RAPD) PCR reactions for associated microbiota are listed in Table 2.2. Primers AP7, ERIC-2 [234] and S [235] were used as previously described [237]. RAPD reactions were carried out using a MyCycler™ Thermal Cycler (Bio-Rad, Hercules, CA) in a 50 µl final volume PCR reaction mixture containing 80 µM of each dNTP (Bio-Rad, Hercules, CA), 5 µl 10X Advanced Taq buffer (5

Prime) (supplemented with 1 mM $MgCl_2$ for reactions with primers AP7 and ERIC-2), 5 µM primer (Thermo Scientific), 2.5 U Taq polymerase (5 Prime) and 200 ng of DNA sample. Conditions for reactions containing primer S consisted of an initial denaturing step at 95 ºC (5 min), followed by 35 cycles of 95 ºC (1 min), 37 ºC (1 min) and 72 ºC (2 min), with a final extension of 5 min at 72 ºC. Conditions for reactions containing primers AP-7 or ERIC-2 included a denaturing step at 94 ºC (4 min), followed by 35 cycles of 94 °C (1 min), 25 °C (1 min) and 72°C (2 min), and a final extension step at 72 ºC for 7 min.

Products were resolved in 1.5 % agarose gels stained with ethidium bromide and bands were visualised using a GelDoc 2000 Apparatus equipped with Quantity One software (Bio-Rad, Hercules, CA).

Setup of biofilm formation

In all cases, work cultures were thawed and subcultured twice in TSB at 37 ºC for *L. monocytogenes* or 25 ºC for associated microbiota prior to use.

Inocula were prepared by adjusting Abs_{700} to 0.1 ± 0.001 in sterile TSB using a 3000 Series scanning spectrophotometer (Cecil Instruments, Cambridge, England), corresponding to a concentration of 10^8 CFU/ml according to previous calibrations. Inocula used for dual-species association assays were further diluted in TSB until obtaining a cellular concentration of 10^4 CFU/ml and 1:1 (v/v) mixed. Controls for these assays were mono-species cultures with the same final concentration.

Biofilms were cultured on 10 x 10 x 1 mm AISI 304 stainless steel (SS) coupons (Acerinox S.A., Madrid, Spain). Coupons were individually cleaned with industrial soap in order to remove any grease residue, thoroughly rinsed with tap water and finally rinsed with distilled water. Coupons were then autoclaved at 121 ºC during 20 min, placed individually into a 24 flat-bottomed well microtiter plate and inoculated with 1 ml of each culture.

L. monocytogenes mono-species biofilms for microscopy were incubated statically at 25 ºC whereas cultures for association assays were incubated statically during 2 hours to allow initial adhesion and then in constant shaking at 100 rpm in saturated humidity conditions at 25 ºC.

Assays to evaluate *Listeria monocytogenes* mixed-species association in biofilms

Two coupons of each culture were harvested at 24, 48 and 72 hours for attached cell number determination. Coupons were briefly immersed in sterile PBS to remove loosely attached cells. Biofilms were then collected by double scrapping using BPW-moistened sterile cotton swabs which were placed in sterile assay tubes containing 2 ml of sterile BPW and vortexed vigorously for 1 min so as to release cells. Cells suspensions were 10-fold diluted in sterile BPW and spread onto agar plates.

Control cultures were spread onto TSA plates (Cultimed, Barcelona, Spain). In mixed-cultures *Listeria*-PALCAM agar (Liofilchem, Italy) was used to select *L. monocytogenes*, *Pseudomonas* Agar Base with CFC Supplement (Liofilchem, Italy) for *Pseudomonas sp.*, Chromogenic *E. coli* agar (Cultimed, Barcelona, Spain) with a 5 mg/l supplement of Vancomycin and Cefsulodin (Sigma-Aldrich) for *Escherichia coli*. TSA medium was used if no selective medium was available, in these cases number of cells of strain co-cultured with *L. monocytogenes* was expressed as the number of colonies present in TSA (total cell counting) minus the number of cells present in PALCAM cultures (*L. monocytogenes* cell counting). Chromogenic and PALCAM plates were incubated at 37 ºC whereas the rest were incubated at 25 ºC for 24-48 hours and results were expressed in log CFU/cm^2.

Fluorescence microscopy assays and image analysis

In order to compare the adhesion dynamics of *L. monocytogenes,* six of the strains isolated were cultured on AISI 304-type SS at 25 ºC in TSB. Two coupons were stained with FilmTracer™ Calcein Green Biofilm Stain (Life Technologies) at 72, 96, 120, 144, 168 and 240 hours and biofilms were visualised with a Leica 4500DM epifluorescence microscope using 10x ocular lenses and 40x objective. From each sample, images of ten randomly chosen fields were taken using a Leica DFC365 FX camera.

Image analysis was performed using BIOFILMDIVER, a MATLAB-based code, in order to perform dynamic analysis [101]. The structural parameters computed on binary images were Average diffusion distance (ADD), Maximum diffusion distance (MDD) [152] and Covered Area (CA). ADD and MDD are defined as the average and maximum euclidean distance from the central foreground pixel of a cell-aggregate to the nearest background pixel of a given image. Combined results of both parameters show the density of the biofilms and the mean and maximum distances covered by cells. CA uses the ratio between the number of foreground pixels and the total number of pixels and the actual area of each pixel to extract the percentage of occupied area by cells.

Results

Detection, isolation and identification of *Listeria monocytogenes* in fish, meat and dairy industry surfaces

270 environmental samples were screened for the presence of *L. monocytogenes* and its accompanying bacterial microbiota collected from a variety of surfaces from food industry.

Enrichment and subsequent Chromogenic (ISO) Listeria Agar cultures of the samples allowed to primary identify putative *L. monocytogenes* by the presence/absence of the halo around the colony, giving positive results in 6.30 % (n = 17) out of the 270 samples. Presumptive *L. monocytogenes* colonies were further analysed so as to obtain an accurate identification by 16S rRNA gene sequencing. Outcomes showed that among Chromogenic (ISO) Listeria Agar-positive isolates only 12 were actually *L. monocytogenes*. False-positive results were subsequently identified as *L. innocua* (n = 2), *L. welshimeri* (n = 2) and *Enterococcus faecalis* (n = 1) giving an overall incidence of *L. monocytogenes* among the checked surfaces of 4.44 %. Distribution of *L. monocytogenes*-positive samples among the different surfaces surveyed shows a higher incidence of positive samples among those samples coming from meat industry (8 %) when compared with those from the fish industry (4.88 %). No *L. monocytogenes*-positive samples were recovered from samples harvested among the surveyed surfaces of cheese factories.

Subtyping of isolated *Listeria monocytogenes*

PFGE and serotyping results

Table 2.1 summarises the results of *L. monocytogenes* isolates' subtyping with regard to their origin. Multiplex PCR serotyping demonstrated that 58.63 % (n = 7) of the isolates belonged to serogroup 1/2a – 3a, 25 % (n = 3) to serogroup 4b – 4d – 4e and 16.67 % (n = 2) to serogroup 1/2b – 3b – 7. Isolates from surveys A, D and E shared the same serogroup whereas in survey F two different serogroups were obtained.

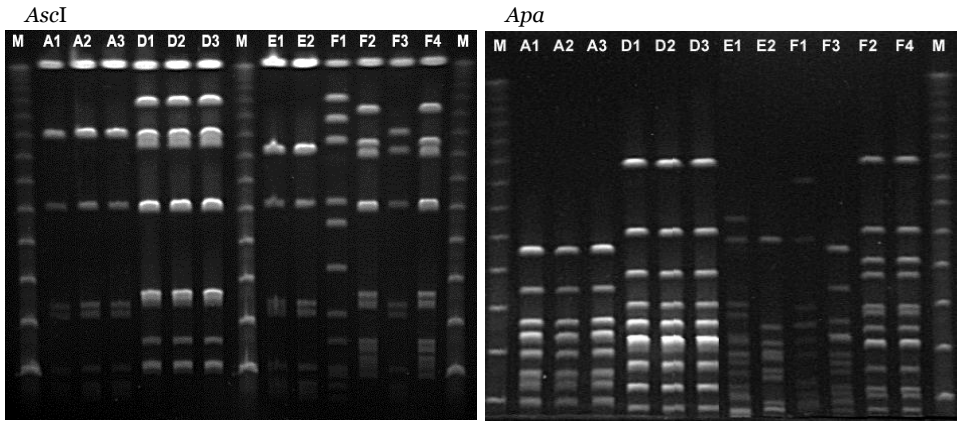

Figure 2.1: *Apa*I and *Asc*I macrorestriction PFGE profiles of *L. monocytogenes* isolates (Lanes: M: Molecular marker; A1 to F4: *L. monocytogenes* samples)

All twelve *L. monocytogenes* isolates were subtyped by PFGE with enzymes *Asc*I and *Apa*I [236] in order to establish molecular relationships among the isolates and also to check the ubiquity of the different subtypes. Composite results of both assays displayed a Simpson's discriminatory index (D.I.) of 0.894 [238]. Among the surfaces belonging to fish industry, *L. monocytogenes*-positive samples from surveys A and D showed a unique band pattern for each assay (Figure 2.1) revealing that in fact, isolates from surveys A and D were the same strain isolated from different points. Contrarily to these situations were samples from survey E and F, both obtained from meat industry, which presented two and three different PFGE profiles, respectively, genetically unrelated based on the "3-band rule" [239]. Among isolates from survey F, *L. monocytogenes* F2 and F4 shared the same subtype, being different from the pattern observed in strains F1 and F3 also different between them.

As shown in Table 2.1, samples of surveys A and D were obtained by surveying just one factory at a time, thus some relationships in terms of ubiquity and strains spreading were feasible to be done. In both cases, *L. monocytogenes* were isolated from surfaces sharing some similarities, being those of survey A from places that appear to be difficult to access to efficient sanitisation procedures whilst in D they were isolated from cleaned surfaces, which are directly in contact with on-process products (Table 2.3).

Fingerprint analysis

UPGMA clustering based on Dice correlation index of strains based on their PFGE profile was undertaken using GelComparII software (Applied Maths NV, Belgium) (Figure 2.2).

In regard to the origins of each strain, correlations between the type of food industry surveyed and the strain subtype could not be established since strains coming from meat and fish industry cluster intermingled among them. Notwithstanding this, it is important to highlight that isolate F1 appeared to be the most unrelated strain with a similarity coefficient below 50% with the closest group.

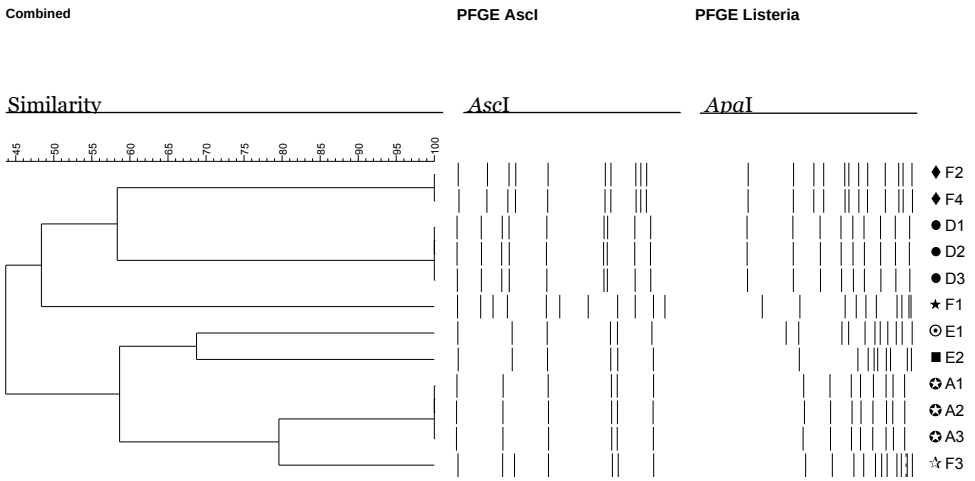

Figure 2.2: *Asc*I and *Apa*I PFGE composite dendogram corresponding to the UPGMA cluster analysis of *L. monocytogenes* isolates.

Characterisation of biofilms formed by *Listeria monocytogenes* isolates by BIOFILMDIVER

L. monocytogenes A1 image analysis parameters (CA, ADD and MDD) suggested a dynamic profile characterised by the presence of one peak at 120 h, reaching the highest values of the study in all parameters measured (Figures 2.3, 2.4 and 2.5). Median values for this isolate ranged between 1.00 % - 39.30 % for CA, 1.65 - 2.78 and 6.70 - 16.00 for ADD and MDD respectively. Maximum values reached by this isolate for CA were 58.32 %, 4.27 for ADD and 23.35 for MDD.

The remaining adhesion kinetics were similar in all other isolates assayed. *L. monocytogenes* D1, E1, F1, F2 and F3 outcomes of ADD, MDD and CA suggested a dynamic profile

characterised by the presence of various peaks rendering values significantly lower and within a narrower range compared to those obtained by *L. monocytogenes* A1 (Figures 2.3, 2.4 and 2.5). However, differences among isolates of this group were also noticeable. *L. monocytogenes* F1 biofilms exhibited the highest values and F2 biofilms the lowest, obtaining the following outcomes: CA_F1: 35.69 % - 2.13 % CA_F2: 3.79 % - 0.02 %, ADD_F1: 1.25 - 1.12, ADD_F2: 1.25 - 1.12, MDD_F1: 16.76 - 5.65 and MDD_F2: 9.21 - 2.82. Nevertheless, minor fluctuations could be observed among isolates thus indicating some sort of dynamics even though in poorly populated biofilms.

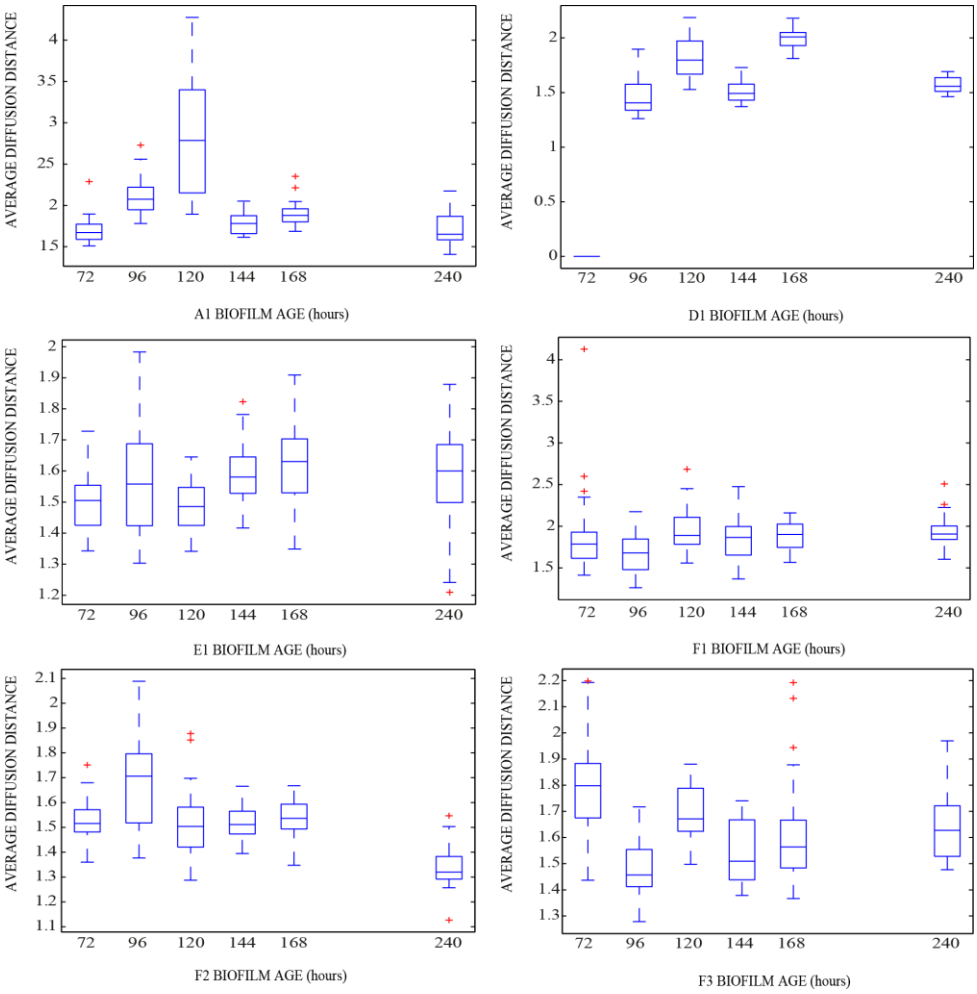

Figure 2.3: Average diffusion distance of biofilms of *L. monocytogenes* onto AISI 304 SS calculated with BIOFILMDIVER.

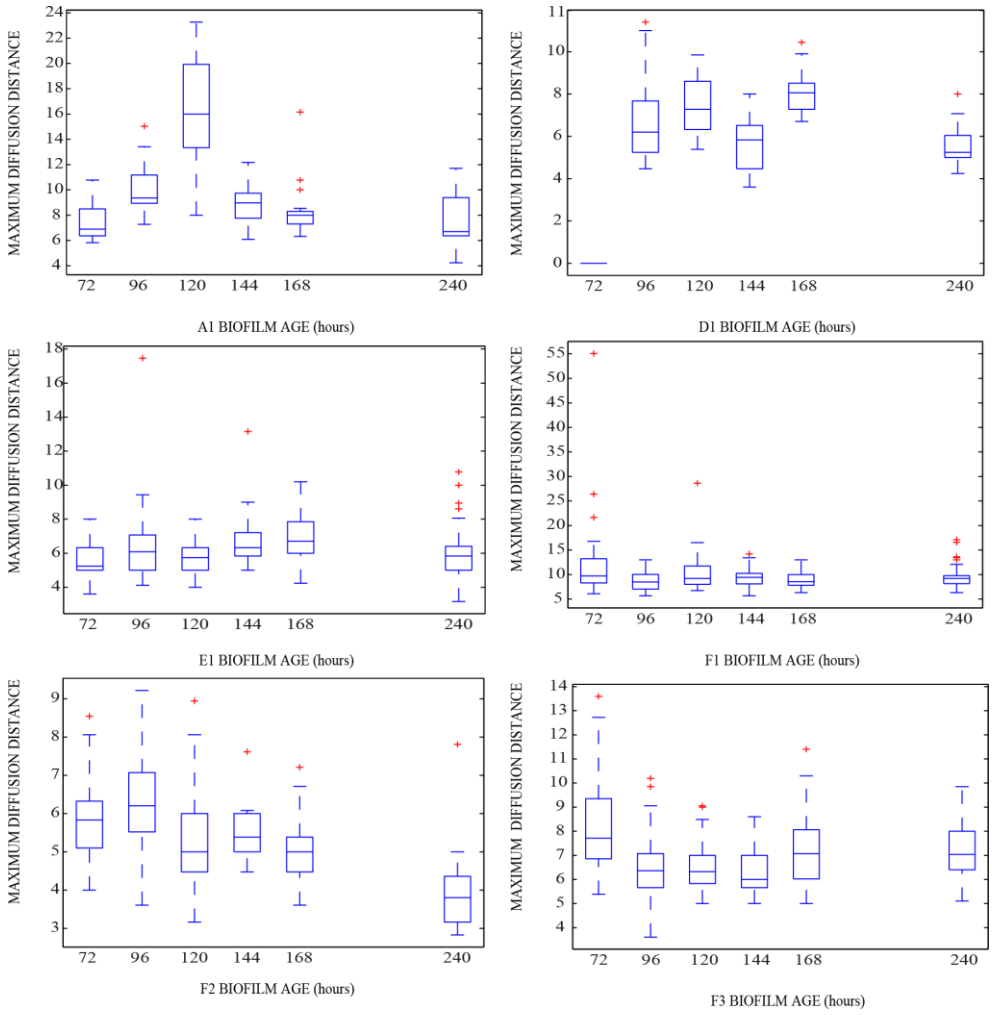

Figure 2.4: Maximum diffusion distance of biofilms of *L. monocytogenes* onto AISI 304 SS calculated with BIOFILMDIVER.

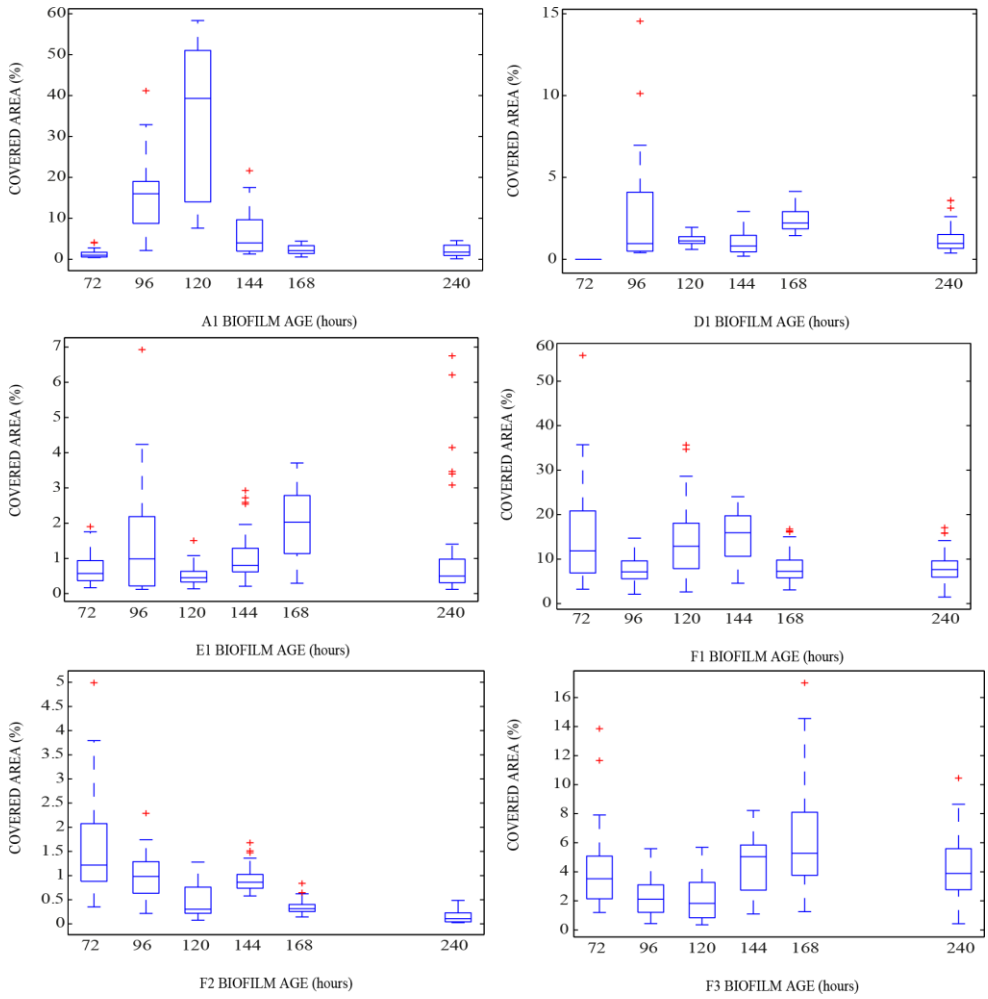

Figure 2.5: Covered area of biofilms of *L. monocytogenes* onto AISI 304 SS calculated with BIOFILMDIVER.

Isolation, characterisation and RAPD-PCR subtyping of accompanying microbiota present in *Listeria monocytogenes*-positive samples

Overall, 18 different species were isolated and molecularly identified, 10 of these were from the fish industry surveys, 7 from the meat industry survey and one species (*Staphylococcus saprophyticus*) was present in both surveys. Table 3 shows the species composition of the *L. monocytogenes*-carrying consortia isolated. In terms of relative presence, *E. coli* appeared to be the most abundant (26.27 %) among the surfaces checked related to the fish industry, whereas among the isolates from meat industry, *Carnobacterium sp*. was the major *L. monocytogenes* accompanying species, found in a 30 % of the isolates assayed.

Survey	Isolate code	Source	Identification
A	A1	Thermal gloves	*Listeria monocytogenes*
	A11		*Staphylococcus saprophyticus*
	A12		*Kokuria varians*
	A13		*Aerococus viridans*
	A14		*Escherichia coli*
	A2	Floor under halibut-defrosting area	*Listeria monocytogenes*
	A22		*Escherichia coli*
	A23		*Microbacterium* sp.
	A24		*Escherichia coli*
	A25		*Corybacterium* sp.
	A3	Sewage channels	*Listeria monocytogenes*
	A31		*Escherichia coli*
	A32		*Staphylococcus scuri*
	A33		*Microbacterium luteolum*
	A35		*Enterococcus aquimarinus*
D	D1	Conveyor belt 1	*Listeria monocytogenes*
	D11		*Staphylococcus* sp.
	D2	Scale line 3	*Listeria monocytogenes*
	D21		*Rothia Terrae*
	D3	Scale line 1	*Listeria monocytogenes*
	D31		*Staphylococcus saprophyticus*

Table 2.3: Microbial composition of isolated consortia.

Survey	Isolate code	Source	Identification
E	E1	Transportation trolley	*Listeria monocytogenes*
	E11		*Serratia* sp.
	E12		*Carnobacterium divergens*
	E2	Metal trolley	*Listeria monocytogenes*
	E21		*Carnobacterium divergens*
	E22		*Staphylococcus saprophyticus*
F	F1	Meat mincer	*Listeria monocytogenes*
	F11		*Pseudomonas* sp.
	F2	Massage drum	*Listeria monocytogenes*
	F21		*Staphylococcus vitulinus*
	F22		*Carnobacterium* sp.
	F3	Mincer	*Listeria monocytogenes*
	F31		*Serratia* sp.
	F4	Drain	*Listeria monocytogenes*
	F41		*Buttiauxella* sp.
	F42		*Carnobacterium* sp.

Table 2.3 (continued)

Association capacity assays of dual-species biofilms onto stainless steel surfaces

Plate counts showed that the presence of certain species caused a deleterious effect in some *L. monocytogenes* isolates significantly reducing the number of attached viable cells (α = 0.05) compared with mono-species biofilms in the same culture conditions (Figure 2.6). *L. monocytogenes* D1 showed a reduction of ~2 log CFU/cm^2 at 24 h when co-cultured with *S. saprophyticus* D31 whilst a reduction of ~1 log CFU/cm^2 at 24 and 48 hours was observed in *L. monocytogenes* E1 and F2 in the presence of *C. divergens* E12 and *S. pulvereri* F21 respectively. Only the pairs *L. monocytogenes* F1 – *Pseudomonas sp*. F11 and *L. monocytogenes* F3 – *Serratia fonticola* F31 showed a significant decrease in all three sampling times being more evident in the latter case (Figure 2.6). Accompanying species viable count also presented differences in some cases. A significant increase was observed in *S. saprophyticus* A11 at 48 hours, in *C. divergens* F22 at 24 and 48 hours and in *S. fonticola* F31 at 72 hours. Contrarily *R. terrae* D21 and *Pseudomonas sp*. F11 displayed a significant reduction at 72 hours (Figure 2.6).

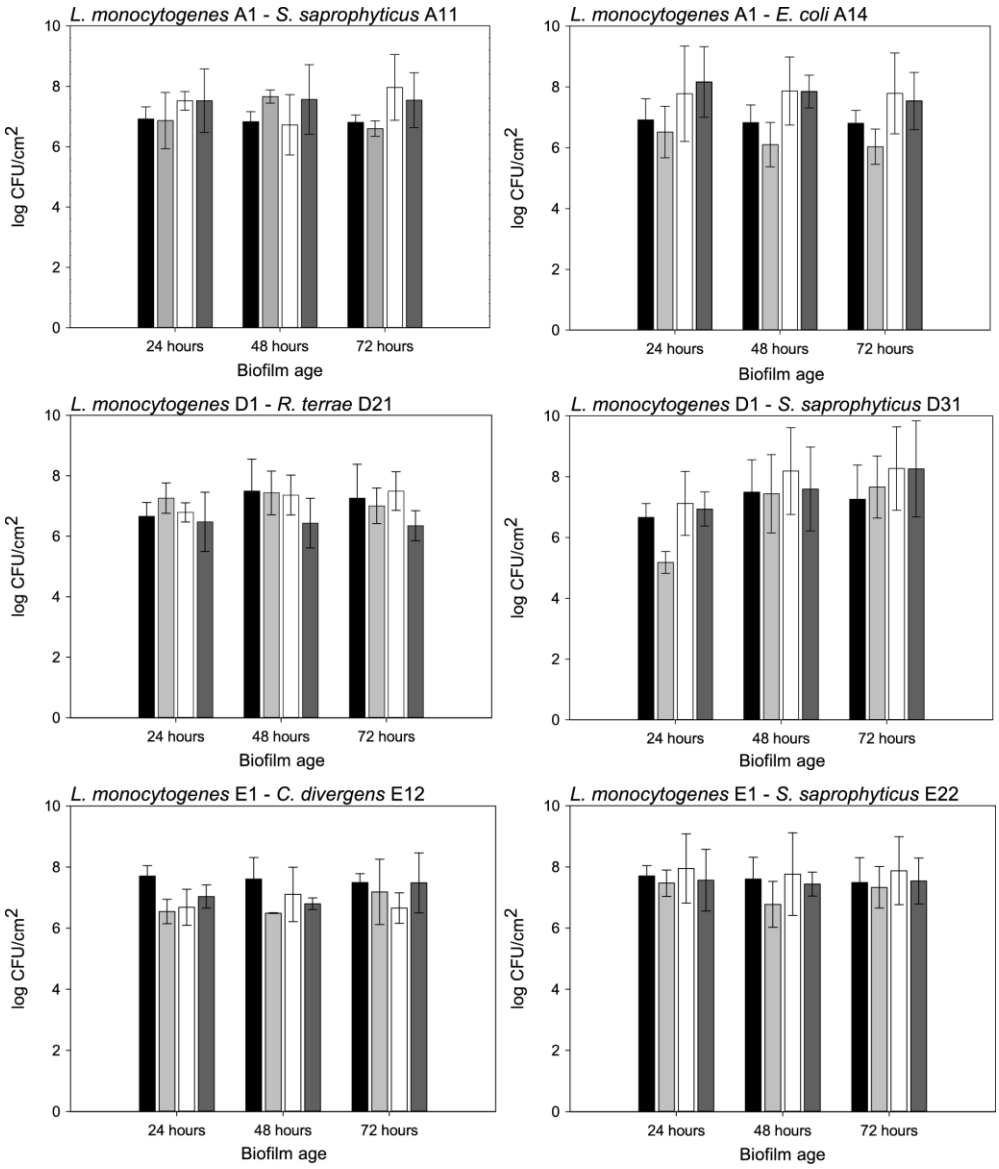

Figure 2.6: Mixed-species biofilm association assays of isolates from food industry onto AISI 304 SS coupons (■ *L. monocytogenes* mono-species culture, ■ *L. monocytogenes* in mixed-species culture, □ Accompanying species mono-species culture, ■ Accompanying species in mixed-species culture).

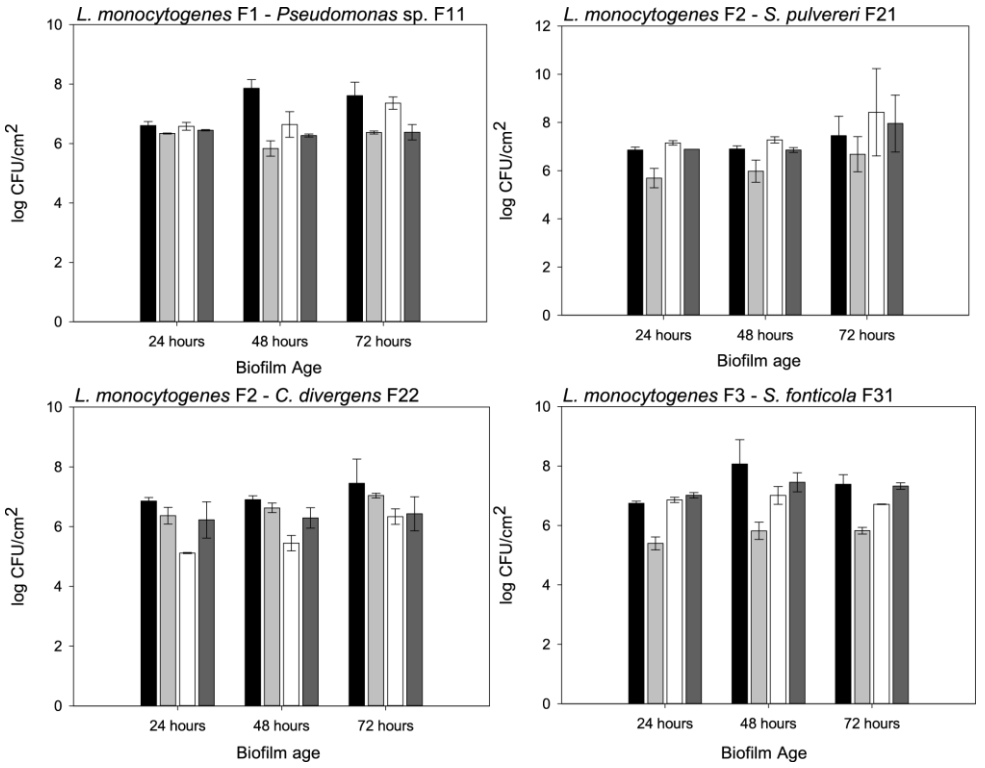

Figure 2.6 (continued)

Discussion

This work presents the detection, identification and ulterior characterisation of bacterial consortia involving the presence of *L. monocytogenes* isolated from surfaces belonging to fish and meat product handling revealing that in addition to *L. monocytogenes* in food-related premises an actual bacterial community is set. It was noticeable that *E. coli* predominates as *L. monocytogenes*-accompanying bacteria among surfaces surveyed regarding fish industry (33.33 %) whereas in meat industry surfaces the predominant genus among the microbiota associated with *L. monocytogenes* was *Carnobacterium sp*. (40 %). The presence of such microorganisms would reflect the own microbial load present on raw fish and meat products as previously reported [240]. It is also known that there is a certain specificity that drives microorganisms to get established in a particular food matrix [241]. Lactic acid bacteria such as *Carnobacterium sp*. and *Carnobacterium divergens* along with

non-fermentative gram negative bacteria such as *Pseudomonas sp.* or *Serratia sp.* are among the main microorganisms present in meat products [242]. Similarly, *E. coli* is known to be associated with faecal contamination [243,244] and it is normally present in seafood products and seafood processing industries [245].

Moreover, obtained results from this surveys have shown an overall incidence of 4.44 %, with higher outcome of *L. monocytogenes* in meat industry (8 %, n = 75) compared with fish industry (4.88 %, n = 125). Obviously, in non-standardized conditions, incidence results can vary noticeably depending on the sampling method used [246], the number of samples analysed, the moment when samples are collected and the size of the surface sampled, among others. Concerning this last, previous authors have obtained values of 7.92 % when sampling surfaces between 50 and 100 cm^2 [247] and reaching values of 17 or even 26 % when 1 m^2 surfaces are sampled [164]. In this article, sponge swabbing with subsequent enrichment was chosen since is the method most commonly used among surveys to determine presence/absence of *L. monocytogenes* [161,164,248–250].

In fish industry results showed that origins of *L. monocytogenes* isolates can be diverse coming from surfaces typically not in contact with raw or processed product in survey A whereas in survey D positive samples were obtained from surfaces typically in contact with fish products. This heterogeneity in the distribution of strains could be of a multifactorial nature and contact with manufactured product may help to maintain *L. monocytogenes* prevalence along food premises [28]. Nevertheless, other authors support the hypothesis that this distribution in a given industrial environment is independent to incoming raw matter contact [251]. In the light of these results and due to the variation of the different surveys, it would be recommendable to establish a common procedure of surfaces sampling in food-related premises, so as to ensure the possibility to compare results between different assays. In addition, identification of contamination *foci* for a particular microorganism taking into account the casuistic of each processing plant may be considered to develop preventive strategies.

When molecular confirmation of *L. monocytogenes* positive colonies was carried out by 16 s rRNA gene sequencing, 29.41 % of positives were demonstrated to be false-positive and subsequently identified as *L. innocua* (n = 2), *L. welshimeri* (n = 2) and *Enterococcus faecalis* (n = 1). This lack of accuracy in regards to classical identification methods is in accordance with results obtained by other authors [252] and highlights once again the combination of classical and molecular methods as the optimal approach for proper bacterial identification.

In order to give further insight on the ecology of *L. monocytogenes* isolates obtained, these were subtyped by serotyping and PFGE yielding a discrimination index (D.I.) of 0.894 which in spite of being below the ideal D.I. value of 0.9 [238], is close enough considering the number of the samples available thus the assay was considered as valid.

Among subtypes obtained from surveys carried out at one single fish-processing premises (surveys A and D), a unique *L. monocytogenes* subtype was present at different locations suggesting that this bacterium may be able to endure and colonise different environmental conditions as was observed in previous studies carried out in fish industries [164]. From an ecological perspective, the fact that *L. monocytogenes* isolates from surveys A and D belonged to a single subtype and shared the same typology of surface may suggest that these isolates have undertaken adaptation phenomena in order to be able to survive in a particular environment [240,242,253].

Molecular serotyping demonstrated that in most cases serovars of isolates belonged to group 1/2a – 3a, followed by serogroup 4b – 4d – 4e and finally by serogroup 1/2b – 3b – 7 being in accordance with previous published studies of surveys performed in Europe which showed that serogroup 1/2a – 3a is the most abundant among environmental samples [254–256].

Two main structural patterns were observed from numerical characterisation of mono-species *L. monocytogenes* biofilms. One was exhibited by *L. monocytogenes* A1. CA, ADD and MDD profiles of this isolate showed one peak that suggest a dynamic evolution characterised by a unique episode of attachment and detachment (Figures 2.3, 2.4 and 2.5). The fact that ADD and MDD increase with CA, until reaching a maximum at 120 h, indicating that A1 form biofilms with a homogeneous distribution of viable cells and high density, according to the CA values obtained.

D1, E1, F1, F2 and F3 isolates shared a common pattern of individual cells that evolved to cell aggregates which finally disappear. The low CA values obtained reflected the incapacity of these isolates to form dense biofilms. However, among them F1 biofilm showed the highest population level of this group. The generation of clusters were noted by ADD and MDD results, however size of those clusters also varied among isolates, being bigger the clusters of F1 regarding those exhibited by the rest. In contrast with results showed by A1, evolution dynamics in this particular case was characterised by several episodes of attachment-detachment. Similar results, two patterns characterised by two different dynamic profiles and parameter values, were reported previously after studying three different *L. monocytogenes* strains under same conditions [101].

Previous studies have demonstrated that biofilm architecture influences the grade in which diffusion takes place. Following this idea Stewart and Franklin [76] stated that physiological heterogeneity and complex structures such as cell clusters, quantified in this study by CA, ADD and MDD, could promote diffusional limitations to antimicrobials via establishment of local gradients being especially remarkable in mature biofilms. In this regard, however, Carpentier and Cerf [35] claimed that the presence of *L. monocytogenes* among surfaces in food-related environments is more likely to be due to an improper design of cleaning and disinfection routines along with erroneous manufacturing practices among plants rather than to an enhanced biofilm forming capability. Nevertheless, recent studies suggest that this theory could be too reductionist and the fact that *L. monocytogenes* can be established in a particular industrial setting appears to be more of a multifactorial phenomenon where genetic and physiological changes may take place [257,258]. In order to elucidate the actual causes of presence and subsequent persistence phenomena in *L. monocytogenes* strains isolated not only further sampling must be carried out at different times but also the assessment of the composition of accompanying microbiota and its contribution to the establishment of stable ecological niches by *L. monocytogenes* in industrial settings.

Interactions among bacteria forming the different consortia appear to be crucial for the fitness of the whole structure [259]. Association capacity assays of dual-species biofilms demonstrated how *L. monocytogenes* isolates were able to form biofilms along with other microorganisms [132,134,260,261]. Outcomes obtained showed that *Pseudomonas sp.* F11 and *Serratia fonticola* F31 seemed to have a deleterious effect at all sampling times, on *L. monocytogenes* present on biofilms. This is in agreement with previous results obtained by Carpentier and Chassaing [129], who demonstrated a 3-log reduction on the number of *L. monocytogenes* adhered cells in presence of *Pseudomonas fluorescens*. *L. monocytogenes* E1 was also affected by *C. divergens* E12 being reduced its number of attached cells at 24 and 48 hours being in accordance with previous studies published showing that lactic acid bacteria (LAB) and LAB-related species such as *Carnobacterium sp.* strains can be used as a strategy so as to control the population of *L. monocytogenes* in order to avoid spoilage and potential foodborne poisoning in meat [262] and in fish products [263]. However, no differences in the number of attached cells of *L. monocytogenes* F2 co-cultured with *C. divergens* F22 were observed. Since isolates E12 and F22 are the same strain according to RAPD subtyping (see results for further detail), these results indicate that different strains of *L. monocytogenes* may respond diversely to the same *C. divergens* strain.

Relative abundances of *L. monocytogenes* and its accompanying strain did not show significant differences except when co-cultured with *S. fonticola* or *Pseudomonas sp.* being in agreement with previous studies [226,264] showing that bacteria such as *Pseudomonas*

spp. in mixed-biofilm culture appear to be dominant in dual-species biofilms with *L. monocytogenes* even though the relative abundances reported by these authors were much lower than the ones obtained in this study.

Recently, the European Food Safety Authority reported a human listeriosis incidence among the European Member States of 0.44 cases per 100000 inhabitants which meant a 8.6 % increase compared with previously published data [265] fact that ratifies that *L. monocytogenes* control systems are, to date, insufficient. Nowadays, bacterial biofilms are well-known to be more resistant to disinfectants and even though it is widely accepted that bacterial species are ubiquitous in the environment, the consideration of multi-species associations for the investigation regarding pathogen control is scarce, despite the fact that the presence of accompanying microbiota producing significant changes in the whole structure has been demonstrated [132,230,266,267]. This work aimed to deepen in the knowledge on sessile bacteria present in an actual food-related industrial context so as to use it as a starting point to perform further investigation of efficient pathogen control strategies regarding not only the composition of the biofilms but also the choice of real targets based on interactions among species.

Although many efforts have been put on the detection and elimination of *L. monocytogenes* in industrial settings, results obtained in this work showed how this pathogen is able to grow and survive in different food industry related surfaces. In addition to this fact, it can be noticed that *L. monocytogenes*-carrying bacterial consortia follow an association pattern from an ecological point of view depending on the industrial setting where they are present, which represents an interesting clue when planning a cleaning and disinfection procedure. Since *L. monocytogenes* has a great impact in the current society at clinical and industrial level, screening of niches of these communities appears to be compulsory in order to identify possible contamination *foci* and to design efficient, target-specific sanitisation methods to ensure proper elimination of undesirable microbiota whereas manufactured products' properties remain unaltered.

3

Enzymes-Benzalkonium chloride combined treatments against *L. monocytogenes*-carrying early-stage biofilms

Introduction

Once the composition of *L. monocytogenes*-carrying communities was determined, the next step in the present work was to carry out a first screening of the combined treatments and their biofilm-removing efficacy.

For this, BAC was chosen since is one of the most used QAC in the food industry causing membrane structure alteration upon bacteria and producing a subsequent cellular leakage [202,203]. However, in biofilms, resistance and tolerance to this biocide has been reported in several microorganisms such as *L. monocytogenes* [67,204], *E. coli* [205] or *Pseudomonas sp.* [134].

Recently, the use of enzymes as an antibiofilm strategy has significantly increased because their dispersal action degrading molecules present in the biofilm matrix, and proven to impede initial adhesion and remove formed structures [175–178]. Nevertheless, enzymes do not have bactericidal properties so they cannot be used as disinfectant compounds [117].

Consequently, in this chapter, the effects of an enzymatic treatment with different enzymes alone and followed by benzalkonium chloride against early-stage *L. monocytogenes* dual-species biofilms grown on stainless steel were assessed by means of classical agar plate counts and epifluorescence microscopy.

Methods

Bacterial strains

Listeria monocytogenes A1 and *Escherichia coli* A14 were isolated from a fish processing plant in a previous survey [37]. *Pseudomonas fluorescens* B52, a strong biofilm former and associated with milk and dairy products spoilage, was kindly provided by Dr. Carmen San José [268].

In all situations, stock cultures were kept at -80 ºC in Brain-Heart infusion broth (BHI; Biolife, Milan, Italy) containing 50% glycerol 1:1 (v/v) mixed. Work cultures were kept at -20 ºC in Tripticase Soy Broth (TSB; Cultimed, Barcelona, Spain) containing 50% glycerol 1:1 (v/v) mixed.

Construction of fluorescent-tagged stains

Genetic modification for constitutive expression of a fluorescent reporter of strains *L. monocytogenes* A1 and *E. coli* A14 was carried out in the laboratory of Prof. Colin Hill (School of Microbiology, University College Cork (UCC), Ireland).

Modification of L. monocytogenes

L. monocytogenes was modified for Green fluorescent protein (GFP) constitutive expression. Briefly, the fragment of pNF8 corresponding to the P*dltΩgfp-mut1* [269] was amplified with primers P*dlt* For-*Kpn*I and GFP pNF Rev-*Pst*I (Table 3.1) containing *Kpn*I and *Pst*I restriction sites, respectively, digested and cloned into pPL2 [270] previously digested with *Kpn*I and *Pst*I and further treated with rAPid Alkaline Phosphatase (Roche) to avoid religation. Ligation was performed using T4-ligase (Roche, Germany) in a PCR thermocycler as follows: 4 ºC for 5 h, 12 h ramp increasing 1 ºC/h, 16 ºC for 2 h and back to 4 ºC giving a plasmid of 7393 bp coded as pROLO1. The plasmid solution was dialysed in sterile deionised water on a 0.025 µm pore nitrocellulose filter (Millipore, Germany) for 30 min and then kept at -20 ºC until use. pROLO1 was then introduced into *E. coli* TOP10 cells (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions and cultured overnight in LB (Merck, Germany) + 10 µg/ml chloramphenicol (Cm; Sigma Aldrich, Saint Louis, MO) at 37 ºC. Plasmid extraction was then performed using a Gene JET Plasmid MiniPrep Kit (Thermo Fisher Scientific, Waltham, MA). P*dltΩgfp-mut1* integration was checked with primers pPL2 MCS-for and pPL2 MCS-rev (Table 3.1).

Electroporation was carried out by mixing 50 µl of electrocompetent cells prepared as previously described [271] with 2 µl of plasmid prep in 2 mm cuvettes using a BTX ECM 630 Generator (Harvard Apparatus, Holliston, MA). Conditions: field strength: 10 kV/cm; time constant: 5 ms; voltage: 2 kV; resistance: 400 Ω; capacitance: 25 µF. Cells were then resuspended in fresh sterile BHI + 0.5M Sucrose, incubated at 37 ºC for 1 h and then plated on BHI + 1,5 % agar + 10 µg/ml Cm and incubated at 37 ºC for 48 h. Colonies were picked and PCR was performed to check for plasmid integration using primers PL95 and PL102 [272] (Table 3.1). The resulting isolate was named *L. monocytogenes* A1-gfp.

Primer	Sequence (5'→3')	Reference
P*dlt* For-*Kpn*I	TGGGTACCATTATACTCGTACCTAC	This study
GFP pNF Rev-*Pst*I	AAACTGCATTTATTTGTATAGTTCATCCATGCCA	This study
MCS for	GACGTCAATACGACTCACTATAGG	This study
pPL2 MCS-rev	GATAATAAGCGGATGAATGGCAG	This study
PL95	ACATAATCAGTCCAAAGTAGATGC	[272]
PL102	TATCAGACCTAACCCAAACCTTCC	[272]

Table 3.1: Sequences of primers used in this work. (For: forward; Rev: Reverse; Underlined: Restriction site)

Modification of E. coli

E. coli was modified for mCherry constitutive expression using the λ-red system [273,274]. *E. coli* A14 electrocompetent cells prepared as previously described [275] using 10 % glycerol for the final cell resuspension. Then, they were transformed with the thermosensitive plasmid pKOBEGA, analogue to pKOBEG [276] in which *cat* gene has been substituted by bla_{amp} gene [277]. This plasmid also contains the genes *exo, bet* and *gam*, necessary for λ-red system-mediated recombination [276]. Electroporation was carried out in 2 mm cuvettes in a BTX ECM 630 Generator. Conditions: field strength: 10 kV/cm; time constant: 5 ms; voltage: 2.5 kV; resistance: 200 Ω; capacitance: 25 μF. Transformants were selected on LB agar + 50 μg/ml ampicillin (Amp; Sigma Aldrich, Saint Louis, MO) at 30 ºC for 24 h.

Then, *E. coli* A14 pKOBEGA electrocompetent cells were prepared as above and newly transformed with pMP7607 miniTn7 [278] carrying the mCherry gene and a streptomycin (Sm) resistance gene. Transformants were selected onto LB agar + 50 μg/ml Amp + 50 μg/ml Sm (Sigma Aldrich, Saint Louis, MO) and incubated at 30 ºC for 24 h. Fifty randomly chosen transformants were picked and spread onto LB agar + 50 μg/ml Sm and incubated at 42 ºC. The resulting isolate was named *E. coli* A14-mChy.

To assess the correct fluorescent signal, ten randomly picked colonies of each modified strain were diluted in a drop of deionised water on a glass slide and visualized under the fluorescence microscope.

Biofilms setup

One hundred microlitres of work cultures was grown overnight at 37 ºC in 5 ml of BHI + 10 µg/ml Cm for *L. monocytogenes* A1-gfp and LB + 50 µg/ml Sm for *E. coli* A14-mChy and subcultured overnight so as to ensure a proper growth.

Inocula preparation was performed following a modification of a protocol previously described [37]. Briefly, cultures were adjusted to Abs_{700} = 0.1 ± 0.001 in sterile phosphate buffer saline (PBS) using a Cecil3000 scanning spectrophotometer (Cecil Instruments, Cambridge, England), corresponding to a concentration of about 10^8 CFU/ml. Adjusted cultures were further diluted in sterile mTSB (TSB supplemented with 2.5 g/l glucose (Vorquímica, S.L., Vigo, Spain) and 0.6 % yeast extract (Cultimed, Barcelona, Spain)) to a final concentration of about 10^4 CFU/ml. Then, equal volumes of these adjusted cultures were mixed to obtain the inoculum for dual-species biofilms.

Biofilms were grown on 10 x 10 x 1 mm AISI 316 stainless steel (SS) coupons (Comevisa, Vigo, Spain). Pre-treatment of coupons included individual washing with industrial soap (Sutter Wash, Sutter Ibérica, S.A., Madrid), rinsing with tap water, a final rinse with deionised water and autoclaved at 121 ºC for 20 min. Coupons were then placed individually into a 24 flat-bottomed well plate and each well was inoculated with 1 ml of the corresponding culture. Plates were incubated in a humidified atmosphere at 25 ºC statically for 2 h so as to allow initial adhesion, and then in constant shaking at 100 rpm.

Biofilm formation kinetics

Samples (SS coupons) were collected at 24, 36, 48, 72 and 100 h and briefly immersed in sterile PBS in order to remove loosely attached cells before any analysis was performed.

Determination of the number of adhered viable cultivable cells (AVC)

Three different coupons were scraped using two cotton swabs pre-moistened with buffered peptone water (BPW; Cultimed, Barcelona, Spain). The swabs were then placed in 2 ml of BPW vigorously vortexed for 1 min to resuspend cells. The cell suspensions were then serially diluted in BPW and spread in duplicates onto agar plates. *Listeria*-PALCAM (Liofilchem, Italy) was used to select *L. monocytogenes* and HiCrome™ Coliform agar (Sigma Aldrich, Saint Louis, MO) with a supplement of 5 µg/ml of Vancomycin and Cefsulodine (Sigma Aldrich, Saint Louis, MO) for *E. coli* selection. Plates were incubated at 37 ºC for 24-48 h and results were expressed as the mean in log CFU/cm² of samples. The accepted limit of

detection for this and all assays involving viable cell counts was at least 25 CFU in the plate of the lowest dilution corresponding to a total of 1.70 log CFU/cm² [149].

Epifluorescence microscopy visualisation

At each sampling time, three coupons were air dried avoiding as much as possible direct light exposure. Samples were then visualised under a Leica DM6000 epifluorescence microscope using a 40x objective and 10x ocular lenses. Microscope was equipped with filter cubes L5 (Excitation 480/40) for A1-gfp and TX2 (Excitation 560/40) for A14-mChy. Images were taken using a Leica DFC365 FX controlled with Metamorph MMAF software (Molecular Devices, Sunnyvale, CA camera from 10 representative fields).

Effect of enzymatic solutions on dual-species biofilms

Enzyme solutions were prepared at concentrations 200, 400, 700 and 1000 μg/ml. Pronase (PRN, from *Streptomyces griseus*, Roche) was dissolved in 0.1 M Tris-HCl (Sigma Aldrich) buffer at pH = 7.5 ± 0.2. Cellulase (CEL, from *Aspergillus niger*, Sigma Aldrich) was dissolved in 100 mM citrate (Sigma Aldrich) buffer at pH = 6.0 ± 0.1. Finally, DNaseI (from bovine pancreas, Sigma Aldrich) was dissolved in 10 mM Tris-HCl (pH = 7.5 ± 0.2) buffer also containing 2.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$. After preparation, all solutions were filter sterilised through a 0.2 μm syringe filter (Sartorius) and kept at -20 ºC until use.

The biofilm removal action of each enzymatic solution was evaluated against 24 h biofilms. Three coupons were washed as before and then placed in a clean well. One millilitre of each enzyme solution was added and allowed to act for 30 min at 37 ºC for PRN and 32 ºC for CEL and DNaseI. Negative controls were run in parallel by adding the corresponding buffer solution without enzyme. Solutions were then gently removed by pipetting and SS coupons were subsequently washed with 1 ml of sterile PBS in order to remove residual enzyme. Determination of remaining adhered cells and visualisation of coupons was performed as described above. Results were expressed as the reduction in log CFU/cm², calculated as the mean of each replica difference in log CFU/cm² before enzymatic and after enzymatic treatment. After this, the two most effective enzymes were used in the following experiments.

Effect of benzalkonium chloride combined with either PRN or DNaseI on *L. monocytogenes-E. coli* biofilms

Benzalkonium chloride solutions (BAC; Guinama, Alboraya, Spain) were prepared in sterile deionised water at concentrations 25, 50, 100, 250 and 500 μg/ml. Each solution was applied after 30 min treatment with 400 μg/ml of either PRN or DNaseI solution against 48 h *L. monocytogenes* A1-gfp-*E. coli* A14-mChy biofilms.

Fourteen different coupons washed with sterile PBS for loosely attached cells removal were used for each enzyme series: two for the negative controls (no treatment), two for enzymes treatment without BAC (only enzyme and deionised water were applied), and two for each BAC concentration after enzymatic treatment performed as described above. In this latter case, 1.5 ml of each BAC solution was added to each coupon for a 10 min contact time at room temperature. For negative controls, buffer without enzymes and deionised water were sequentially used. Coupons were then transferred to a new well and immersed for 30 s in 1 ml of a neutralising solution (composition per litre: 10 ml of a 34 g/l KH_2PO_4 buffer (pH = 7.2); soybean lecithin: 3 g; Tween 80: 30 ml; $Na_2S_2O_3$: 5 g; L- histidine: 1 g) at room temperature followed by a final 10 s wash by immersion with sterile PBS to remove any neutraliser residues.

Following its application, neutralising solution was serially diluted in BPW and spread in duplicate onto appropriate agar media to determine the number of released viable cells (RVC) after treatments. Outcomes were expressed as mean of log CFU/ml. Microscopic visualisation and determination of the remaining attached cells were performed as described above. In the latter case, results were expressed as percentage of biofilm removal with respect to the log CFU/cm^2 obtained in control samples.

Determination of BAC effect: Calculation of lethal dose 90 (LD_{90})

LD_{90}, defined as the dose of an antimicrobial required to achieve a 90 % kill of the initial bacterial population, was used as a parameter to determine the effect of BAC on dual-species biofilms. To assess this, a modified logistic model proposed by Cabo et al. [279] was used. Logistic equations are widely recognised as suitable for describing dose-response kinetics [280,281]. Firstly, outcomes were obtained by fitting of the experimental data obtained in plate count assays, expressed in percentage of biofilm removal according to following equation (1) using the least-squares method (quasi-Newton) of the SOLVER tool of Microsoft Excel 2016:

$$BR = K\left(\frac{1}{1+0.11e^{r(LD_{90}-D)}} - \frac{1}{1+0.11e^{rLD_{90}}}\right) \quad (1)$$

where BR = biofilm removal expressed in percentage; LD_{90} = dose of BAC that removes 90% of the initial adhered population; D = dose of BAC used; K = maximum percentage of biofilm removal (asymptote); and r = specific inhibition coefficient (dimensions: inverse of the dose).

Since the equation [1] modifies the resulting Dose/Response parameters by subtracting the intercept of the original logistic equation, results were further adjusted to obtain the new K value (K'):

$$BR_{max} = K' = \lim_{D\to\infty} BR \quad (2)$$

Then, the real LD_{90} (RD_{90}) was determined according to a modification of an equation described previously [282]:

$$RD_{90} = \frac{1}{r}\ln(9 + e^{rD}) \quad (3)$$

Influence of *L. monocytogenes* accompanying species in the resistance to DNaseI-BAC treatments

Two different 48 h dual-species biofilms were used: *L. monocytogenes* A1-*E. coli* A14 and *L. monocytogenes* A1-*P. fluorescens* B52 to evaluate sequential DNaseI-BAC treatments.

400 µg/ml DNaseI + 100 µg/ml BAC treatments and plate count analysis for attached and released cells determination were performed as described above. For *P. fluorescens* selection Pseudomonas Agar Base (PAB; Liofilchem, Italy) supplemented with CFC supplement (Liofilchem, Italy) was used and incubated at 30 ºC for 48 h.

For microscopic visualisation, samples were stained using LIVE/DEAD Bacterial viability kit (Life Technologies) to distinguish total cells with undamaged membranes (green fluorescence) and damaged cells (red fluorescence). Staining solution was prepared by mixing 0.25 µl of Propidium iodide and 0.75 µl of Syto9 in 1 ml of filter sterilized deionised water. Fifty microlitres of this solution was then poured onto each coupon and allowed to dwell for 15 min in the dark. Coupons were then washed three times in 1 ml of sterile milliQ water, air dried and visualised under the epifluorescence microscope to obtain images of representative fields.

Statistical analysis

Experimental results were analysed for statistical significance using IBM SPSS Statistics 23. An independent-samples two-tailed Student's *t* test was performed to assess differences between species in the biofilm formation kinetics and the effects of BAC in RVC after PRN and DNaseI treatments. Differences among the effects of the different enzymatic treatments and treatments' effects in different dual-species biofilms were determined using a one-way ANOVA with a *post hoc* Bonferroni test. In all cases, significance was expressed at the 95 % confidence level ($\alpha = 0.05$) or greater.

In RD_{90} determination, correlation coefficient (r^2) was calculated to quantify the discrepancy between the observed experimental values and those expected according with the model.

Results

L. monocytogenes-E. coli biofilm formation kinetics on AISI 316 stainless steel

Dual-species biofilm formation dynamics are depicted in Figure 3.1. Plate count assays showed a significantly higher number of AVC in *E. coli* with respect to that obtained for *L. monocytogenes* at 24 and 100 hours of growth yielding differences of 3.11 and 2.63 log CFU/cm^2 respectively. No significance was observed among the values of the rest sampling times.

Microscopic images displayed in Figure 3.2 showed a uniform distribution of *E. coli* and *L. monocytogenes* over the coupon. In spite of this uniform distribution, at 24 h *E. coli* presented about 3 log higher AVC counts compared to *L. monocytogenes* (Figure 3.1). A tendency for aggregation was observed at 24 and 36 h yielding a final composite structure with both species intermingled therein (Figure 3.2). From this point onward, the amount of cells increased and the biofilm developed a cloud-shape structure which was maintained in the last three sampling times (Figure 3.2).

Effects of pronase, cellulase and DNaseI on the elimination of mixed biofilms formed by *L. monocytogenes-E. coli*

The effects of the application of PRN, CEL and DNaseI on the number of AVC of 24 h *L. monocytogenes – E. coli* biofilm were compared. Results were expressed in terms of log CFU/cm² reduction (Figure 3.3).

In general terms, *L. monocytogenes* was more sensitive than *E. coli* to treatments used yielding higher log reductions in most of the concentrations and enzymes used with exception of DNaseI at 1000 µg/ml where *E. coli* log reductions were significantly higher (Figure 3.3). Comparing the effects of the enzymes, higher concentrations were required to achieve a comparable log reduction of AVC in *E. coli* being especially relevant in the case of PRN and CEL (Figure 3.3).

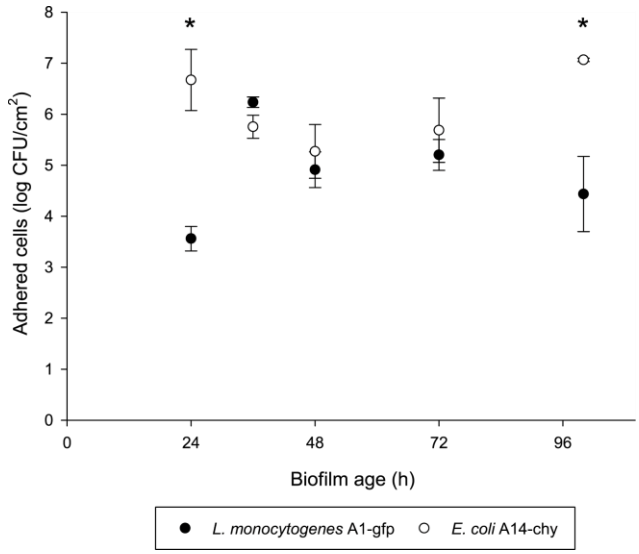

Figure 3.1: Growth dynamics of the *L. monocytogenes*-gfp-*E. coli*-mChy dual-species biofilm. Asterisks indicate statistically significant differences (α = 0.05). Error bars = SD values of each sampling time dataset (n =3).

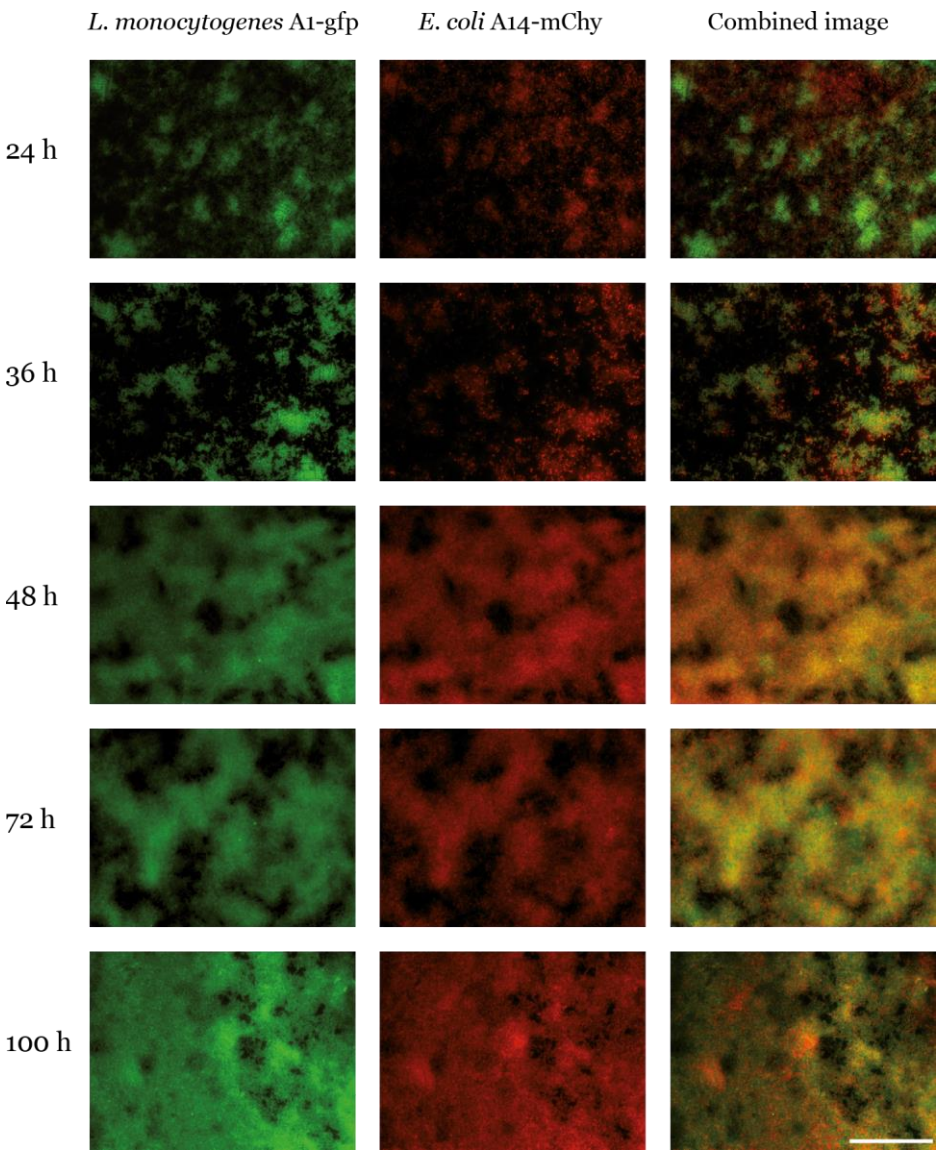

Figure 3.2: Fluorescence microscopy 40x-field images of *L. monocytogenes* A1-gfp, *E. coli* A14-mChy and combined fields in dual-species biofilm formation kinetics. Scale bar = 100 µm.

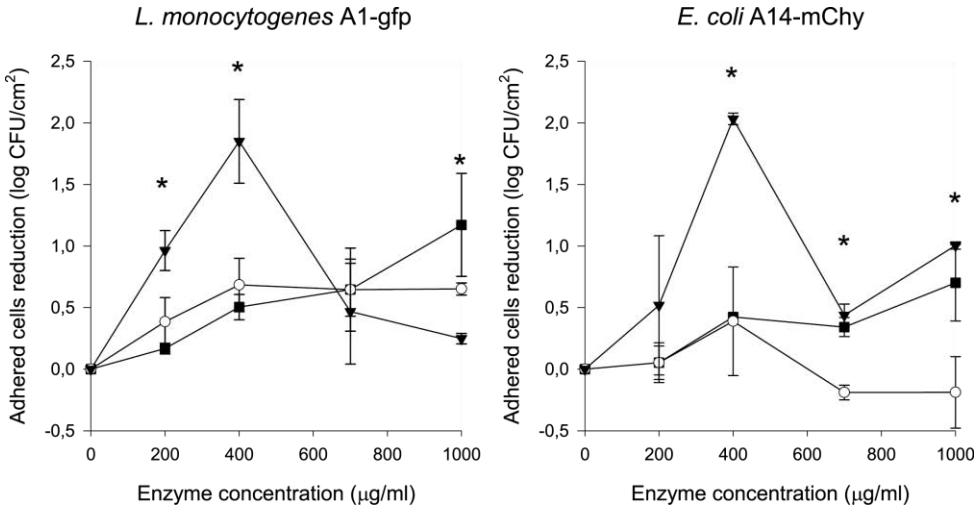

Figure 3.3: Logarithmic reductions of adhered cells obtained on 24 h *L. monocytogenes* A1-gfp-*E. coli* A14-mChy dual-species biofilms after an enzymatic treatment with PRN (■), CEL (○) or DNAseI (▼). Asterisks indicate statistically significant differences in any of the treatments at a given concentration. Error bars = SD of each dataset (n = 3).

In both species, maximum effects (about 2 log reduction) were obtained after the application of 400 μg/ml of DNaseI. In *L. monocytogenes*, log reduction value was significantly higher when treated with DNaseI as compared to treatments with PRN and CEL in 2 out of 4 concentrations tested (200 and 400 μg/ml) (Figure 3.3). On the other hand, considering *E. coli* removal by DNaseI, significance was only observed after applying a 400 μg/ml solution (Figure 3.3). In both species, application of higher concentrations of this enzyme resulted in a lower log reduction. In fact, biofilm removal decreased about 1.5 log CFU/cm² when the DNaseI concentration applied increased from 400 to 600 μg/ml.

The application of CEL resulted in lower log reductions in both species tested compared to outcomes obtained after treatment with DNaseI with exception of 1000 μg/ml against *L. monocytogenes* where CEL significantly performed better than DNaseI (Figure 3.3).

Finally, results displayed a concentration-dependent increase in log reduction in both species when PRN was used with maximum log reductions at 1000 μg/ml of 1.17 ± 0.42 and 0.70 ± 0.31 log CFU/cm² for *L. monocytogenes* and *E. coli*, respectively (Figure 3.3).

Combined effects of BAC and PRN or DNaseI solutions for 48 h *L. monocytogenes-E. coli* biofilm elimination

Maximum percentage of biofilm removal (K') and lethal doses 90 (RD_{90}) values for *L. monocytogenes* and *E. coli* were calculated according to equations [1] to [3] after sequential treatment with 400 μg/ml of either PRN or DNaseI followed by disinfection with different concentrations of BAC. In these experiments 48 h biofilms were preferred to provide a more challenging scenario to enzyme-BAC treatments.

Results showed a satisfactory fitting of experimental data ($r^2 = 0.984$) and demonstrated a higher efficacy of both combined treatments removing *L. monocytogenes* with respect to *E. coli* as indicated by K' values (Figure 3.4, Table 3.2). Whereas in the case of *L. monocytogenes* BAC performed better after DNaseI treatment compared to PRN, in *E. coli* RD_{90} values showed a higher effect of BAC after PRN treatment compared to DNaseI (Table 3.2).

	***L. monocytogenes* A1-gfp**		***E. coli* A14-chy**	
	K' (%)	BAC RD_{90} (mg/Kg)	K' (%)	BAC RD_{90} (mg/Kg)
Pronase	100.00	82.28	42.06	38.90
DNaseI	94.59	16.74	41.39	82.10

Table 3.2: Parameters obtained after fitting biofilm removal experimental data to equations 1 to 3. Maximum percentage of reduction (K') and real lethal dose 90 (RD_{90}) values obtained due to BAC action after a single application of 400 μg/ml solution of either PRN or DNaseI.

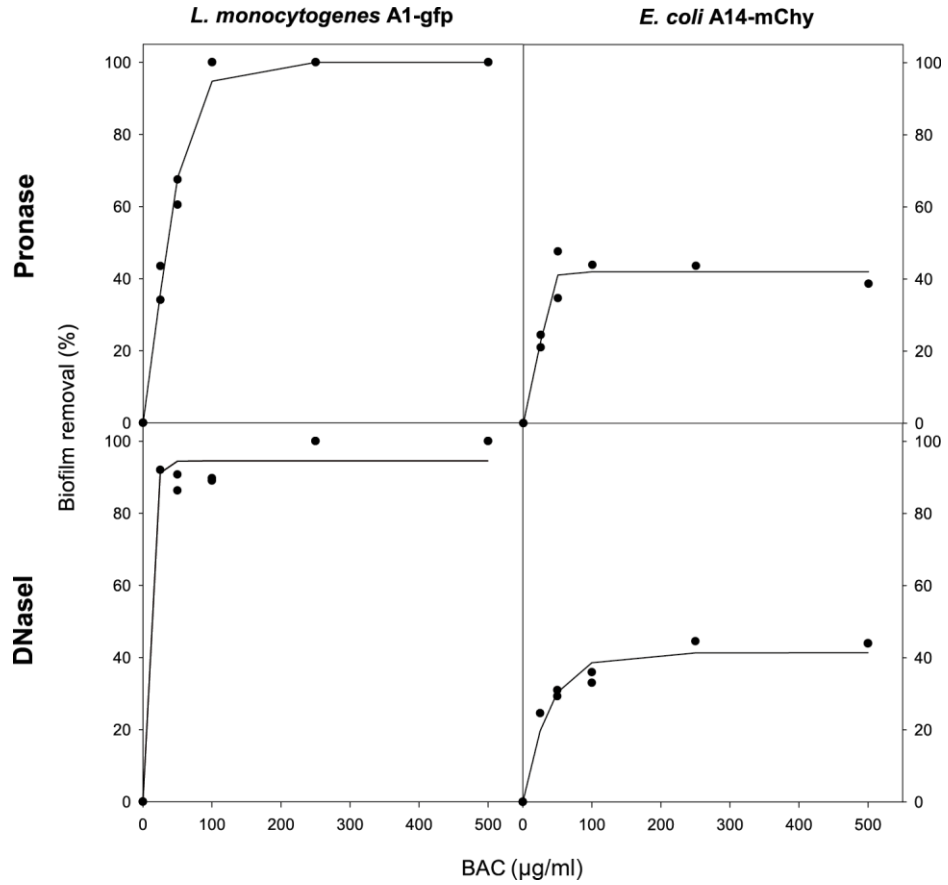

Figure 3.4: Lethal dose 90. Fit of biofilm removal values against *L. monocytogenes-E. coli* mixed biofilms obtained after the application of PRN-BAC or DNaseI-BAC treatments according to equation 1.

Outcomes of RVC (*L. monocytogenes* and *E. coli*) demonstrated a high level of cell dispersion after the application of sequential enzyme-BAC treatments, with values ranging from about 3 to 5 log CFU/ml (Figure 3.5). Student's *t* test showed significance ($P < 0.05$) between treatments at BAC concentrations of 25, 50 µg/ml in *L. monocytogenes* and 25 and 100 µg/ml in *E. coli*, with a general tendency to lower RVC values as the BAC concentration increased (Figure 3.5). If only RVC values of *L. monocytogenes* are considered, is important to highlight that no viable cells were detected after ≥ 100 µg/ml BAC neither in PRN nor in DNaseI-treated samples (Figure 3.5).

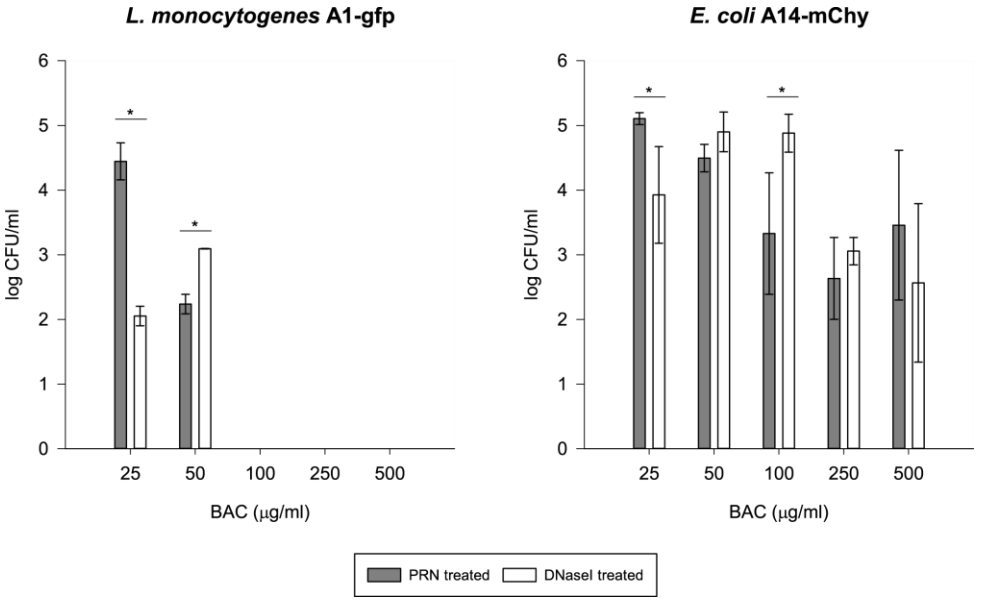

Figure 3.5: Released viable cells of *L. monocytogenes* (left) and *E. coli* (right) coming from 48 h dual-species biofilms after the application of different BAC solutions following a single dose of a 400 µg/ml solution of pronase (filled bars) or DNaseI (void bars). Error bars = SD of each sample set. Asterisks indicate statistically significant differences between enzymatic treatments at each BAC concentration (α = 0.05).

Role of the accompanying species (*E. coli, P. fluorescens*) in the adhesion and resistance of *L. monocytogenes* to DNaseI and DNaseI-BAC treatments in dual-species biofilms

Cell counts demonstrated that *L. monocytogenes* was able to achieve significant higher number of adhered cells in presence of *P. fluorescens* compared to co-culture with *E. coli* reaching values of 7.23 ± 0.04 and 5.48 ± 0.05 log CFU/cm², respectively (Figs. 6A, B).

The application of a 400 µg/ml DNaseI solution gave higher *L. monocytogenes* log reduction values in co-culture with *E. coli* (2.47 log CFU/cm²) compared to that obtained in co-culture with *P. fluorescens* (0.58 log CFU/cm²) (Figure 3.6 A , B). Combined treatments (400 µg/ml DNaseI + by 100 µg/ml BAC) also produced a significant reduction in *L. monocytogenes* compared to controls, being of 3.24 and 2.83 log CFU/cm² in co-culture with *E. coli* and *P. fluorescens*, respectively (Figure 3.6 A, B). Nevertheless, if only BAC effects on *L. monocytogenes* are considered, by comparing the log reductions of DNaseI alone and

DNaseI-BAC treatments, these were higher in *L. monocytogenes-P. fluorescens* biofilms (2.55 log CFU/cm²) compared to *L. monocytogenes-E. coli* samples (0.77 log CFU/cm²) (Figure 3.6 A, B).

L. monocytogenes RVC after DNaseI-BAC treatment did not present significant differences comparing both dual-species biofilms (4.23 ± 0.41 log CFU/ml in *L. monocytogenes-P. fluorescens* and 3.65 ± 0.41 log CFU/ml in *L. monocytogenes-E. coli*). Notice that *E. coli* presented a significant higher number of RVC (6.22 ± 0.09 log CFU/ml) after DNaseI-BAC combined treatment compared with *P. fluorescens* and *L. monocytogenes* in both dual-species biofilms (Figure 3.6 C, D).

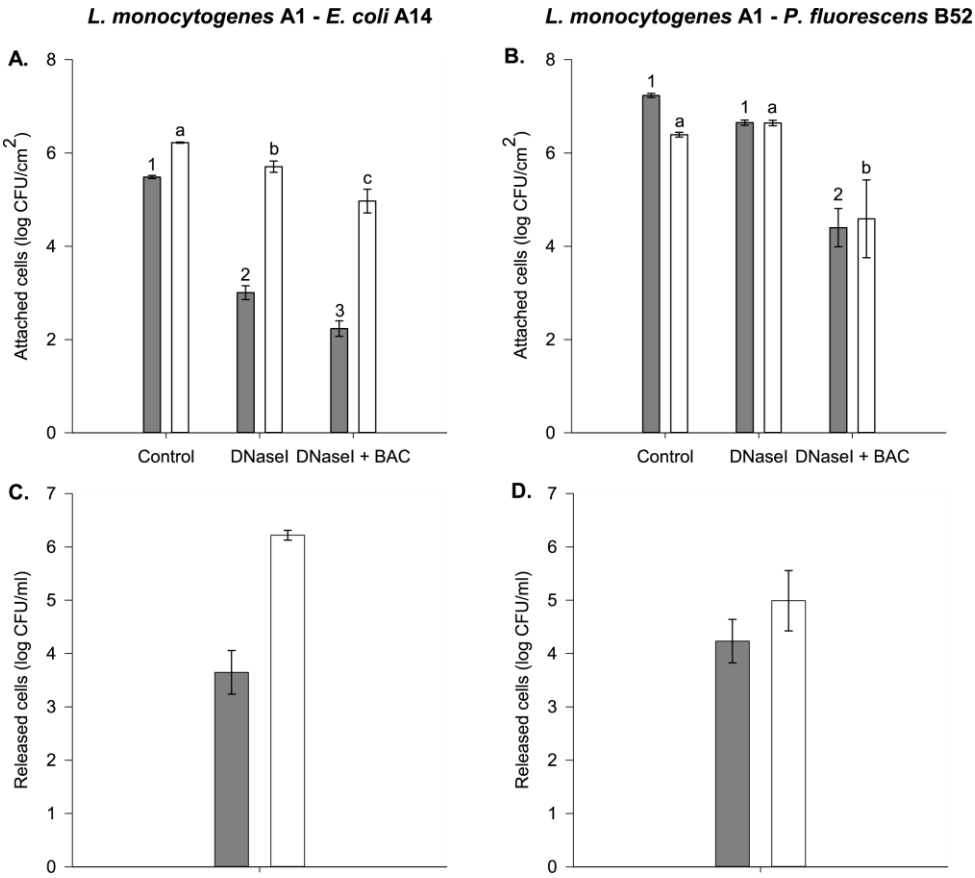

Figure 3.6: Sensitivity of 48 h *L. monocytogenes* A1 dual-species biofilms to the application of DNaseI and DNaseI-BAC. A, B: Number of viable attached cells of *L. monocytogenes* (filled bars) and of *E. coli* A14 (A) and *P. fluorescens* B52 (B) (void bars). For each species separately, bars with different number

or letter indicate significant differences (α = 0.05). C, D: Number of viable released cells after the DNaseI–BAC treatment. Error bars represent the standard deviation of each sample set (n = 3).

Microscopic analysis showed that both biofilms presented remarkable differences in their 2D-morphologies (Figure 3.7). While *L. monocytogenes-E. coli* biofilms showed a reticular distribution in all biofilm, *L. monocytogenes-P. fluorescens* biofilms were characterised by the presence of microcolonies surrounded by small cell groups. These microcolonies presented a local accumulation of damaged cells compared to the rest of the sample, as observed by a higher red signal in the central part of the microcolony. The same microcolony formation tendency was also observed in our laboratory with other *L. monocytogenes* strains when co-cultured with *P. fluorescens* B52 (data not shown).

Sequential DNaseI-BAC treatments produced a significant increase of the red cell signal especially in *L. monocytogenes-E. coli* samples pointing out the BAC killing effects. Besides, noticeable structural changes were observed in samples of both dual-species biofilms, especially in *L. monocytogenes-P. fluorescens* biofilms in which the cellular groups surrounding the microcolonies were substituted by sparsely distributed cells (Figure 3.7).

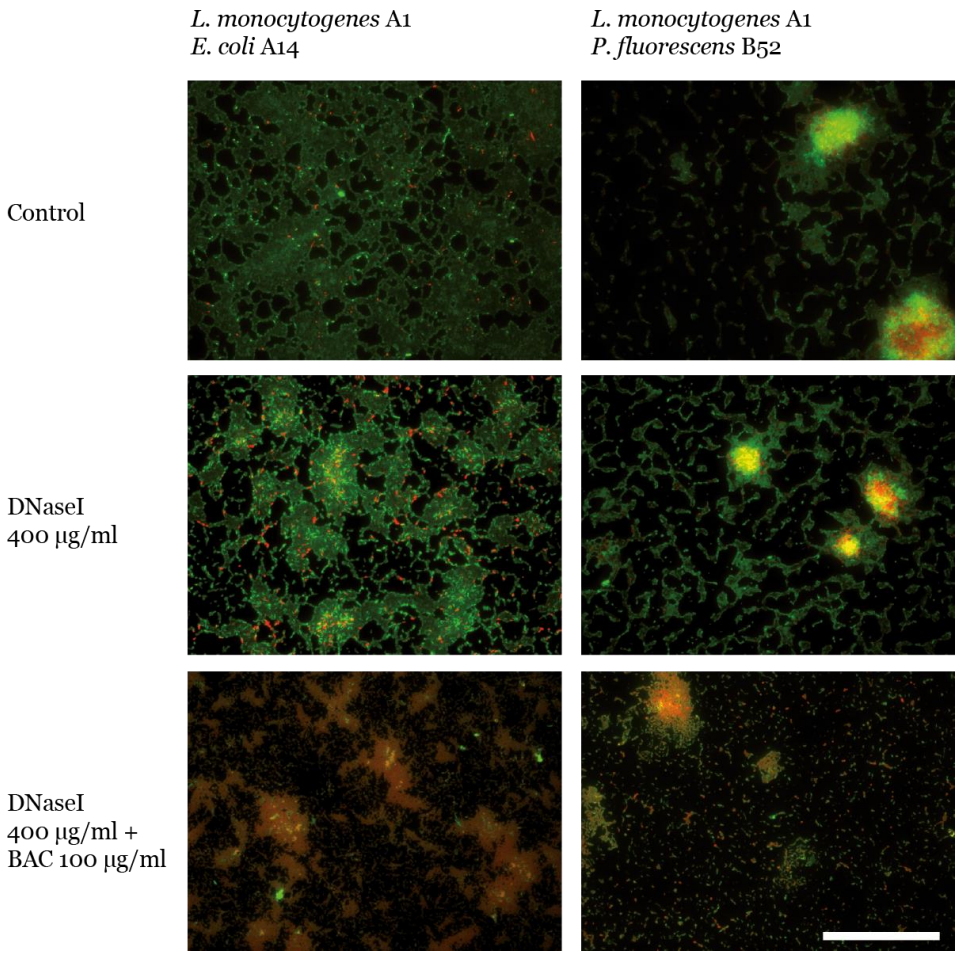

Figure 3.7: Fluorescence microscopy 40x-field images for comparison of the effects of DNaseI-BAC combined treatments in two different 48 h *L. monocytogenes* dual-species biofilms. Scale bar = 100 µm.

Discussion

Biofilm kinetics of the *L. monocytogenes* A1-gfp-*E. coli* A14-chy biofilm showed a typical biofilm fit-curve with minor fluctuations (Figure 3.1). *E. coli* viable counts were significantly higher than *L. monocytogenes* at 24 and 100 h. Differences in AVC counts at 24 h could be attributed to a better initial adhesion of *E. coli* compared with *L. monocytogenes* as previously reported [283]. However, AVC values of both species were equilibrated at 36, 48 and 72 hours (Figure 3.1).

Microscopic images showed a uniform distribution of *E. coli* and *L. monocytogenes* over the coupon despite the differences up to 3 log present between these species at 24 h (Figs. 1, 2). Almeida et al. reported that in 48 h *L. monocytogenes-E. coli* biofilms grown on stainless steel and plastic, species presented this sort of uniform distribution with *E. coli* being present in higher numbers [146]. The fact that green fluorescence, corresponding to *L. monocytogenes* cells, was similar to red despite viable counts (Figure 3.1), could have been caused in part because a fraction of this green signal was emitted by cells in the viable but non culturable (VBNC) state. Previous authors have observed that 24 h-old *L. monocytogenes* biofilms present a part of VBNC [284]. In such condition, GFP remains totally functional and fluoresces even though cells are not able to grow in solid media [285,286].

Enzymes have been previously used as a biofilm removal strategy due to their specificity and their low environmental impact [175,181,287]. In this work, comparison between the effects of cellulase (CEL), DNaseI and pronase (PRN) demonstrated a maximum effect of a 400 μg/ml of DNaseI solution reducing about 2 log CFU/cm² the number of AVC in *L. monocytogenes* and *E. coli* (Figure 3.3). This reduction was followed by that produced by PRN and CEL, despite no broad differences were observed between these two (Figure 3.3).

It has been reported that extracellular DNA (eDNA) is present in considerable amounts of the extracellular matrix and considered as a requisite for biofilm formation in *L. monocytogenes* [118] as well as in other Gram-positives [288,289]. Hence, DNaseI has been proposed as an antibiofilm enzyme cleaving eDNA and thus interfering in biofilm development. As an example, Harmsen et al. [118] observed that 100 μg/ml DNaseI solution at 37 ºC, completely prevented *L. monocytogenes* EGDe biofilm formation if applied up to 24 h after strain inoculation and, from that point onwards, DNaseI antibiofilm capacity was reduced. In other Gram-positives such as *S. aureus,* 1 h contact time at 37 ºC of a 100 μg/ml DNaseI solution significantly reduced the biomass of 24 h biofilms grown on polystyrene plates [290]. Despite this previously reported data, no complete removal with DNaseI was achieved among the experiments performed in this work. This could be due to the application

of a more realistic time of action (30 min) or to the biofilm age, which could affect DNaseI biofilm removal activity [118]. Experimental data also showed an inverted effect of DNaseI at concentrations higher that 400 μg/ml (i.e. higher doses produced a lower log reduction), both in *L. monocytogenes* and *E. coli* (Figure 3.3). Nguyen and Burrows [117] demonstrated a similar enzymatic stimulatory effect on planktonic *L. monocytogenes* cells in which the more proteinase K present in the culture, the more stimulated its growth was. Focusing in our experimental approach, these effects in the number of cells in the planktonic state, could have had eventually provoked an upturn in the number of cells adhered to the biofilm detected in AVC assays.

Proteases have also been proved to be effective in removing biofilms. In this line, Nguyen and Burrows [117] demonstrated that the addition of 100 μg/ml of proteinase K for 24 h is able to disperse 72 h *L. monocytogenes* biofilms grown on polystyrene up to undetectable levels. In *S. aureus* it has been recently reported that active proteases remove biofilms formed in polystyrene plates [208]. However, PRN effects against *L. monocytogenes* were lower than expected compared with DNaseI considering the proteinaceous nature of *L. monocytogenes* biofilm matrix [116,117] even though it has been demonstrated that teichoic acids are also present [119].

Previous investigations have reported that interspecies interactions that take place within multi-species biofilms significantly modify the matrix composition if compared with monocultures [142,291]. This differential composition can affect, among others, the efficacy of enzymes as well as several antimicrobial compounds [291]. In our particular case, the dominance of *E.coli* in 24 h biofilms (Figure 3.1) could have given rise to a matrix with a higher polysaccharide content as proposed for most Gram-negative bacteria [292]. Nevertheless, CEL showed the lowest effects against *L. monocytogenes-E. coli* biofilms perhaps because polysaccharide constituents interacted among themselves and among other molecules present thus concealing enzyme targets or they simply lack on glucose-glucose bonds susceptible to cleavage by CEL specific ß (1→4) endoglucanase activity.

Considering the aforementioned results, it is logical to think that the use of dual-species biofilms represents a more challenging environment for biofilm-degrading enzymes due to a higher matrix complexity. Thus, the idea of a combination of enzymes would be an interesting option to be considered for proper biofilm removal [44,181] especially when dealing with Gram-negatives such as *Pseudomonas* sp. [208]. Efficacy of enzymatic mixtures have been previously reported by Orgaz et al. [178] using proteinase, cellulase, pectinesterase, pectin lyase and alginate lyase derived from fungal cultures against 24-hour-

old *P. fluorescens* B52 biofilms on glass achieving removal values up to an 84 % of the total biomass.

In any case, enzymatic solutions show only dispersing-but-not-killing effect as previously reported [117]. As a consequence, enzyme based disinfection may need to be performed in combination with biocides that are able to kill the cells avoiding the dispersion of live cells released from the biofilm [181,208].

In food related premises, RVC could provoke a pathogen thus enhancing the formation of new reservoirs and increasing the probability of product contamination. Also, pathogens could be easily spread through rinse after disinfection via water or aerosols produced [293] or by means of typical cleaning tools such as sponges or wipes [294]. Therefore, controlling RVC after cleaning and disinfection treatments appears to be as an interesting topic to consider for further investigation.

Enzyme-BAC combined treatments showed a differential effect on *L. monocytogenes-E. coli* biofilms depending on the species. More specifically, BAC performed better against *L. monocytogenes* when preceded by DNaseI whereas removal of *E. coli* from the coupon was higher after PRN-BAC treatment (Table 3.2, Figure 3.4). In *L. monocytogenes* the lower BAC RD_{90} values obtained after DNaseI treatment indicated that despite proteins are considered the main fraction in *L. monocytogenes* biofilm matrix [116], eDNA degradation by DNaseI provokes a higher decrease in *L. monocytogenes* AVC counts thus confirming the key role of eDNA to maintain already formed biofilms [117,118]. This biofilm-dispersing capacity of DNaseI to facilitate BAC access into the biofilm is especially relevant in *L. monocytogenes*-carrying biofilms as this bacterium is usually located in the bottom layers [146] . In *E. coli*, a better performance of BAC after enzymatic dwelling was also observed but to a lesser extent (Figure 3.4). This can be attributed to its intrinsic higher resistance to QACs [201,295] and also because of the possible presence of protective colanic acid capsules [296].

It is important to remark the fact that BAC effects against 48 h samples were different depending on the species (Figure 3.4) whereas in 24 h biofilms DNaseI was the most efficient enzyme in both species of the mixed biofilms (Figure 3.3). This points out that the biofilm matrix varies its molecular composition along time. So, if proper enzyme-based biofilm cleaning strategies are intended to be designed it is important to determine the constituents (proteins, eDNA and polysaccharides) of the matrix of the target sessile community.

A release of live cells of both species is observed from biofilms after PRN-BAC or DNaseI-BAC treatments, especially at low BAC concentrations (Figure 3.6). Pathogen dispersal after sanitation is a factor to take into account in cleaning and disinfection methodologies

[117,175]. This fact can be minimised by using appropriate effective concentrations of disinfectants (e.g. BAC) after dispersing agents, enzymes in this particular case, to avoid dissemination of live cells in adjacent areas after biofilm removal.

Another important issue to be assessed in dual-species biofilms of *L. monocytogenes* is the role of the accompanying species. Significant differences were observed in the *L. monocytogenes* AVC counts, as well as in the effect of the enzyme and enzyme-BAC treatment depending on the accompanying bacterium (Figure 3.6). Regarding the first, a higher number of *L. monocytogenes* A1 cells was attached to stainless steel after 48 h in presence of *P. fluorescens* respecting to *E. coli*, probably due to an entrapping of the *L. monocytogenes* into the polymeric matrix secreted by the *P. fluorescens*. Morphological features agreed with previously reported data in which *L. monocytogenes-E.coli* biofilms appeared as uniform layers [146] whereas *L. monocytogenes-P. fluorescens* were characterised by local microcolony formation surrounded by smaller biofilm aggregates randomly distributed (Figure 3.7) [144].

DNaseI produced a significant decrease of *L. monocytogenes* only in the mixed biofilm with presence of *E. coli*, probably because matrix composition differently affected its diffusion and effectiveness (Figure 3.6) [297]. Nevertheless, the application of BAC against *L. monocytogenes* was more effective when co-cultured with *P. fluorescens* despite the latter is considered a strong biofilm former (Figure 3.6) [157,268].

In summary, in this work the effectiveness of treatments with an enzyme solution alone and combined with a BAC dose on *L. monocytogenes* dual-species biofilms was demonstrated. In addition to this, results demonstrated that the removal efficacy of a combined enzyme-BAC treatment against mixed biofilms depends not only on the enzyme chosen but also on the biofilm species composition. Following this idea, for proper biofilm removal in food related surfaces as well as in others capable of harbour bacterial biofilms, customised treatments depending on the species composition should be considered when developing new cleaning and disinfection methodologies. This would be intended not only to impede biofilm formation but also to significantly remove already present structures while minimising the amount of live cells released.

4

Pronase-Benzalkonium chloride combined treatments against *L. monocytogenes*-carrying late-stage biofilms

Introduction

This part of the thesis goes one step further in the assessment of the biofilm-removing properties of combined treatments. Specifically, this study aimed to study the effectiveness of combining PRN and BAC for removal of late-stage *L. monocytogenes* dual-species biofilms grown on stainless steel, mimicking real industrial conditions where biofilms are formed after long periods. PRN was selected since it has been reported that *L. monocytogenes* biofilm matrix is mainly constituted by proteins [117,298] and gave the highest maximum biofilm removal percentages in the assays performed in the previous chapter (chapter 3) . Enzymes solutions were applied at room temperature to further simulate realistic environmental conditions. The assessment of the effects was performed combining microscopy and image analysis with classical microbiology methods.

Methods

Bacterial strains

Two different consortia were used. The first was formed by *L. monocytogenes* A1-*E. coli* A14, both isolated from a fish processing plant in a previous survey [37]. The second one was formed by a strain of *L. monocytogenes* G1, isolated from a cheese processing plant, kindly provided by Dr. Luisa Brito [299] and *Pseudomonas fluorescens* B52, as one of the species commonly isolated in dairy industry, was kindly provided by Dr. Carmen San José [268]. These consortia were chosen based on their relevance in fish and dairy industries, and their capability to form dual-species biofilms. From now on consortia used will be referred as fish industry and dairy industry consortia for A1-A14 and G1-B52 biofilms, respectively.

In all cases, stock cultures were maintained at -80 ºC in Brain-Heart infusion broth (BHI; Biolife, Italy) containing 50% glycerol 1:1 (v/v) mixed. Work cultures were kept at -20 ºC in Tripticase Soy Broth (TSB; Cultimed, Barcelona, Spain) containing 50% glycerol 1:1 (v/v) mixed.

Setup of dual-species biofilms

100 µl of work cultures was cultured overnight in 5 ml sterile TSB at 37 ºC for *L. monocytogenes* and *E. coli* and 25 ºC for *P. fluorescens* and subcultured once so as to ensure a proper activation.

Inocula preparation was performed as follows: briefly, Abs_{700} of cultures was adjusted to 0.1 ±0.001 in sterile phosphate buffer saline (PBS), corresponding to a bacterial concentration of about 10^8CFU/ml according to previous calibrations. Adjusted cultures were further diluted in sterile mTSB (TSB (Cultimed, Barcelona, Spain) supplemented with 2.5 g/l glucose (Vorquímica, S.L., Vigo, Spain) and 0.6 % yeast extract (Cultimed, Barcelona, Spain)) until obtaining a final concentration of about 10^4 CFU/ml and 1:1 mixed.

Biofilms were grown on 10x10x1 mm AISI 316 stainless steel (SS) coupons (Comevisa, Vigo, Spain). Pre-treatment of coupons included individual washing with industrial soap to remove grease residues, thoroughly rinsing with tap water with a final rise with deionized water and sterilized by autoclaving them at 121 ºC for 20 min. Coupons were individually placed into a 24 flat-bottomed well plate and each well was inoculated with 1 ml of the corresponding culture. Plates were incubated in a humidified atmosphere at 25 ºC statically for 2 h for initial adhesion, and then in constant shaking at 100 rpm.

In all samplings, coupons were aseptically collected and briefly immersed in 1 ml sterile PBS to remove loosely attached cells before any assay was performed.

Plate count assays

Attached viable cultivable cells (AVC) were harvested from coupons by scrapping using two sterile cotton swabs moistened in sterile buffered peptone water (BPW). Swabs were then suspended in 2 ml of BPW and vortexed vigorously for 1 min in order to release cells, serially diluted in BPW and spread onto agar plates for number of AVC determination. In reproducibility, repeatability and biofilm formation kinetics assays AVC values were expressed in CFU/cm^2 whereas in the PRN-BAC assays they were expressed in log CFU/cm^2.

The number of released viable cells (RVC) into neutraliser was determined after treatments performing direct serial dilution of the neutralising solution in BPW and spread onto appropriate solid media. Outcomes were expressed in log CFU/ml.

In all cases, *Listeria*-PALCAM (Liofilchem, Italy) was used to select *L. monocytogenes,* Chromogenic *Escherichia coli* agar (Cultimed, Barcelona, Spain) with a supplement of 5 mg/l of Vancomycin and Cefsulodine (Sigma- Aldrich) to isolate *E. coli* and *Pseudomonas* agar base (PAB) with CFC supplement (Liofilchem, Italy) for *P. fluorescens*. Chromogenic agar and PALCAM plates were incubated at 37 ºC whereas 25 ºC was preferred for PAB for 24-48 h.

Fluorescence microscopy and image analysis assays

After applying the corresponding treatment, coupons were washed by immersion in 1 ml sterile PBS for 10 s. Samples were then stained using FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Life Technologies). Staining solution contained 0.75 µl Syto9 and 0.25 µl propidium iodide in 1 ml of filter sterilised deionised water. Fifty microlitres of this solution were used for sample staining and allowed to remain 15 min in the dark. Then, coupons were washed three times in 1 ml of sterile MilliQ water. Samples were air dried and visualised in a Leica 6000DM epifluorescence microscope using 10x ocular lenses and 40x objective.

From each sample, a randomly chosen field was considered as start point to automatically acquire images using a Leica DFC365 FX camera. Each image set was composed by 3 mosaics of five 12-bit images covering a total surface of 1.92×10^6 µm². Image analysis was then performed using the Integrated Morphometry Analysis (IMA) module of the Metamorph MMAF software (Molecular Devices) in order to determine the occupied area (OA) by undamaged (green) cells.

Results of image analysis in biofilm formation were expressed as the percentage of occupied area (POA) of the mosaic whilst in repeatability, reproducibility and enzyme-disinfectant experiments outcomes of OA were expressed in mm².

Repeatability and reproducibility assays

Repeatability is defined as the ability of a particular method to generate the same outcomes over a short period of time under the same conditions [300]. It was obtained by calculating the intra-assay variation among images (3 x 25-field mosaics) and plate counts of 9 different coupons of A1-A14 and G1-B52 samples harvested at 24 and 168 h.

Reproducibility is defined as the variation values obtained among analysts [300]. It was obtained by comparing the values of the occupied area by the undamaged cells of a 24 h A1-A14 biofilm calculated by 3 analysts with different level of expertise in microscopy and image analysis. Data sets comprised the images of 9 different coupons, each one processed as described above.

Accuracy of both methods was evaluated by determining the coefficients of variation (CV) of the values of AVC obtained by plate count and POA obtained by image analysis. Taking as a reference the quantitation limit in analytical chemistry, a CV value ≤ 20% was considered as acceptable measurements whereas a value > 20% was considered as low precision values which can only be used with descriptive purposes [301].

Dual-species biofilm formation kinetics

Samples of both consortia were collected at 24, 48, 72, 96 and 168 h of incubation. In each sampling time, 3 coupons were used for plate count and 3 more for microscopy analysis as described above. For comparison of the accuracy of both techniques, the coefficients of variation (CV) were also calculated dividing the standard deviation by the mean of the sample set.

Effects of sequential pronase-benzalkonium chloride treatments on 168 h A1-A14 biofilms.

Preparation of the solutions

Pronase (PRN; from *Streptomyces griseus*, Roche) was prepared at concentrations listed in Table 1 using 0.1 M Tris-HCl (Sigma Aldrich) buffer at pH = 7.5 ± 0.2 and then filter sterilised through a 0.2 μm pore diameter syringe filter (Sartorius). Solutions were kept at -20 ºC until use. Benzalkonium chloride (BAC; Guinama, Alboraya) was prepared at concentrations listed in Table 1 dissolving the stock solution in sterile distilled water according to the concentrations needed, and kept at 4 ºC until use.

Neutralising solution was prepared with following composition per litre: 10 ml of a 34 g/l KH_2PO_4 solution adjusted to pH = 7.2 with $NaOH_{(aq)}$, 3 g soy lecithin, 5 g $Na_2S_2O_3$, 1 g L-histidine, 30 ml Tween 80 and deionised water [230]. This solution was sterilised by autoclaving at 121 ºC for 20 min and kept at 4 ºC until use.

Experimental design

A first order factorial design [302,303] with 4 combinations of variables and 4 replicates in the centre of the domain was carried out. The independent variables were the concentration of PRN and the concentration of BAC. Natural and encoded values used are listed in Table 4.1. This sort of design permits to obtain information about various factors without increasing the size of the assay. Besides, they are useful to quantify individual and synergistic effects among different treatments in a given experimental ambit [302,303].

Encoded values	Natural values	
	PRN (IU/l)	BAC (mg/l)
[-1,-1]	700	50
[-1,1]	700	2000
[1,-1]	7000	50
[1,1]	7000	2000
[0,0]	3850	1025

Table 4.1: PRN and BAC concentrations and their corresponding encoded values used in the factorial experimental approach.

PRN-BAC combinations (Table 4.1) were sequentially applied on samples. Briefly, after washing the coupons, 1 ml of each enzymatic solution was applied for 1 h contact time statically at room temperature. Then, 1.5 ml of the corresponding BAC concentration was allowed to dwell for 10 min at room temperature. Treated coupons were then transferred to new a well containing 1 ml of neutralising solution and immersed for 30 s. Untreated biofilm samples were used as controls. Finally, quantification of AVC, RVC and the OA by undamaged attached cells were carried out as described above.

Statistical analysis

For the factorial design, least-squares method (quasi-Newton) was used for model fits to experimental data. Significance of the coefficients obtained in the empirical equation was determined by a Student's *t* test ($\alpha = 0.05$). A Fisher test ($\alpha = 0.05$) was employed to test the consistency of the models.

In POA, AVC and RVC determinations, a one-way ANOVA with a *post–hoc* Bonferroni test was used. Significance was expressed at the 95 % confidence level ($\alpha = 0.05$) or greater.

Results

Repeatability and reproducibility of OA and agar plate count assays in quantification of *L. monocytogenes* mixed-species biofilm formation

Two different *L. monocytogenes*-carrying consortia were used in this study: *L. monocytogenes* A1-*E. coli* A14, as representative of *L. monocytogenes* associations potentially present in fish industry, and *L. monocytogenes* G1-*P. fluorescens* B52, as representative of *L. monocytogenes* associations present in dairy industry.

Repeatability assays showed that in both consortia data dispersion was larger in AVC values compared with OA (Figure 4.1). If each consortium is individually compared, AVC dispersion was higher in A1-A14 whereas in OA, G1-B52 samples presented less dispersed values. In all cases, interquartile range (IQR) values regarding agar plating gave higher values compared to those obtained in OA determinations. So, OA determination could be considered repeatable when comparing with the determination of the number of adhered cells by the classical method of swabbing and plate count.

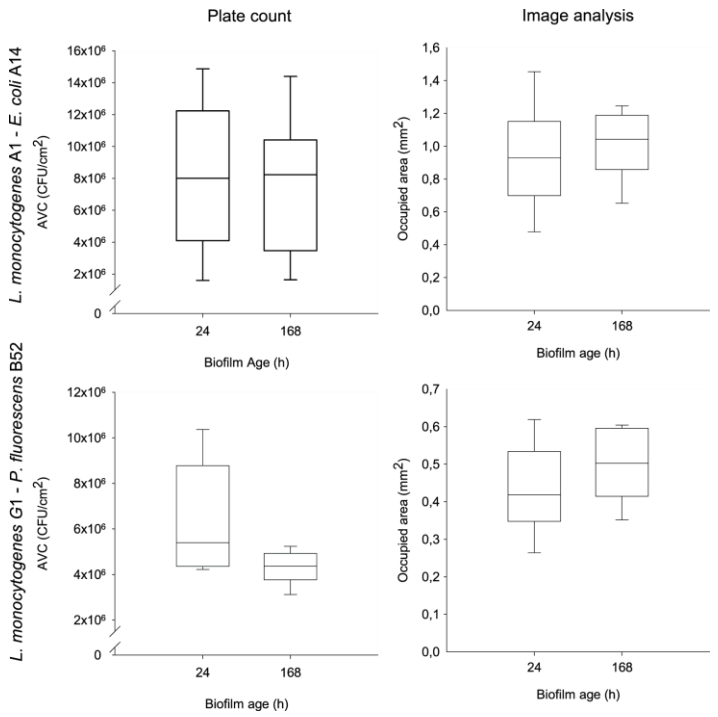

Figure 4.1: Boxplot and whiskers diagrams showing the distribution of values obtained in repeatability assays in fish and dairy industry consortia (n = 9). Bottom, middle and top lines represent Q1, median and Q3, respectively.

Blind assays to determine the reproducibility of the image analyses were carried out. In an initial phase, occupied area values of 24 h A1-A14 biofilms consortium were independently calculated by three different technicians (Figure 4.2). Analyst 1 was a technician who had performed some image analyses previously, analyst 2 an experienced technician and analyst 3 an untrained technician with basic knowledge in microscopy image analysis. Results showed that OA values obtained by analyst 2 were significantly higher than those obtained by analysts 1 and 3. Besides, it was observed that outcomes obtained by analyst 3 presented the highest dispersion. In a second phase, analyst 3 was in-house trained by analyst 2 in image analysis during a period of about a month. OA values of the same images set were re-calculated by analyst 2 and outcomes were compared again. As observed in Figure 4.2, values of occupied area obtained after training were not significantly different to those obtained by analyst 2 although a high dispersion was still observed.

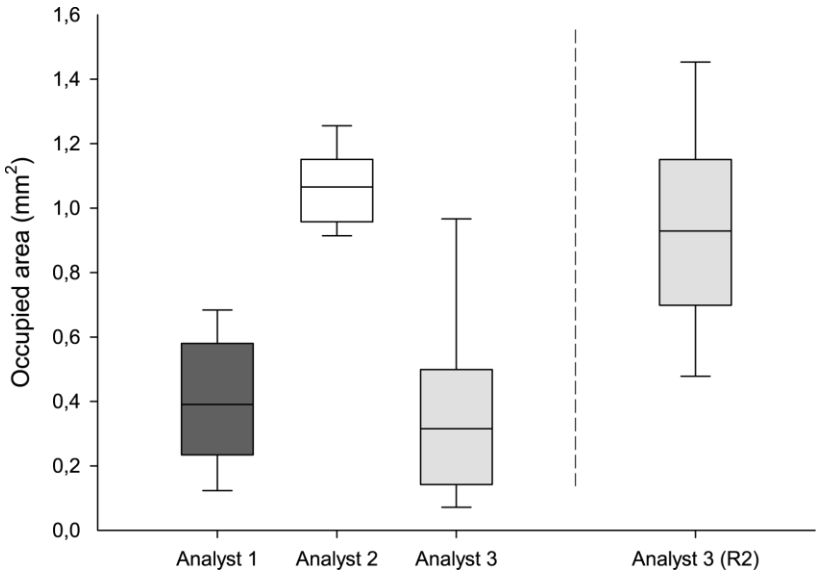

Figure 4.2: Boxplot and whiskers diagrams showing the distribution of values obtained by different analysts in reproducibility assays (n = 9). Box named as *Analyst 3 (R2)* corresponds to the values obtained by analyst 3 after in-house training.

Combination of OA and plate count assays for determination of *L. monocytogenes* mixed-species biofilm formation dynamics

Once the reproducibility and repeatability of the method were checked, it was used to assess the formation of two different *L. monocytogenes* mixed-species biofilms.

In A1-A14 samples, AVC values showed an increasing tendency up to a peak at 72 h of about 1.21 x 10^8 CFU/cm² followed by a decrease in the last two times of sampling reaching a minimum of about 1.91 x 10^6 CFU/cm² at 168 h (Figure 4.3). POA dynamics showed also some fluctuations although not as accused as in AVC. Besides, the 72 h peak observed in AVC was not present in POA quantification.

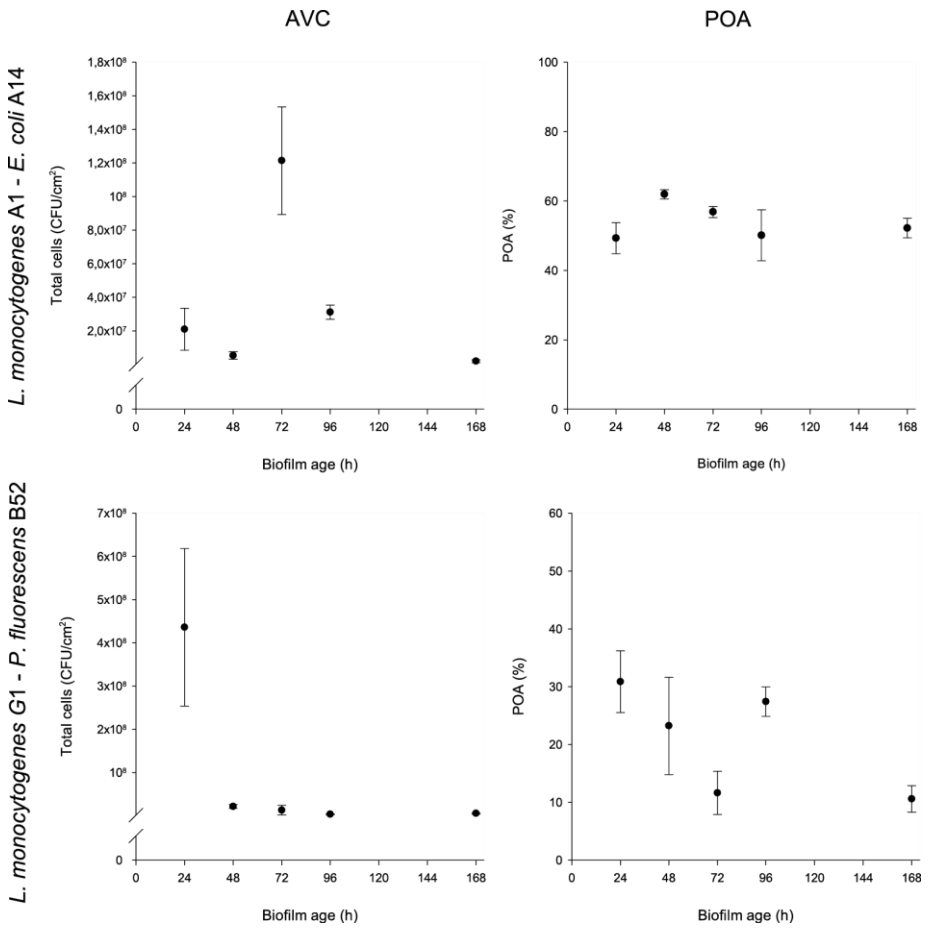

Figure 4.3: Representation of AVC and POA values of fish and dairy industry consortia obtained in biofilm formation kinetics. Error bars represent SD values (n = 3, for each assay).

Analysis of the microscopic mosaics showed a clear predominance of undamaged (green) cell population of the sample (Figure 4.4). However, green and red cells were equally distributed along the SS surface forming a uniform surface in which cluster formation started to be evident at 48 h (Figure 4.4). Clusters present at 72 h presented an intense fluorescence signal suggesting that these structures were formed by superposition of cellular layers (Figure 4.4). From that point onwards, these cellular aggregates became denser and more packed up to 168 h.

In G1-B52 samples, significance ($P < 0.05$) corresponding to maximum AVC values were obtained at 24 h of growth (about 4.36×10^8 CFU/cm^2). From that point, AVC outcomes decreased in the following sample times until 96 h where the minimum AVC value was obtained (about 8.12×10^5 CFU/cm^2) (Figure 4.3). No statistically significant differences were observed in AVC values between 48 to 168 h. POA values also displayed similar dynamics where a gradual decrease from 24 h (POA = 30.86 %) until 72 h (POA = 11.64 %) occurred (Figure 4.3). Microscopy images gave evidence of cluster formation where high-density groups of red-fluorescent cells were present surrounded by a network of green-fluorescent cells (Figure 4.4).

Generally, acceptable CV values were obtained in occupied area when analysing fish and dairy consortia. More specifically, results of POA in A1-A14 samples rendered CV values below 10% in all experimental times except at 96 h (21.17%). In plate counts, CV values were above 20% in all sample times but at 96 h (13.55%) (Appendix, Figure S1). In G1-B52 biofilms, CV values obtained in POA were around 20% at 24, 48 and 168 h whereas in plate counts, CV values were all above 20 % excepting at 96 h. Besides, even though above the threshold, CV value at 168 h was still around the threshold value (Appendix, Figure S1).

Taking all these results together indicate that, numerically, the occupied area can be considered a reliable 2D-structural parameter to quantify the dynamics of *L. monocytogenes* mixed-species biofilm formation. Besides, it provides easy-to-interpret biological information regarding morphology and distribution of the biofilm along the surface.

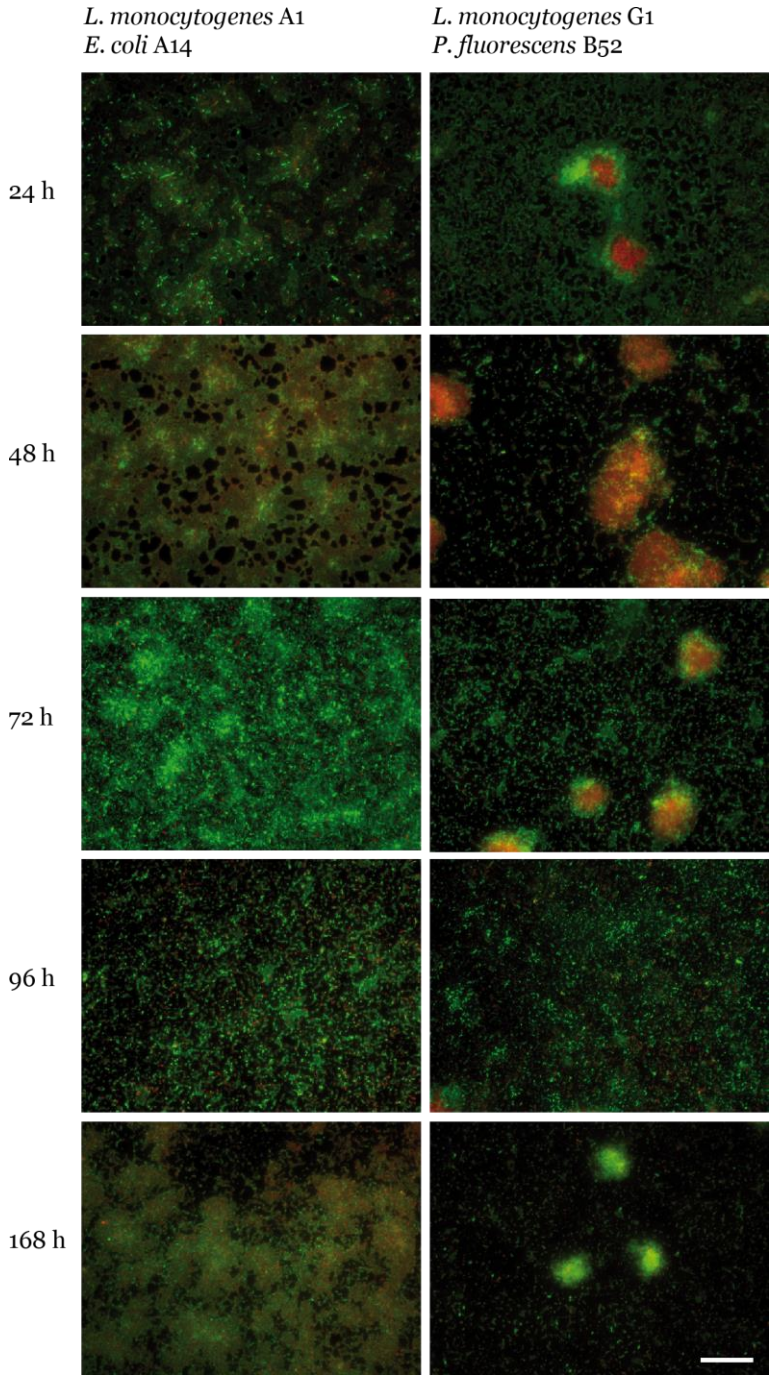

Figure 4.4: Formation kinetics of *L. monocytogenes* mixed-species biofilms. Fluorescence microscope 40x-field images obtained after LIVE/DEAD staining. Green cells represent undamaged (live) cells whereas red cells represent either damaged or dead cells (Scale bar = 50 µm).

Effectiveness of PRN-BAC sequential treatments on the removal of 168 h *L. monocytogenes* A1-*E. coli* A14 biofilms grown on SS

Different combinations of PRN-BAC according with a first order factorial design (see methods above) were applied on 168 h biofilms. Quantification of the effects was carried out by combining microscopy and image analysis and agar plate count.

Occupied area

Empirical equation (4) significantly (r^2 = 0,927) described the combined effects of PRN-BAC sequential treatment on the occupied area by undamaged cells (according to LIVE/DEAD staining) in 168 h A1-A14 biofilms:

$$OA\ (mm^2) = 0.46 - 0.12\ \mathbf{BAC} - 0.06\ \mathbf{PRNBAC} \quad (4)$$

Expected OA data according with the equation (4) after the application of PRN-BAC together with illustrative microscopy images are showed in Figure 4.5. Additionally, complete statistical data of the model can be found in the appendix (Table S1).

Statistically significant coefficients in the equation indicated a negative individual effect of BAC against the occupied area by undamaged cells within the biofilm, thus corroborating the effectiveness of BAC as a disinfectant.

No significant effect of the application of PRN alone was demonstrated although the negative interaction PRN-BAC proved a synergistic effect of these two components. Whereas the effect of PRN increased the occupied area by undamaged cells of the biofilm at low BAC concentrations; this value was reduced as the enzyme was combined with higher BAC concentrations. Thus, the green signal (undamaged cells) was higher in the experimental point [1,-1] compared to [-1,-1] whereas in the latter a higher red signal was observed (Figure 4.5). To check this effect, factorial design was repeated yielding a similar increase in OA value (data not shown). Regardless of OA outcomes, in both experimental points, an altered structure was evident compared to control (Figure 4.5). Conversely, at points [-1,1] and [1,1] a higher proportion of red (damaged/dead) cells was observed produced by higher BAC concentrations if compared to the aforementioned points. In the latter, large voids with absence of cells were also present pointing out a deep removal of the biofilm caused by the treatment.

The lowest expected value of OA according to equation (4) (46 % respecting to that obtained in absence of treatments) was obtained when PRN and BAC were applied at the highest

concentrations. Moreover, at point [1,1] the majority of the remaining cells emitted a red fluorescence indicating that those were either damage or death (Figure 4.5).

Adhered viable cultivable cells (AVC)

Kinetics showed that both *L. monocytogenes* A1 and *E. coli* A14 AVC were present on the coupons at 168 h (5.13×10^5 and 1.40×10^6 CFU/cm^2, respectively. Table S2). Nevertheless, no *L. monocytogenes* A1 AVC were recovered from the coupons after the application of the combinations PRN-BAC corresponding to the extreme values of the experimental domain assayed to 168 h A1-A14 biofilms (Figure 4.6). Conversely, *E. coli* A14 AVC were detected in the experimental points where BAC concentrations were low (Figure 4.6) indicating a higher degree of resistance of this bacterium to the treatments. Statistical analysis demonstrated that adhered cells of control samples (5.12 ± 0.06 log CFU/cm^2) presented significant differences with experimental point [-1,-1] (4.17 ± 0.05 log CFU/cm^2). At high PRN but low BAC concentrations (point [1,-1]) a higher number of *E. coli* AVC remained attached to the coupon (5.10 ± 0.21 log CFU/cm^2). Nonetheless, this value was not significantly different compared to control samples but it did to point [-1, -1] (Figure 4.6). This suggested, together with the outcomes obtained in OA, that at low BAC but high PRN concentrations the quantity of biofilm on the coupon increased compared with the other points of the experimental plan (Figures 4.5, 4.6). At points [-1,1] and [1,1], A14 viable cells were below the level of detection thus indicating that the elevated BAC concentrations affected the viability of the remaining attached cells (Figure 4.6).

Released viable cells (RVC)

No A1 strain RVC from the biofilm were recovered into the neutralising solution after the application of the PRN-BAC treatments as similar as in the adhered cells values. Contrarily, A14 strain viable cells were detected in the treatments with low concentration of BAC, experimental points [-1,-1] and [1,-1], giving significantly different values of 4.90 ± 0.19 and 5.29 ± 0.10 log CFU/ml at low and high PRN concentration, respectively (Figure 4.7), thus indicating that PRN used significantly increases *E. coli* cells detachment from the biofilm.

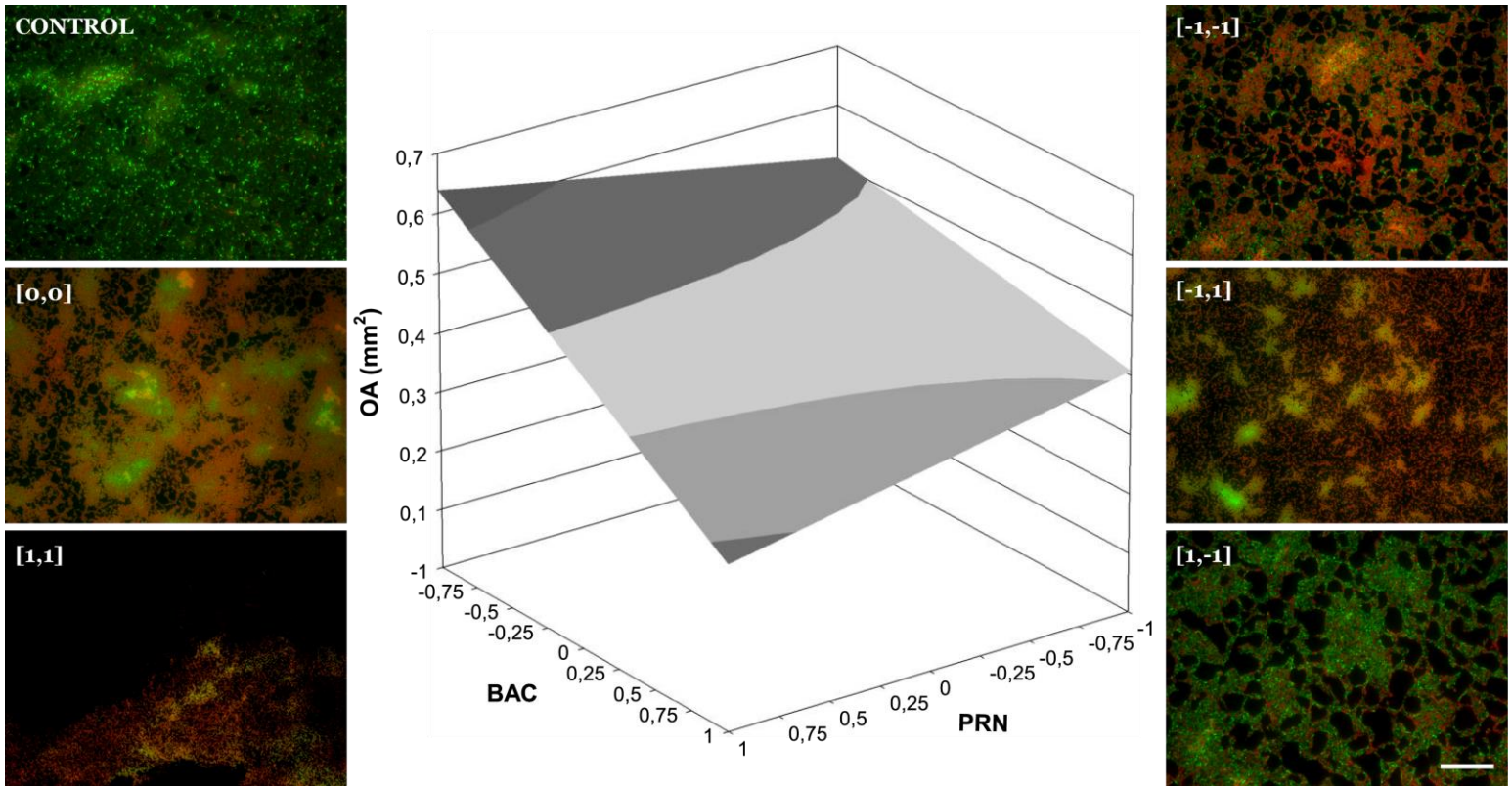

Figure 4.5: Central: Values of occupied area (OA) expressed in mm^2 after the combined sequential application of pronase (PRN) and benzalkonium chloride (BAC) on 168 h *L. monocytogenes-E. coli* biofilms. Sides: Representative epifluorescence 40x field images of control and PRN-BAC treated samples stained with live/dead staining (Scale bar = 50 μm).

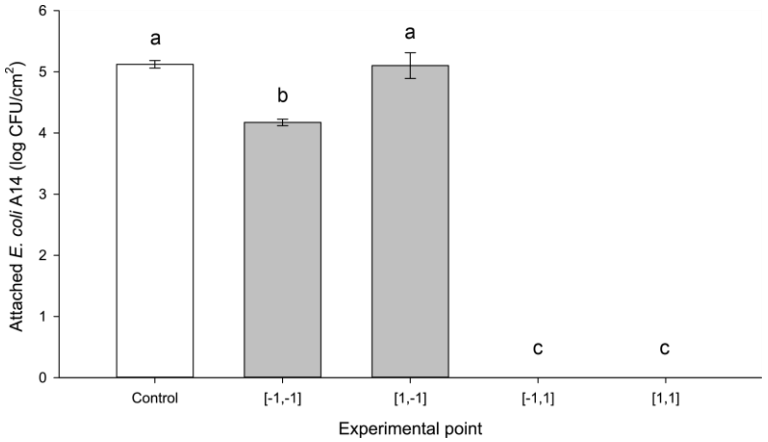

Figure 4.6: Remaining *E. coli* A14 attached cells after PRN-BAC treatments obtained in the factorial design. Error bars represent SD values. Different letters indicate statistical different value (one-way ANOVA, $\alpha = 0.05$)

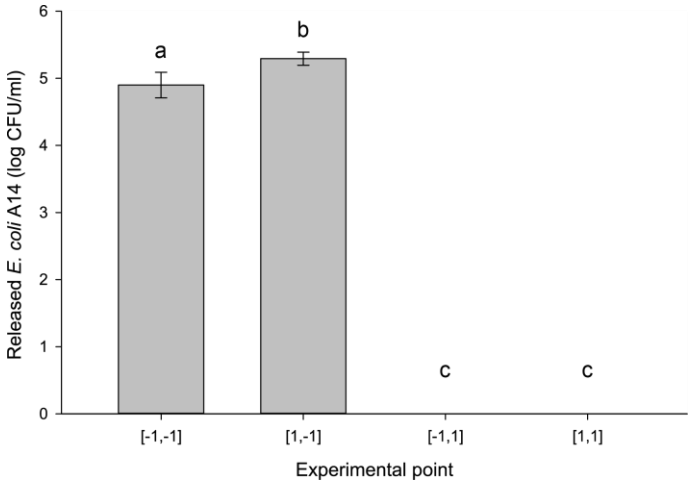

Figure 4.7: *E. coli* A14 cells recovered in the neutraliser after PRN-BAC treatments obtained in the factorial design. Error bars represent SD values. Different letters indicate statistical different value (one-way ANOVA, $\alpha = 0.05$)

Discussion

2D areal parameters have been considered as good biofilm descriptors giving biologically meaningful information [152,153,157,160]. In the present study, accuracy of the OA for quantifying biofilms was evaluated in terms of repeatability and reproducibility. This was intended not to obtain the same results from both methods, but to evaluate their applicability in biofilm studies from a numerical/statistical point of view.

Outcomes showed lower data dispersion and CV values in OA regarding those obtained by the classical method (Appendix, Figure S1). Besides, it was demonstrated that after a period of training the method is reproducible (Figure 4.2) making it suitable for assessment of biofilm formation and to evaluate the biofilm removal effects of antimicrobials measuring the ability to produce biofilm-free gaps on the surface after a given treatment.

In biofilm formation, fluorescence microscopy analysis provided information regarding 2D structural features not detected by plate count. However, accuracy of results depends on the uniformity of the structure, giving higher CV values in heterogeneous biofilms. This was of a special relevance in G1-B52 samples in which a clear tendency to form microcolonies in most cases was observed (Figure 4.4). These structures were formed by dense groups of red (damaged/dead) cells surrounded by a network of undamaged (green) cells (Figure 4.4). Bayles [304] pointed out the importance of dead cells as a biofilm support, anchoring the structure to the surface, improving its stability.

The effects of PRN-BAC sequential treatments were assessed on late-stage *L. monocytogenes*-*E. coli* biofilms using a first-order factorial design. Empirical equation (4) demonstrated a significant individual effect of BAC and a synergistic effect between PRN and BAC. Nevertheless, no individual effect of PRN on the occupied area by the mixed biofilm was demonstrated in the experimental conditions used.

PRN is a mixture of various endo- and exo-peptidases [305]. However, although *L. monocytogenes* biofilm matrix has a high protein content [117,298], in 168 h samples, *L. monocytogenes* A1 population was about 2 log CFU/cm² lower than *E. coli* A14 (data not shown) thus the contribution for the final matrix composition of A1 strain may be significantly lower if compared with A14 strain. Hence, the absence of individual PRN effect could be related to the presence of soluble protective polysaccharides in the matrix secreted by *E. coli*, becoming richer in sugar residues [292]. Besides, *L. monocytogenes* could have also promoted this sugar-rich environment by secreting soluble polysaccharides such as teichoic acids equal to those present in the cell membrane [119].

So as to mimic the environmental conditions found in industrial premises, treatments were applied at room temperature, below its optimal [306], that may have produced a lower activity of the enzyme. Optimal temperatures have been used in previous biofilm-removal studies involving PRN [307] as well as for other protein hydrolases [117,298]. Despite this, Orgaz et al. [308] demonstrated the effectiveness of PRN at 25 ºC against *P. fluorescens* biofilms, however, the concentration of PRN used were about 4 times more than the maximum concentration used in this work. Nevertheless, among microscopy images it was observed that, compared with controls, biofilm structure was affected by PRN-BAC treatments regardless of BAC concentrations (Figure 4.5).

The unexpected increase of OA and the number of viable and attached cells of *E. coli* by PRN in presence of low BAC concentrations can be explained by the dispersant effect of the enzyme. Hence, the enzyme could provoke cell disaggregation in the biofilm, and the released cells could subsequently re-adhere during the time of exposition. This hypothesis would explain, by one hand, the observed increase in the occupied area by the re-adherence and, on the other hand, the observed increase in the number of AVC and RVC detected as cell aggregates give rise to less number of colonies in the plate counts.

BAC interacts with cell membranes promoting disruption of their integrity and cellular content leakage [203,309]. According to results, it seems to be clear that PRN-BAC acted in a synergic manner being this in agreement with a recent opinion of Meireles et al. [181], who stated that a combination of enzymes and biocidal agents is desirable to obtain a good biofilm biomass removal. Besides, PRN could have provoked a certain level of cell lysis as reported for other hydrolytic enzymes [175,181,310] further enhancing the removal and biocidal effects of the combined treatment at high BAC concentrations (Figure 4.5).

No adhered or released viable cells of *L. monocytogenes* or *E. coli* were detected in those experimental points with the highest BAC concentrations (Figures 4.7, 4.8). However, values of occupied area indicated the presence of undamaged cells on the coupon. Two main reasons can explain the observed discrepancy. Firstly, the lower limit of detection of the microscopic method (1 cell/field) respecting to the plate count method (1.70 log CFU/cm^2), and secondly, the presence of viable but non-cultivable cells (VBNC). In fact, considering that our experimental system consisted of 168 h biofilms that have been exposed to PRN-BAC treatments, it should be expected that in those biofilms the pool of VBNC cells will be significant. In this state cells do not grow in solid media and microscopy assays are the only alternative to detect them [284].

Moreover, it is becoming clear among microbiologists that microbial pathogens survive to environmental stresses by entering into the VBNC state [284,311]. This status is reversible

under appropriate stimuli so, undetected pathogens can resuscitate from this dormant state thus entailing several public health concerns [312]. In *L. monocytogenes* this process is multifactorial [313]. Indeed, a recent study carried out in biofilms grown in tap water showed that *L. monocytogenes* VBNC state depends not only on the nutrient availability but also on the temperature [284].

Cell dispersion is an intrinsic process in the life-cycle of any biofilm [79]. Nevertheless, this phenomenon can be accelerated if an antimicrobial treatment is applied due to an alteration of the structural integrity. This has been considered as a topic of concern since it can facilitate the dissemination of pathogens into the environment becoming a feasible cause of contamination [181,314].

Enzymatic-based treatments have been used to sensitise biofilm so as to reduce the dose of antibiotics [315] and antiseptics [211] needed for treatment of biofilm-colonised medical devices. A recent study conducted by Stiefel et al., (2016) demonstrated that the use different species-specific enzyme mixtures increased the efficacy of commercially available cleaners to remove biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* potentially present in endoscopes. Besides, once bacteria are removed from the biofilm, pathogenicity factors may also be affected by enzymatic treatments. As an example, Longhi et al., (2008) observed how the infectiveness of planktonic *L. monocytogenes* in Caco-2 cells was significantly reduced after 24 h treatment with 200 U/ml of serratiopeptidase. This phenomenon demonstrated that beside live cells are dispersed after enzyme contact, these may represent a lower health threat.

In summary, it has been empirically demonstrated how PRN-BAC combined treatments can synergically interact the biofilm removal of the system on *L. monocytogenes-E. coli* dual-species biofilms grown on stainless steel. This approach performed in a straightforward manner with a pre-treatment with PRN combined with a high dose of BAC. Besides, at high BAC concentrations the quantity of RVC from the biofilm is also significantly diminished thus avoiding potential pathogen spread into the environment. Moreover, it has been demonstrated that microscopy 2D-image analysis combined with plate count may represent a helpful tool in assays dealing with multispecies biofilms. It provides biologically meaningful and easy-to-interpret data for quantification of biofilm development as well as empirical determination of antimicrobial treatments' effects.

Appendix

PRN	BAC	OA_{obs}	OA_{exp}	Coefficients	t	Model
1	1	0.258	0.28	0.46	35.00	0.46
1	-1	0.617	0.64	-0.02	0.94	-
-1	1	0.421	0.41	-0.12	6.24	-0.12 BAC
-1	-1	0.525	0.51	-0.06	3.44	-0.06 PRNBAC
0	0	0.440	0.46	Average value =		3.673
0	0	0.414	0.46	Expected average value =		3.67
0	0	0.510	0.46	Var (Ee) =		0.0014
0	0	0.428	0.46	t (α = 0.05; υ = 3) =		3.182

	SS	υ	MS				
				MSM/MSE =	31.943	F 0.05 {2;5} =	5.786
M	0.070	2	0.035	**MSMLF/MSM =**	0.510	F 0.05 {4;2} =	19.247
E	0.005	5	0.001	**MSE/MSEe =**	0.795	F 0.05 {5;3} =	9.117
Ee	0.004	3	0.001	**MSLF/MSEe =**	0.487	F 0.05 {2;3} =	9.552
LF	0.001	2	0.001			**r^2 =**	0.927
Total	0.075	7	0.011			**Corrected r^2 =**	0.898

Table S1. Effects of PRN and BAC treatments on the occupied area (OA) in mm^2 on 168 h *L. monocytogenes* A1-*E. coli* A14 biofilms. Results of factorial design and test of significance for model in equation [4]. (**SS**: Sum of squares; **υ**: Degrees of freedom; **MS**: Minimum squares; **M**: Model; **E**: Error; **Ee**: Experimental error; **LF**: Lack of fitting**; MSM**: Minimum squares model; **MSE**: Minimum squares error; **MSMLF**: Minimum squares model lack of fitting; **MSEe**: Minimum squares experimental error)

	***L. monocytogenes* A1**		***E. coli* A14**	
Age (h)	CFU/cm²	SD	CFU/cm²	SD
24	3.69×10^6	5.65×10^5	1.38×10^7	7.08×10^6
48	1.19×10^6	3.24×10^5	4.14×10^6	2.29×10^6
72	8.33×10^5	2.63×10^5	1.33×10^8	2.40×10^7
96	1.50×10^6	1.70×10^5	2.96×10^7	2.82×10^6
168	5.13×10^5	2.32×10^5	1.40×10^6	6.84×10^5

	***L. monocytogenes* G1**		***P. fluorescens* B52**	
Age (h)	CFU/cm²	SD	CFU/cm²	SD
24	3.88×10^7	6.53×10^6	3.70×10^8	1.76×10^8
48	6.06×10^6	2.73×10^6	2.56×10^7	1.13×10^7
72	3.90×10^6	2.74×10^6	1.33×10^8	1.72×10^7
96	1.49×10^6	4.46×10^5	2.96×10^7	6.71×10^5
168	3.23×10^5	1.43×10^5	1.40×10^6	1.40×10^6

Table S2. Separate values of AVC and standard deviations obtained of the dual-species biofilms (n = 3) corresponding to the kinetics depicted in Figure 4.3.

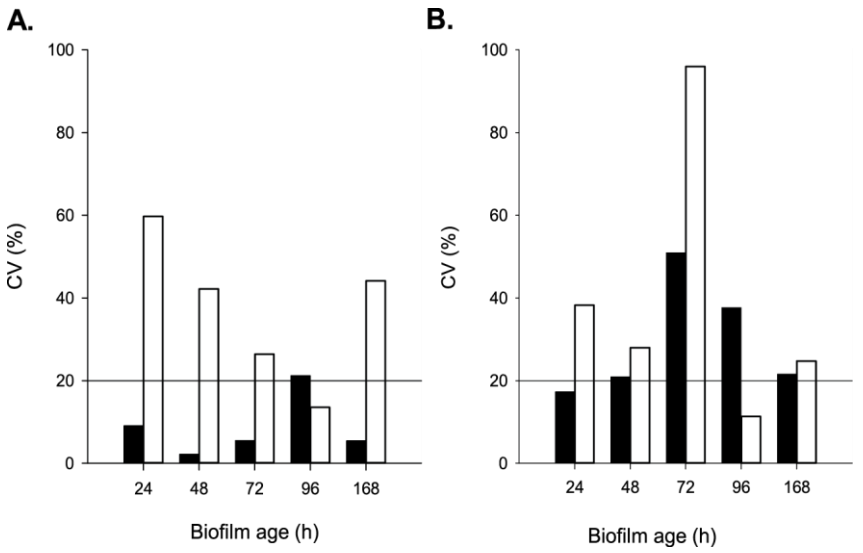

Figure S1. Coefficients of variation of fish (A) and dairy (B) industry consortia obtained with image analysis (filled bars) and plate count (void bars) (n = 3, for each assay).

5

Tolerance development to PRN-BAC combined treatments in *L. monocytogenes* dual-species biofilms

Introduction

Tolerance development after sublethal exposure to BAC and other quaternary ammonium compounds has extensively reported in planktonic cells of *L. monocytogenes* [219,221,223,316] and *E. coli* [317,318]. However, a significant smaller number of studies involving tolerance development after BAC sublethal exposure in mixed-species biofilms is currently found in the scientific literature and, among them, no studies were found assessing the tolerance to enzyme-BAC combined strategies. The only evidence found to BAC tolerance development in mixed-species biofilms was the study performed by Machado et al. [206]. They demonstrated that sublethal exposure to BAC in *Pseudomonas aeruginosa-E. coli* 6-day-old biofilms in polystyrene plates, presented a higher biomass compared with unexposed samples despite the number of adhered cells was similar in both cases.

One of the main advantages of applying enzymes is that they do not suppose selective pressure on bacteria [181]. However, if it is combined with biocides like BAC, it seems to be important to consider both agents for a correct evaluation of tolerance development.

Therefore, the main aim of this las part of the present thesis was to assess the tolerance development to PRN-BAC treatments after sublethal exposure in *L. monocytogenes-E. coli* dual-species biofilms grown on SS coupons. The exposures were carried out following three different approaches based on the concentrations of the antimicrobials used, the intervals of dosage and the duration of the whole cycle.

Methods

Bacterial strains

Dual-species biofilms were formed by *L. monocytogenes* A1 and *Escherichia coli* A14, both isolated in a previous survey performed among pre-sanitised surfaces in a fish processing plant [37].

Stock cultures of all strains were maintained at – 80 ºC in brain-heart infusion broth (BHI; Biolife, Italy) containing 50 % glycerol 1:1 (v/v) mixed. Work cultures were kept at -20 ºC in Trypticase Soy Broth (TSB, Cultimed, Barcelona) containing 50 % glycerol 1:1 (v/v) mixed.

Set-up of dual-species biofilms

100 µl of work cultures of *L. monocytogenes* and *E. coli* were cultured overnight in 5 ml sterile TSB at 37 ºC and subcultured overnight in order to ensure a proper growth.

Inocula preparation was performed as described previously [145]. Briefly, Abs_{700} of each culture was adjusted to 0.1 ± 0.001 in sterile phosphate buffer saline (PBS) using a 3000 Series scanning spectrophotometer (Cecil Instruments, Cambridge, England) corresponding to a bacterial concentration of about 10^8 CFU/ml. Adjusted cultures were 1:10^4 diluted in sterile mTSB (TSB supplemented with 2.5 g/l glucose (Vorquímica, S.L., Vigo, Spain) and 0.6 % yeast extract (Cultimed, Barcelona, Spain) to obtain a final concentration of about 10^4 CFU/ml. Then, equal volumes of these adjusted cultures were mixed to obtain the inoculum for dual-species biofilms.

Biofilms were grown on 10x10x1 mm AISI 316 stainless steel (SS) coupons (Comevisa, Vigo, Spain). Coupon pre-treatment included individual washing with industrial soap (Sutter Wash, Sutter Ibérica, S.A., Madrid, Spain), rinsing with tap water, a final rinse with deionised water and autoclaving at 121 ºC for 20 min. After this, coupons were individually placed into a 24 flat-bottomed well plate and each well was inoculated with 1 ml of the inoculum. Plates were incubated in a humidified atmosphere at 25 ºC statically for 2 h for initial adhesion and then in constant shaking at 100 rpm.

Before any assay was performed, samples (SS coupons) were aseptically collected and briefly immersed in 1 ml sterile PBS to remove loosely attached cells.

Adhered viable cultivable cells (AVC) quantification

After PBS washing, adhered viable cultivable cells (AVC), were collected from three different coupons by swabbing using two sterile cotton swabs moistened in buffered peptone water (BPW, Cultimed, Barcelona) per coupon. Swabs were resuspended in 2 ml of BPW and vortexed vigorously for 1 min in order to release cells. Suspensions were serially diluted in BPW and spread onto agar plates. *Listeria*-PALCAM (Liofilchem, Roseto degli Abruzzi, Italy) was used for *L. monocytogenes* selection whereas HiCrome™ Coliform Agar (Sigma-Aldrich, St Louis, MO, USA) supplemented with 5 µg/ml of vancomycin and cefsulodine (Sigma-Aldrich) was used for *E. coli*. Plates were incubated at 37 ºC for 24-48 h and results were expressed as the mean in log CFU/cm² or log CFU/cm² reduction depending on the assay. The accepted limit of detection was 25 CFU in the plate of the lowest dilution corresponding to 1.70 log CFU/cm² [149].

Microscopy assays

After washing, coupons were stained using LIVE/DEAD Bacterial viability kit (Life Technologies) that allows distinguishing total cells with undamaged membranes (green fluorescence) and damaged membranes (red fluorescence). Staining solution was prepared mixing 0.25 µl of propidium iodide and 0.75 µl of Syto9 in 1 ml of filter sterilised distilled water. 50-60 µl of this solution was poured onto each coupon for 15 min contact time. Subsequently, staining solution was carefully removed and samples were washed three times by immersion in 1 ml of sterile milliQ water for 30 s. Coupons were then air dried and visualised under a Leica DM 6000 epifluorescence microscope (Leica, Wetzlar, Germany) using a 40x objective and 10x ocular lenses. In each sample, a randomly chosen field was considered as start point to acquire 12 bit images using a Leica DFC365 FX camera. Each image set was composed by three 5 x 5 mosaics each one covering a total surface of 1.92×10^6 μm^2. Image analysis was then performed using the Integrated Morphometry Analysis (IMA) module of Metamorph MMAF software (Molecular Devices, Sunnyvale, CA, USA) in order to determine the occupied area (OA) by undamaged (green) cells. Results were expressed in mm^2.

Enzymes, BAC and neutralising solutions preparation

Pronase (PRN, from *Streptomyces griseus*, Roche, Mannheim, Germany) stock solutions were prepared at concentrations 100, 1000 and 2000 µg/ml dissolved in 0.1 M Tris-HCl (Sigma Aldrich) buffer at pH = 7.5 ± 0.2. After preparation, solutions were filter sterilised through a 0.2 µm pore diameter syringe filter (Sartorius, Göttingen, Germany) and kept at -20 ºC until use.

Benzalkonium chloride (BAC, Guinama, Alboraya, Spain) stock solutions were prepared at concentrations 50, 2000 and 4000 µg/ml diluting the commercial solution in sterile distilled water. Solutions were kept at 4 ºC until use.

Neutralising solution to stop BAC biocidal effects had the following composition per litre: 10 ml of a 34 g/l KH_2PO_4 solution adjusted to pH = 7.2 with $NaOH_{(aq)}$, 3 g soy lecithin, 5 g $Na_2S_2O_3$, 1 g L-histidine, 30 ml Tween 80 and adjusted with distilled water. This solution was autoclaved at 121 ºC for 20 min and kept at 4 ºC until use.

Experimental design

A two-phase experimental procedure was designed to evaluate the tolerance development (TD) in dual-species *L. monocytogenes-E. coli* biofilms to the application of PRN-BAC combined treatments.

Phase 1: sublethal expositions.

In this phase, 24 h *L. monocytogenes-E. coli* biofilms were exposed to a different number of consecutive PRN-BAC sublethal treatments with or without medium renovation as schematized in Figure 5.1. Sublethal concentrations were determined in previous assays (data not shown). Both PRN and BAC stock solutions were diluted in mTSB at concentrations for sublethal exposure (Figure 5.1). Additionally, a negative control experimental series in which PRN and BAC solutions were substituted by equal volumes of sterile deionised water was included in each experiment. Thus, the experimental approaches and concentrations used were as follows:

i). *Experimental approach 1 – Short term exposure:* In this approach, two consecutive expositions without medium renovation were carried out. At 24 h, the bulk phase was carefully pipetted out and dual-species biofilms were exposed to 1 ml of 50 µg/ml PRN for 1 h contact time without agitation. Then, 1.5 ml of 25 µg/ml BAC were added and plates were placed back at 25 ºC/100 rpm. At 48 h, bulk phase was removed and dual-species biofilms were exposed to 1 ml of 100 µg/ml PRN, let to dwell 1 h statically, followed by 1.5 ml of 50 µg/ml BAC, and placed back at 25 ºC/100 rpm until next step. In this approach, TD quantification in exposed biofilms was determined after 72 h.

ii). *Experimental approach 2 – Short term exposure with medium renewal:* In this second scheme, exposure to treatments was carried out as described for approach 1 but after every PRN-BAC exposition the bulk phase was pipetted out and substituted by 1 ml of fresh mTSB (i.e. at 48 and 96 h), and incubated at 25 ºC/100 rpm for 24 h (Figure 5.1). In this case, final treatment and TD quantification to PRN-BAC acquired resistance was carried out at 120 h (Figure 5.1).

iii). *Experimental approach 3 – Long term exposure:* In this last approach, a discontinuous exposure to 1 ml 50 µg/ml PRN followed by 1.5 ml 25 µg/ml of BAC was undertaken at 24, 48, 72, 96 and 168 h. At 190 h, final treatment and quantification of the TD to PRN-BAC was carried out (Figure 5.1).

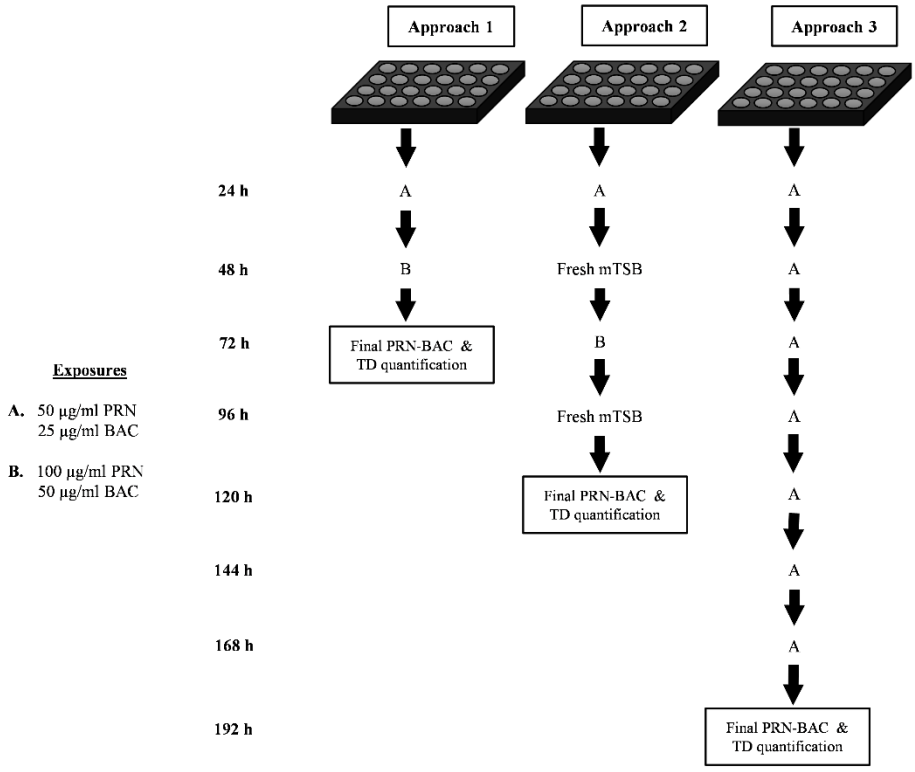

Figure 5.1: Experimental approaches used for biofilm exposure to PRN-BAC solutions and tolerance development (TD) evaluation (see text for further details).

Phase 2: Quantification of tolerance development (TD) in L. monocytogenes-E. coli biofilms to PRN-BAC combined treatments

After growth in absence and presence of PRN-BAC, the dual-species biofilms were evaluated by assessing the state of the biofilm and the TD after previous exposure to combined treatments.

Biofilm condition was assessed by quantifying the number of AVC and the OA by undamaged (green) cells on the SS coupons in both experimental series as detailed above. Results were expressed in log CFU/cm² and in mm² for AVC and OA, respectively

TD was quantified comparing the resistance of the mixed biofilm previously exposed to sublethal PRN-BAC concentrations with that obtained in controls after final PRN-BAC

treatment. Specifically, 3 new coupons per series were transferred to a new well and treated again with 1 ml of a 100 μg/ml PRN solution in 0.1 mM Tris-HCl for 1 h contact time followed by 1.5 ml of 50 μg/ml BAC solution in deionised water and let to dwell statically for 10 min. To stop BAC effects, samples were newly transferred to a well containing 1 ml of sterile neutraliser and immersed for 10 s. After neutralisation, coupons were processed for AVC and microscopy assays as above. In this part, an additional visual comparison between the 2D structure of the biofilms before and after the final treatment was carried out.

Statistical analysis

An independent-samples Student's *t* test was performed to determine differences between values using Microsoft Excel 2016. Significance was expressed at the 95 % confidence level ($\alpha = 0.05$) or greater.

Results

Approach 1: short term exposure

Comparison between the condition of *L. monocytogenes-E. coli* dual-species biofilm formed without any exposition to PRN-BAC and after two consecutive exposures to double-fold increasing PRN-BAC concentrations was carried out according to experimental approach 1 (Figure 5.1). Obtained results showed that the number of *L. monocytogenes* adhered in exposed biofilms was significantly lower comparing with that obtained in the controls (3.95 ± 0.48 and 6.37 ± 0.46 log CFU/cm^2, respectively) (Figure 5.2 A). No significant differences were obtained in the number of *E. coli* in unexposed and exposed biofilms (Figure 5.2A).

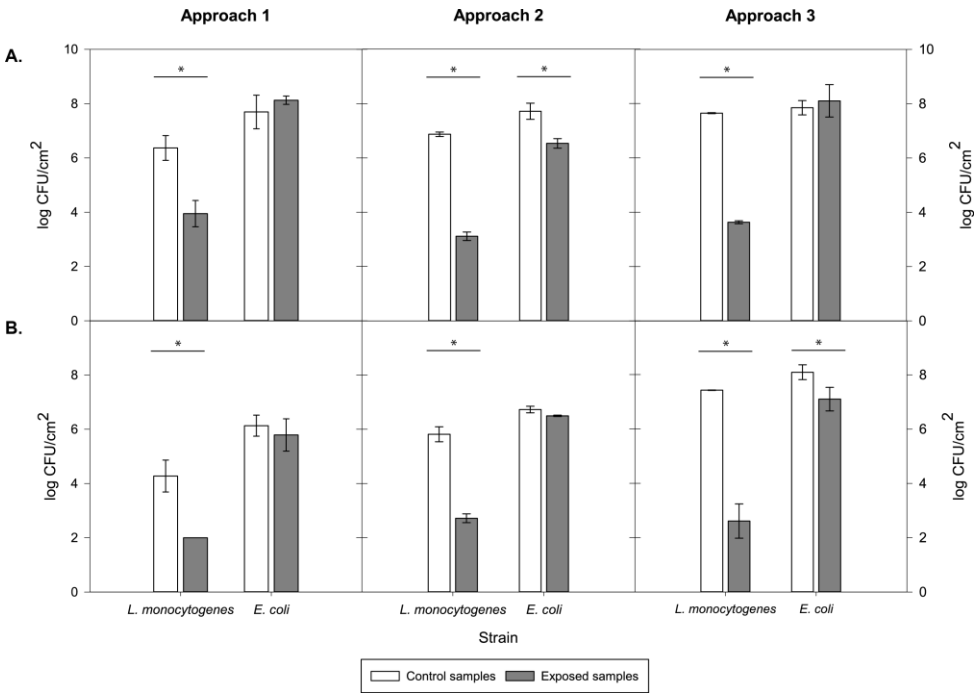

Figure 5.2: Adhered viable cultivable (AVC) counts obtained in all experimental approaches A) before final and B) after final 100 μg/ml pronase followed by 50 μg/ml benzalkonium chloride combined treatment. Asterisks indicate statistical significance ($P < 0.05$).

The final treatment with PRN-BAC decreased the number of adhered cells in all experimental series assayed about 1.5-2 log CFU/cm² (Figure 5.2B). However, when the resistance of exposed and control biofilms was compared in terms of log reduction of the number of adhered cells of both species, no significant differences ($P > 0.05$) were obtained (Figure 5.3). Taken together, these results seem to indicate that *L. monocytogenes-E. coli* dual-species biofilms did not acquire any tolerance to PRN-BAC treatment after exposure following approach 1.

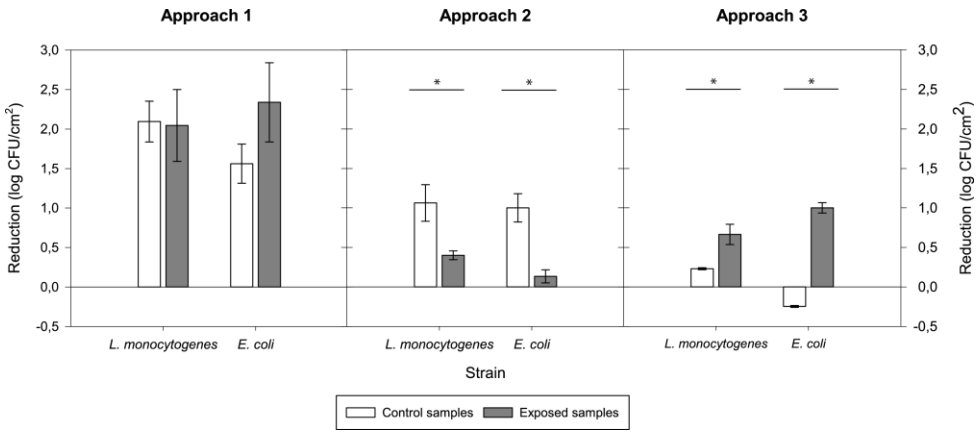

Figure 5.3: Log reductions obtained in all experimental approaches after the application of a final 100 µg/ml pronase followed by 50 µg/ml benzalkonium chloride combined treatment. Asterisks indicate statistical significance ($P < 0.05$).

Microscopic images showed that denser, with significantly higher values of OA by undamaged (green) cells were formed by control biofilms (1.20 ± 0.08 mm²) compared to PRN-BAC exposed samples (0.72 ± 0.09 mm²) (Figure 5.4). Last PRN-BAC treatment applied to the samples obtained with approach 1 had different effects against control and exposed biofilms both in OA values and architecture of the biofilm. PRN-BAC application deeply altered the 2D structure of the biofilm making it to lose the uniform distribution of the cells among the surface giving rise to a disorganised structure (Figure 5.4). Despite this, some cellular clusters with a mixture of green and red-fluorescent cells were still present (Figure 5.4). This loss of 2D structure among controls was concomitant with a decrease in the OA value (0.91 ± 0.15 mm²) (Figure 5.4). On the other hand, in exposed samples, final PRN-BAC treatment produced a counter-effect in the OA values yielding a uniform, yet slightly altered, and presenting a higher green signal (1.16 ± 0.15 mm²) (Figure 5.4).

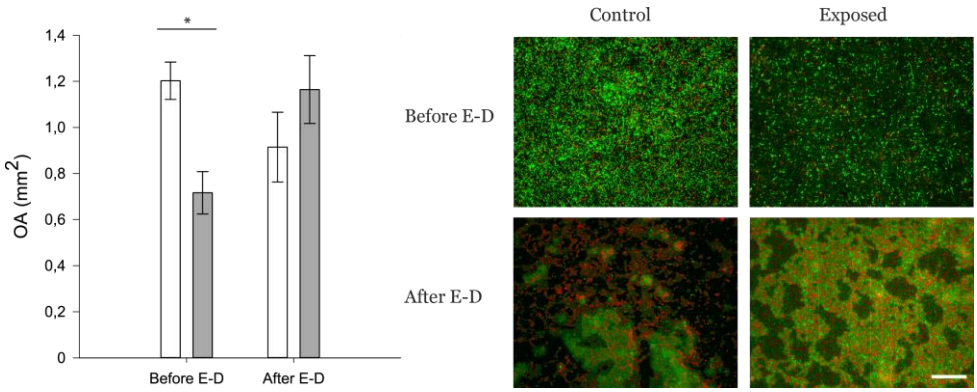

Figure 5.4: Values of occupied area obtained after analysis of 25-field mosaics of approach 1 before and after the application of PRN-BAC final treatment. E-D: Enzyme-Disinfectant. Asterisk indicates statistical significance ($P < 0.05$) Scale bar = 50 μm.

Approach 2: short term exposure with medium renewal

This second approach was specifically designed to check whether *L. monocytogenes-E. coli* dual-species biofilm was able to develop tolerance to PRN-BAC treatments when incorporating 24 h-recovery periods after each of the two exposures scheduled in the approach 1 using fresh culture medium (Figure 5.1).

No significant differences ($P > 0.05$) were obtained in AVC values of control samples neither in *L. monocytogenes* nor in *E. coli* (6.87 ± 0.08 and 7.72 ± 0.30 log CFU/cm², respectively) compared to those obtained in controls of approach 1 (Figure 5.2) meaning that the inclusion of recovery periods in between expositions did not produce a higher number of cells to be attached to the surface. On the other hand, AVC outcomes in exposed samples were lower in both species compared to those obtained in approach 1 (3.11 ± 0.16 CFU/cm² for *L. monocytogenes* and 6.53 ± 0.18 CFU/cm² for *E. coli*) (Figure 5.2).

Quantification of the acquired resistance to PRN-BAC of the dual-species biofilms of approach 2 was then assessed in terms of log reduction and compared to results obtained in approach 1. Outcomes demonstrated that the renewal of the medium caused a drop in the log reduction values in both unexposed (control) and exposed dual-species biofilms (Figure 5.3). Considering that AVC values before final PRN-BAC treatment between approaches 1 and 2 were not significantly different in neither species (Figure 5.2), that meant that the overall resistance of the cells of *L. monocytogenes-E. coli* biofilms to the PRN-BAC treatment was higher, even in the case of the control (Figure 5.3). Indeed, whereas the log reduction

values obtained in approach 1 ranged from 1.56 ± 0.25 to 2.34 ± 0.50 log CFU/cm², using this second exposure schedule the variation among log reduction outcomes was from 0.13 ± 0.08 to 1.06 ± 0.23 log CFU/cm² (Figure 5.3). This increased resistance was observed in both species, even though *L. monocytogenes* presented higher differences than *E. coli* if values between approaches are compared (Figure 5.3). Surprisingly, a higher resistance to PRN-BAC treatments was also observed in control biofilms respecting to those values obtained in exposed biofilms of approach 1 (Figure 5.3).

Approach 3: long term exposition without medium renewal

In order to check if longer incubation times with the sublethal treatments would influence the final resistance of the biofilm, a third experimental approach consisting in five consecutive exposures to sublethal PRN-BAC concentrations was used (Figure 5.1).

After sublethal exposures, control samples did not present significance in *E. coli* (7.85 ± 0.26 log CFU/cm²) respecting to previous approaches whereas in *L. monocytogenes* the AVC value increased slightly (7.65 ± 0.02 log CFU/cm²) (Figure 5.2A). In exposed biofilms, AVC values were significantly ($P < 0.05$) higher than those of approach 2 (3.63 ± 0.05 and 8.10 ± 0.59 log CFU/cm² for *L. monocytogenes* and *E. coli*, respectively) (Figure 5.2A).

TD quantification demonstrated the lowest log reductions among control samples in *L. monocytogenes* (0.23 ± 0.12 log CFU/cm²) even presenting negative values (i.e. a slight AVC increase) in *E. coli* (-0.25 ± 0.01 log CFU/cm²) (Figure 5.3). Among exposed samples, the log reductions were higher than those of approach 2 but significantly lower than those of approach 1 (Figure 5.3), with final AVC values of 2.61 ± 0.63 and 7.10 ± 0.43 log CFU/cm² in *L. monocytogenes* and *E. coli*, respectively (Figure 5.2).

Microscope images acquired before PRN-BAC treatment for TD evaluation showed a dense biofilm in exposed biofilms (Figure 5.5). Besides, the amount of live (green) cells was visibly lower compared to controls, concomitant with a lower OA value (0.41 ± 0.03 and 0.18 ± 0.05 mm² for controls and exposed biofilms, respectively). Final PRN-BAC treatment provoked an increase in the OA values in both series (Figure 5.5). On the other hand, exposed samples images appeared much more clear before and after PRN-BAC application (Figure 5.5).

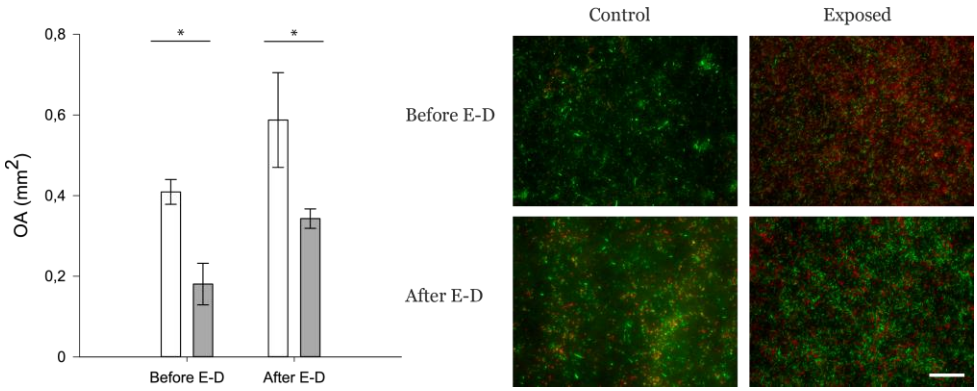

Figure 5.5: Values of occupied area obtained after analysis of 25-field mosaics of approach 3 before and after the application of PRN-BAC final treatment. E-D: Enzyme-Disinfectant. Asterisks indicate statistical significance ($P < 0.05$) Scale bar = 50 μm.

Discussion

In the present study, the capability of *L. monocytogenes-E. coli* biofilms to grow and develop tolerance to PRN-BAC combined treatments after sublethal exposure following three different approaches was assessed.

Before any antimicrobial treatment can be applied, its efficacy against a particular must be tested. Despite this, to date, no standardised methods for antibiofilm testing are available [319] and none of them considers TD as a parameter, which is determinant to determine the right dose to apply to avoid bacterial resistance especially when treatments are intended to be used for long periods.

Results showed that the number of *L. monocytogenes* AVC decreased after sublethal exposure to PRN-BAC regardless of the exposure scheme used (Figure 5.2A). Contrarily, in *E. coli* the presence of PRN-BAC did not affect the number of AVC on SS coupons in approaches 1 and 3 (Figure 5.2A). In approach 2, the number of AVC of *E. coli* in exposed samples was slightly lower compared to control samples but the differences between series were smaller than those obtained in *L. monocytogenes* (Figure 5.2A). These results were not surprising considering that Gram-negatives are generally more resistant to QACs than Gram-positives [201] and were in accordance with results obtained by Machado et al. [206] who observed that the number of adhered cells in 6-day old dual-species biofilms of *Pseudomonas aeruginosa-E. coli* was not affected by presence of 328.5 μg/ml BAC in the culture medium.

Obtained results showed that the acquired tolerance to PRN-BAC by *L. monocytogenes-E. coli* dual-species biofilms is the result of the following two interdependent factors: i) the effects produced by the cultural features of each experimental approach giving rise to a different *L. monocytogenes-E. coli* dual-species biofilms, and ii) the specific physiological effects provoked by the sublethal PRN-BAC expositions. Both effects can be straightforwardly individually analysed comparing, in the first case, the acquired tolerance in control biofilms after the application of antimicrobials in the three different experimental approaches and, in the second case, comparing the tolerance developed by controls and by exposed biofilms in each experimental approach.

Regarding the first comparison, significant higher values of log reduction were obtained in the dual-species biofilms subjected to short-term approach (approach 1) compared to those obtained when short-term with medium renewal and long-term exposures (approaches 2 and 3, respectively) were applied (Figure 5.3). In other words, biofilms grown in approach 1 were much less tolerant to PRN-BAC. This fact, together with the absence of significance between AVC values in all the experimental approaches before and after the application of the last PRN-BAC treatment, pointed out that longer incubation times (approaches 2 and 3) and the incorporation of recovery periods (approach 2) gave rise to structures with higher PRN-BAC tolerance.

The TD in terms of the number of AVC that resist the last PRN-BAC treatment after sublethal exposure was only observed in approach 2, in which significant differences ($P < 0.05$) in the log reduction between control and exposed dual-species biofilms were observed in both strains.

In approach 1, microscopy images showed a deep alteration in the 2D structure especially in control biofilms, not detected in AVC values (Figure 5.4). Contrarily, in approach 3 an unexpected increase in OA values of control samples was observed (Figure 5.5) which could be caused by the increment in the number of *E. coli* AVC (Figures 5.2A, B). Further experimentation needs to be carried out, including additional parameters, in order to determine the actual causes of such increase.

Bacterial recovery after sublethal injured has been reported to contribute to adaptation and hardening of cells after stresses [320]. Among foodborne pathogens, this recovery period is rather short; around 1 to 5 hours at 25-37 ºC in rich broth [321]. Thus, in biofilms, these factors can promote the appearance of persistent strains in food-related environments [322]. Considering this, it is logical to think that the incorporation of recovery periods in approach 2, allowed injured bacteria to repair damaged structures, and develop stress-induced strategies to prepare the cells for further external aggressions.

Membrane alterations together with the expression of BAC-induced efflux pumps, have been previously reported by several authors as the main responsible of BAC-sensitivity reduction both in *L. monocytogenes* [219,223,323,324] and *E. coli* [317,318,325]. Following these ideas, the recovery periods in approach 2 could have permitted the surviving subpopulation after PRN-BAC exposure to undergo mechanisms for membrane damage repair, as previously observed in *L. monocytogenes* after the application of sanitisers [326].

Additionally, these periods could have allowed cells in the bulk phase to have enough time to repair possible damaged cellular structures and express chromosome and plasmid-encoded efflux pumps, therefore causing the extrusion of BAC outside of the cell. With this regard, Tamburro et al. [327] observed an alteration in the gene expression pattern concomitant with a higher tolerance to BAC in *L. monocytogenes* after exposure to 10 μg/ml BAC, highlighting a significant increase in the expression of *mdr*L (efflux pump) and *sig*B (transcription factor) genes. Similar phenomena have also been reported in *E. coli* after BAC sublethal exposure [318]. This increased tolerance is even more relevant if we consider that these pumps can be effective against other molecules therefore promoting BAC-induced multidrug resistance [323,328].

In addition to this, expression of genes related to physiological changes and metabolic pathways, as a consequence of BAC exposure are also altered in both species. Bore et al. [318] demonstrated an upregulation of the porin OmpC and the *sox*S gene, related with protection against oxidative stress, in *E. coli* K-12 after BAC exposure. In *L. monocytogenes* it has been observed that fatty acid composition, and therefore membrane fluidity and permeability, was altered after BAC exposure which made cells to be less sensitive to BAC [223,324].

Nevertheless, the specific effect of the recovery periods has not been previously described in biofilms. In planktonic cells of *Pseudomonas spp*. it was previously observed that the adaptive resistance acquired after short term exposition to 200 μg/ml of BAC was rapidly lost after overnight incubation in absence of the disinfectant [329]. Interestingly, the authors also demonstrated that the level of tolerance of *Pseudomonas sp*. to the application of BAC and BAC-EDTA was conditioned by the treatment applied during the sublethal exposition.

Besides, sublethal expositions to PRN-BAC would probably determine important specific biological modifications in the final L. *monocytogenes-E. coli* dual-species biofilm. In fact, previous studies have demonstrated that the presence of BAC during biofilm formation by *E. coli* gives rise to a denser matrix richer in proteins and polysaccharides [206]. In this latter case, the possible presence of high amounts of protective colanic acid would make the matrix barrier to be very difficult to penetrate [120,330].

The way that all these biological processes takes places in industrial settings, are directly related with the way sanitation procedures are applied. Insufficient rinsing is one the most common causes of presence of sublethal amounts among surfaces treated with QACs in the food industry [224]. This fact together with the time in-between scheduled cleaning and disinfection protocols applied in a processing plant, would permit bacteria to recover from biocide injury eventually boosting their resistance to chemicals as demonstrated by the results obtained.

Summarising, outcomes in this work have demonstrated that the manner in which *L. monocytogenes-E. coli* biofilms are exposed to sublethal concentrations of PRN-BAC influences the subsequent resistance to this combined treatment. Firstly, it was observed that recovery periods lead to a selection of a resistant subpopulation compared to unexposed samples. In addition to this, it was also observed that longer incubation times also influenced the resistance of the biofilm. Therefore, for biofilm eradication, a thorough optimisation not only of the right amounts of antimicrobial compounds utilised but also a proper time scheduling would necessary prior to the application of any sanitation procedure in order to obtain proper bactericidal effects while avoiding the selection of resistant variants.

General discussion

Listeria monocytogenes is considered one of the pathogens of a major relevance in Europe with an increasing incidence according to the EFSA [39].

The present thesis deals with the study of the effectiveness of the combined application of enzymes and benzalkonium chloride (BAC) to remove *L. monocytogenes*-carrying biofilms present in food-related industrial environments. Chapter 2 deals with the detection and characterisation of *L. monocytogenes*-carrying consortia present in food industry surfaces. Chapters 3 and 4 aimed to evaluate the effectiveness of the combined application of enzymes and benzalkonium chloride against the removal of biofilms formed by the representative bacterial associations characterized in chapter 2. Finally, in chapter 5, the development of tolerance to the application of sublethal concentrations of enzymes and benzalkonium chloride by one of the mixed biofilm was carried out.

In the present document, fluorescence microscopy together with image analysis were used for visual characterisation of mixed species biofilms formation (chapters 3, 4 and 5) but also for quantification of 2D structure related parameters of *L. monocytogenes* monospecies biofilm formation using BIOFILMDIVER (chapter 2). Besides, the occupied area (OA) by the biofilm determined by microscopy image analysis was successfully used to study the effects of pronase (PRN)-BAC combined treatments (chapter 4). For that purpose, microscopic studies were combined with agar plating to quantify the number of adhered viable culturable cells (AVC) and the number of released viable cells (RVC). Biologically speaking, high amounts of RVC after a sanitation treatment would indicate biofilm displacement, but not bacterial killing. **For this reason, RVC should be considered as a relevant parameter when evaluating the efficacy of a particular hygienisation treatment.**

2D areal parameters have been considered good biofilm descriptors giving biologically meaningful information [152,153,157,160]. In the present study, it has been demonstrated that microscopy 2D-image analysis together with plate count may represent a helpful tool in assays dealing with multispecies biofilms. It provided biologically meaningful and easy-to-interpret data for quantification of biofilm development as well as empirical determination of antimicrobial treatments' effectiveness.

Previous studies have demonstrated how different strains isolated from industrial environments, are able to interact with *L. monocytogenes* forming multispecies biofilms [129]. However, the number of studies dealing with the detection and characterisation of *L. monocytogenes*-carrying present in food industry are still scarce.

Results in chapter 2 demonstrated an incidence of *L. monocytogenes* of a 4.44 % among surfaces surveyed, with an heterogeneous distribution, remarking thus the capacity of this

pathogen to adapt to different ecological niches [240,242,253]. Serogroup 1/2a–3a was the most abundant among *L. monocytogenes* positive samples as previously reported [254–256]. Despite this, no relationship between the sort of industry and the *L. monocytogenes* serogroup was found.

The composition of *L. monocytogenes*-carrying communities present in food processing plants keeps a direct relationship with the typical indigenous microbiota present in the environment. *Escherichia coli* was found as the most frequent accompanying species in fish industry, while *Carnobacterium* sp. was the most representative among meat industry samples being in agreement with previous authors [242,245] and mostly related with the microbiota present in raw products [240].

The species variety and the ubiquitous distribution of isolated consortia demonstrated in the present thesis pointed out the capacity of ecological adaptation of these microorganisms and the inefficacy of cleaning and disinfection treatments applied. In fact, one of the main concerns in the food industry is the appearance of resistant bacteria derived from the biocide misuse [73]. For this reason, the introduction of innovative sanitation strategies could be an effective alternative to avoid, or at least reduce, the risk of biofilm formation and antimicrobial resistance.

In this line, as an environmentally, worker-friendly alternative, several authors have recommended the utilisation of enzymes breaking down the biofilm matrix [175] with chemical biocidals [181], in order to improve the efficacy of the latter. With this regard, in this thesis the effectiveness of the combined application of enzymes and BAC against early and late-stage *L. monocytogenes*-carrying biofilms representative of the bacterial associations present in the food industry was investigated.

In early-stage biofilms (chapter 3), comparison between the effects of cellulase (CEL), DNaseI and PRN against *L. monocytogenes-E. coli* was assessed. Maximum effect (~ 2 log) was obtained after the application of 400 μg/ml of DNaseI, followed by PRN and CEL solutions on 24 h mixed biofilms. Previous authors have already proposed DNaseI as an antibiofilm enzyme cleaving extracellular DNA (eDNA) that is present in the biofilm matrix and considered as a requisite for biofilm formation in *L. monocytogenes* as well as in other Gram-positives [288,289]. Proteases have been also proved to be effective in removing biofilms with matrices of proteinaceous nature, like *L. monocytogenes* [117]. However, in this particular case, the dominance of *E. coli* in 24 h biofilms (Figure 3.1) could have given rise to a matrix with higher polysaccharide content [292].

Although in 24 h biofilms, DNaseI was the most efficient enzyme in both species of the mixed biofilm (Figure 3.3), BAC effects after 48 h samples varied depending on the species and the enzyme applied. Thus, accordingly with the RD_{90} values showed in Table 3.2, in the case of *L. monocytogenes* BAC performed better after DNaseI treatment, in *E. coli* obtained results showed a higher effect of BAC after PRN treatment. These results reflected that the composition of the biofilm matrix varies with time and also that the action of BAC is conditioned by the enzymatic pre-treatment. In fact, subsequent studies carried out in late-stage biofilms will demonstrate a synergic action between PRN and BAC against mixed *L. monocytogenes* biofilms.

The role of the accompanying species on the effectiveness of the combined application of DNaseI-BAC treatments against *L. monocytogenes-E. coli* and *L. monocytogenes-P. fluorescens* mixed biofilms was also studied. A significant higher number of adhered cells of *L. monocytogenes* was obtained in presence of *P. fluorescens*, probably due to entrapping of *L. monocytogenes* into the polymeric matrix secreted by *P. fluorescens*. Regarding the effect of the treatments, DNaseI produced a significant decrease of *L. monocytogenes* only in the mixed biofilm with the presence of *E. coli*, probably because the matrix composition affected its diffusion [297]. On the contrary, higher effectiveness of BAC against *L. monocytogenes* was demonstrated in mixed biofilms with *P. fluorescens*. Additionally, microscopic analysis demonstrated remarkable differences in their 2D structures. **This variation in efficacy derived from bacterial composition, pointed out the importance of considering primary characterisation and specific target determination for sanitation procedures design.**

As mentioned above, when considering RD_{90} values, DNaseI was more effective against *L. monocytogenes-E. coli* 48 h biofilms than PRN when combined with BAC (Figure 3.4). However, PRN, a mixture of exo- and endo-peptidases [305], followed by BAC achieved theoretical maximum biofilm removing values (K') of 100% in *Listeria monocytogenes* (Table 3.2), whereas 94% was reached in presence of DNaseI. So, PRN was next combined with BAC to assess biofilm removal of late stage mixed *L. monocytogenes* biofilms. The effects of the treatments against biofilm removal were evaluated in terms of the occupied area (OA) by the mixed biofilm, the number of remaining adhered cells and the number of released viable cells of both species after the treatment.

The effects of the combined treatment against the OA by the mixed biofilm were quantified following a factorial experimental design. A significant empirical equation that describes the individual and combined effects of PRN and BAC was obtained (Equation 4). Contrary to the effects obtained against 24 and 48 h *L. monocytogenes-E. coli* biofilms (chapter 3), no

significant individual effects of PRN were observed against late-stage dual-species biofilms (chapter 4). This could have been due to the fact that matrix composition could have become richer in protective polysaccharides such as colanic acid [331] that could have concealed targets susceptible to be cleaved by PRN.

However, significant terms of the equation 4 demonstrated a synergistic effect between PRN and BAC against 168 h *L. monocytogenes*-*E. coli* dual-species biofilms achieving log reductions in OA of 54 % compared with control samples (Figure 4.5). One possible hypothesis to explain this is that BAC interaction with cell membranes could expose peptide bonds susceptible to be hydrolysed by PRN inducing subsequent cell lysis. In fact, this cell-lytic properties have been previously described for other enzymes [175,181,310]. Moreover, the PRN efficacy in polysaccharide-rich matrices has been previously reported by Orgaz et al. [308]. Considering this, **the observed synergy of PRN and BAC against *L. monocytogenes* mixed biofilms opens a new perspectives of research dealing with specific degradation of biofilm components prior to disinfection by QACs or other biocides**.

One of the most important outcomes derived from the studies performed in this thesis, was the determination of the number of RVC after the application of the treatments. This cellular pool is especially significant for food safety, since dispersion produced by antimicrobials application, rinsing, etc. could increase the probability of generation of new contamination foci.

Regarding this, obtained results demonstrated that unless high concentrations of enzyme and biocide were applied, a high number of RVC was observed in biofilm removal assays (chapters 3 and 4), thus corroborating that enzyme based disinfection need to be performed in combination with biocides [181]. This was especially relevant in *E. coli* that was detected even after application of a 500 μg/ml solution of BAC (Figure 3.5) which confirms that this microorganisms can endure the concentrations of this biocide normally used in industry that are between 200 and 100 μg/ml [332] despite the previous enzymatic treatment. The higher sensitivity of *L. monocytogenes* to the combined treatment compared to *E. coli* was no surprising since Gram-negatives are generally more resistant to quaternary ammonium compounds (QACs), such as BAC, than Gram-positives [201]. Therefore, **RVC should be included as an additional parameter in the normal design of novel cleaning and disinfection studies**.

Detection of *L. monocytogenes*-carrying consortia in chapter 2 was performed after application of routine sanitation protocols. Therefore, it is logical to think that these consortia could have developed adaptive strategies to overcome. It has been widely reported

that in food industry, constant exposure of bacteria to sublethal concentrations of biocides due to either their misuse [73] or to residues left after rinsing [333,334] are among the main causes of resistance generation. One of the main purposes for including enzymes in hygienisation systems is that, it could improve the efficacy of disinfectants and, theoretically, bacteria are unable to develop resistance to such molecules. The present thesis presents the first evaluation of tolerance development in *L. monocytogenes-E. coli* dual-species biofilms to PRN-BAC combined treatments. Outcomes obtained demonstrated how, in exposed biofilms, the periods in-between sublethal exposures significantly decreases ($P < 0.05$) the level of log reduction obtained after PRN-BAC treatments (Figure 5.3). These periods could have permitted injured bacteria to repair cellular damage while synthesising structures, such as efflux pumps, to prepare the cell for possible subsequent aggressions as reported in *L. monocytogenes* [223,323,324] and *E. coli* [317,318]. Contrarily, in unexposed samples, an effect of time was observed achieving lower log reduction values presumably because the matureness, and therefore the level of resistance of the dual-species biofilms was higher. Taken together, results obtained in chapter 5 indicate that tolerance development in *L. monocytogenes-E. coli* biofilms is of a multifactorial nature. Additionally, **they suggest that the development of resistances should be incorporated to the tests performed in investigations regarding novel sanitation systems.**

Conclusions

The main conclusions derived from this PhD dissertation are the following:

1. The composition of *L. monocytogenes*-carrying communities present in food processing plants keeps a direct relationship with the typical indigenous microbiota present in the environment. Particularly, *Escherichia coli* and *Carnobacterium sp.* were found to be the most representative accompanying strains in fish and meat industry, respectively. These communities are able to survive in different zones of a processing plant thus pointing out the outstanding ecological adaptation capacity of these microorganisms. There was no evidence about the relation of the *L. monocytogenes* ecological distribution and its serogroup.

2. The effectiveness of hydrolytic enzymes targeting different components of the biofilm matrix combined with benzalkonium chloride against early-stage *Listeria monocytogenes* dual-species biofilms formed in stainless steel is demonstrated. In addition, obtained results demonstrate that the removal efficacy depends on the species composition and on the age of the biofilm pointing out the necessity to develop *ad hoc* cleaning and disinfection systems.

3. The combined application of pronase and benzalkonium chloride can synergically decrease the occupied area by *Listeria monocytogenes-Escherichia coli* late-stage biofilms grown on stainless steel. Besides, high doses of benzalkonium chloride must be applied to ensure proper bactericidal effects upon adhered and released cells. It has been also demonstrated that fluorescence microscopy 2D-image analysis together with plate count may represent a helpful tool in assays dealing with multispecies biofilms.

4. Released viable cells after sanitation treatments may be included as a parameter in studies regarding design of novel strategies due to its potential consequences, in terms of pathogen spreading and food safety, among food processing plants.

5. It is demonstrated that the recovery periods between antimicrobial exposures favours the development of tolerance of *Listeria monocytogenes-Escherichia coli* biofilms to pronase-benzalkonium chloride combined treatments.

The experimental design regarding the combination of enzymes and benzalkonium chloride, and its efficacy against *L. monocytogenes* dual-species biofilms isolated from food processing environments detailed in this PhD dissertation, open new perspectives in the design of novel sanitation strategies for pathogen control in the food industry.

Reference list

1. McLauchlin J, Rees CED, Dodd CER. Listeria monocytogenes and the genus Listeria. The Prokaryotes: Firmicutes and Tenericutes. 2014. pp. 241–259. doi:10.1007/978-3-642-30120-9

2. Weller D, Andrus A, Wiedmann M, den Bakker HC. Listeria booriae sp. nov. and Listeria newyorkensis sp. nov., from food processing environments in the USA. Int J Syst Evol Microbiol. 2015;65: 286–292. doi:10.1099/ijs.0.070839-0

3. Orsi RH, Wiedmann M. Characteristics and distribution of Listeria spp., including Listeria species newly described since 2009. Appl Microbiol Biotechnol. Applied Microbiology and Biotechnology; 2016;100: 5273–5287. doi:10.1007/s00253-016-7552-2

4. den Bakker HC, Warchocki S, Wright EM, Allred AF, Ahlstrom C, Manuel CS, et al. Listeria floridensis sp. nov., Listeria aquatica sp. nov., Listeria cornellensis sp. nov., Listeria riparia sp. nov. and Listeria grandensis sp. nov., from agricultural and natural environments. Int J Syst Evol Microbiol. 2014;64: 1882–1889. doi:10.1099/ijs.0.052720-0

5. Murray EGD, Webb RA, Swann HBR. A disease of rabbits characterized by a large mononuclear leucocytosis caused by a hitherto undescribed bacillus Bacterium monocytogenes (n.sp.). J Pathol Bacteriol. 1926;29: 407–439. doi:10.1002/path.1700290409

6. Pirie JHH. Listeria: Change of Name for a Genus Bacteria. Nature. 1940;145: 264. doi:10.1038/145264a0

7. Seeliger HPR, Jones D. Genus Listeria. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG, editors. Bergey's manual of systematic bacteriology, vol 2. The Williams and Wilkins Co.; 1986. pp. 1235–1245.

8. Paterson JS. The antigenic structure of organisms of the genus Listerella. J Pathol Bacteriol. 1940;51: 427–436.

9. Seeliger HPR, Höhne K. Chapter II Serotyping of Listeria monocytogenes and Related Species. Methods Microbiol. 1979;13: 31–49. doi:10.1016/S0580-9517(08)70372-6

10. Liu D. Identification, subtyping and virulence determination of Listeria monocytogenes, an important foodborne pathogen. Journal of Medical Microbiology. 2006. pp. 645–659. doi:10.1099/jmm.0.46495-0

11. Doumith M, Buchrieser C, Glaser P, Jacquet C, Martin P. Differentiation of the major Listeria monocytogenes serovars by multiplex PCR. J Clin Microbiol. 2004;42: 3819–22. doi:10.1128/JCM.42.8.3819-3822.2004

12. Wiedmann M, Bruce JL, Knorr R, Bodis M, Cole EM, McDowell CI, et al. Ribotype diversity of Listeria monocytogenes strains associated with outbreaks of listeriosis in ruminants. J Clin Microbiol. American Society for Microbiology (ASM); 1996;34: 1086–1090.

13. Jacquet C, Gouin E, Jeannel D, Cossart P, Rocourt J. Expression of ActA, Ami, InlB, and Listeriolysin O in Listeria monocytogenes of human and food origin. Appl Environ Microbiol. American Society for Microbiology (ASM); 2002;68: 616–622. doi:10.1128/AEM.68.2.616-622.2002

14. Wiedmann M, Bruce JL, Keating C, Johnson AE, McDonough PL, Batt CA. Ribotypes and virulence gene polymorphisms suggest three distinct Listeria monocytogenes lineages with differences in pathogenic potential. Infect Immun. American Society for Microbiology; 1997;65: 2707–2716.

15. Nadon CA, Woodward DL, Young C, Rodgers FG, Wiedmann M. Correlations between molecular subtyping and serotyping of Listeria monocytogenes. J Clin Microbiol. 2001;39: 2704–2707. doi:10.1128/JCM.39.7.2704-2707.2001

16. Van Der Veen S, Moezelaar R, Abee T, Wells-Bennik MHJ. The growth limits of a large number of Listeria monocytogenes strains at combinations of stresses show serotype- and niche-specific traits. J Appl Microbiol. Blackwell Publishing Ltd; 2008;105: 1246–1258. doi:10.1111/j.1365-2672.2008.03873.x

17. Blackman IC, Frank JF. Growth of Listeria monocytogenes as a Biofilm on Various food-processing surfaces. J Food Prot. 1996;59: 827–831.

18. Gandhi M, Chikindas ML. Listeria: A foodborne pathogen that knows how to survive. Int J Food Microbiol. 2007;113: 1–15. doi:10.1016/j.ijfoodmicro.2006.07.008

19. Moltz AG, Martin SE. Formation of biofilms by Listeria monocytogenes under various growth conditions. J Food Prot. 2005;68: 92–97.

20. Vivant A-L, Garmyn D, Piveteau P. Listeria monocytogenes, a down-to-earth pathogen. Front Cell Infect Microbiol. Frontiers Media SA; 2013;3: 87. doi:10.3389/fcimb.2013.00087

21. Dowe M, Jackson E, Mori J, Colin B. Listeria monocytogenes Survival in Soil and Incidence in Agricultural Soils. J Food Prot. International Association for Food Protection; 1997;10: 1158–1286.

22. Linke K, Rückerl I, Brugger K, Karpiskova R, Walland J, Muri-Klinger S, et al. Reservoirs of Listeria species in three environmental ecosystems. Appl Environ Microbiol. American Society for Microbiology; 2014;80: 5583–5592. doi:10.1128/AEM.01018-14

23. Locatelli A, Spor A, Jolivet C, Piveteau P, Hartmann A. Biotic and Abiotic Soil Properties Influence Survival of Listeria monocytogenes in Soil. Freitag NE, editor. PLoS One. Public Library of Science; 2013;8: e75969. doi:10.1371/journal.pone.0075969

24. Orsi RH, den Bakker HC, Wiedmann M. Listeria monocytogenes lineages: Genomics, evolution, ecology, and phenotypic characteristics. Int J Med Microbiol. Elsevier GmbH.; 2011;301: 79–96. doi:10.1016/j.ijmm.2010.05.002

25. Lomonaco S, Nucera D, Filipello V. The evolution and epidemiology of Listeria monocytogenes in Europe and the United States. Infect Genet Evol. 2015;35: 172–183. doi:10.1016/j.meegid.2015.08.008

26. Söderqvist K, Thisted Lambertz S, Vågsholm I, Boqvist S. Foodborne Bacterial Pathogens in Retail Prepacked Ready-to-Eat Mixed Ingredient Salads. J Food Prot. 2016;79: 978–985. doi:10.4315/0362-028X.JFP-15-515

27. Gombas DE, Chen Y, Clavero RS, Scott VN. Survey of Listeria monocytogenes in ready-to-eat foods. J Food Prot. 2003;66: 559–69.

28. Pagadala S, Parveen S, Rippen T, Luchansky JB, Call JE, Tamplin ML, et al. Prevalence, characterization and sources of Listeria monocytogenes in blue crab (Callinectus sapidus) meat and blue crab processing plants. Food Microbiol. Elsevier Ltd; 2012;31: 263–70. doi:10.1016/j.fm.2012.03.015

29. Goh SG, Leili A-H, Kuan CH, Loo YY, Lye YL, Chang WS, et al. Transmission of Listeria monocytogenes from raw chicken meat to cooked chicken meat through cutting boards. Food Control. Elsevier Ltd; 2014;37: 51–55. doi:10.1016/j.foodcont.2013.08.030

30. Chasseignaux E, Toquin M-T, Ragimbeau C, Salvat G, Colin P, Ermel G. Molecular epidemiology of Listeria monocytogenes isolates collected from the environment,

raw meat and raw products in two poultry- and pork-processing plants. J Appl Microbiol. 2001;91: 888–899. doi:10.1046/j.1365-2672.2001.01445.x

31. Thévenot D, Dernburg a, Vernozy-Rozand C. An updated review of Listeria monocytogenes in the pork meat industry and its products. J Appl Microbiol. 2006;101: 7–17. doi:10.1111/j.1365-2672.2006.02962.x

32. Ajayeoba TA, Atanda OO, Obadina AO, Bankole MO, Adelowo OO. The incidence and distribution of *Listeria monocytogenes* in ready-to-eat vegetables in South-Western Nigeria. Food Sci Nutr. 2016;4: 59–66. doi:10.1002/fsn3.263

33. Oliveira M, Viñas I, Colàs P, Anguera M, Usall J, Abadias M. Effectiveness of a bacteriophage in reducing Listeria monocytogenes on fresh-cut fruits and fruit juices. Food Microbiol. Elsevier Ltd; 2014;38: 137–42. doi:10.1016/j.fm.2013.08.018

34. Viswanath P, Murugesan L, Knabel SJ, Verghese B, Chikthimmah N, Laborde LF. Incidence of Listeria monocytogenes and Listeria spp. in a small-scale mushroom production facility. J Food Prot. 2013;76: 608–15. doi:10.4315/0362-028X.JFP-12-292

35. Carpentier B, Cerf O. Review - Persistence of Listeria monocytogenes in food industry equipment and premises. Int J Food Microbiol. Elsevier B.V.; 2011;145: 1–8. doi:10.1016/j.ijfoodmicro.2011.01.005

36. Ferreira V, Wiedmann M, Teixeira P, Stasiewicz MJ. Listeria monocytogenes persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. J Food Prot. 2014;77: 150–70. doi:10.4315/0362-028X.JFP-13-150

37. Rodríguez-López P, Saá-Ibusquiza P, Mosquera-Fernández M, López-Cabo M. Listeria monocytogenes – carrying consortia in food industry. Composition, subtyping and numerical characterisation of mono-species biofilm dynamics on stainless steel. Int J Food Microbiol. Elsevier B.V.; 2015;206: 84–95. doi:10.1016/j.ijfoodmicro.2015.05.003

38. Scallan E, Hoekstra RM, Angulo FJ, Tauxe R V., Widdowson MA, Roy SL, et al. Foodborne illness acquired in the United States-Major pathogens. Emerg Infect Dis. 2011;17: 7–15. doi:10.3201/eid1701.P11101

39. EFSA. The European Union summary report on trends and sources of zoonoses,

zoonotic agents and food-borne outbreaks in 2014. EFSA J. 2015;13: 4329. doi:10.2903/j.efsa.2015.4329

40. Vázquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Domi G, González-zorn B, et al. Listeria Pathogenesis and Molecular Virulence Determinants Listeria Pathogenesis and Molecular Virulence Determinants. Clin Microbiol Rev. 2001;14: 584–640. doi:10.1128/CMR.14.3.584

41. Vázquez-Boland JA, Domínguez-Bernal G, González-Zorn B, Kreft J, Goebel W. Pathogenicity islands and virulence evolution in Listeria. Microbes Infect. 2001;3: 571–584. doi:10.1016/S1286-4579(01)01413-7

42. Brouwer MC, van de Beek D, Heckenberg SGB, Spanjaard L, de Gans J. Community-acquired Listeria monocytogenes meningitis in adults. Clin Infect Dis. Oxford University Press; 2006;43: 1233–8. doi:10.1086/508462

43. Farber JM, Peterkin PI. Listeria monocytogenes, a food-borne pathogen. Microbiol Rev. 1991;55: 476–511. doi:10.1104/pp.110.161547

44. Simões M, Simões LC, Vieira MJ. A review of current and emergent biofilm control strategies. LWT - Food Sci Technol. 2010;43: 573–583. doi:10.1016/j.lwt.2009.12.008

45. Bille J, Blanc DS, Schmid H, Boubaker K, Baumgartner A, Siegrist HH, et al. Outbreak of human listeriosis associated with tomme cheese in northwest Switzerland, 2005. Euro Surveill. 2006;11: 91–93.

46. Vít M, Olejník R, Karpíšková R, Částková J, Príkazský V, Príkazská M, et al. Outbreak of listeriosis in the Czech Republic, late 2006 – preliminary report. Euro Surveill. 2007;12: pii=3132.

47. Koch J, Dworak R, Prager R, Becker B, Brockmann S, Wicke A, et al. Large listeriosis outbreak linked to cheese made from pasteurized milk, Germany, 2006-2007. Foodborne Pathog Dis. Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA; 2010;7: 1581–1584. doi:10.1089/fpd.2010.0631

48. CDC. Outbreak of Listeria monocytogenes Infections Associated with Pasteurized Milk from a Local Dairy --- Massachusetts, 2007. Morb Mortal Wkly Rep. 2008;57: 1097–1100.

49. Johnsen BO, Lingaas E, Torfoss D, Strøm EH, Nordøy I. A large outbreak of Listeria

monocytogenes infection with short incubation period in a tertiary care hospital. J Infect. 2010;61: 465–470. doi:10.1016/j.jinf.2010.08.007

50. Canada G of. Report of the Independent Investigator into the 2008 Listeriosis Outbreak. 2009. pp. 1–95. doi:10.1017/CBO9781107415324.004

51. Fretz R, Sagel U, Ruppitsch W, Pietzka A, Stoger A, Huhulescu S, et al. Listeriosis outbreak caused by acid curd cheese Quargel , Austria and Germany 2009. Euro Surveill. 2010;15.

52. Magalhães R, Almeida G, Ferreira V, Santos I, Silva J, Mendes MM, et al. Cheese-related listeriosis outbreak, Portugal, March 2009 to February 2012. Euro Surveill. 2015;20: pii=21104. doi:10.2807/1560-7917.ES2015.20.17.21104

53. CDC. Multistate outbreak of listeriosis associated with Jensen Farms Cantaloupe -- - United States, August--September 2011. Morbidity and Mortality Weekly Report. 2011. pp. 1357–1358.

54. CDC. Listeria outbreaks [Internet]. [cited 8 Nov 2016]. Available: https://www.cdc.gov/listeria/outbreaks/

55. Ruppitsch W, Prager R, Halbedel S, Hyden P, Pietzka A, Huhulescu S, et al. Ongoing outbreak of invasive listeriosis, Germany, 2012 to 2015. Euro Surveill. 2015;20: pii=30094. doi:10.2807/1560-7917.ES.2015.20.50.30094

56. Pérez-Trallero E, Zigorraga C, Artieda J, Alkorta M, Marimón JM. Two outbreaks of listeria monocytogenes infection, Northern Spain. Emerg Infect Dis. 2014;20: 2155–2157. doi:10.3201/eid2012.140993

57. Anonymous. Listeria. In: Wingstrand A, Sorensen A, Helwigh B, editors. Annual Report on Zoonoses in Denmark 2014. National Food Institute, Technical University of Denmark; 2015. pp. 14–17. doi:10.1128/AEM.02991-09

58. Swaminathan B, Gerner-Smidt P. The epidemiology of human listeriosis. Microbes and Infection. 2007. pp. 1236–1243. doi:10.1016/j.micinf.2007.05.011

59. Schlech IWF, Lavigne PM, Bortolussi RA. Epidemic listeriosis - Evidence for transmission by food. N Engl J Med. Massachusetts Medical Society; 1983;308: 203–206. doi:10.1056/NEJM198301273080407

60. Gilbert RJ, McLauchlin J, Velani SK. The contamination of paté by Listeria monocytogenes in England and Wales in 1989 and 1990. Epidemiol Infect.

Cambridge University Press; 1993;110: 543–51. doi:10.1017/S0950268800050962

61. Salamina G, Dalle Donne E, Niccolini A, Poda G, Cesaroni D, Bucci M, et al. A foodborne outbreak of gastroenteritis involving Listeria monocytogenes. Epidemiol Infect. 1996;117: 429–436. doi:10.1017/S0950268800059082

62. Dalton CB, Austin CC, Sobel J, Hayes PS, Bibb WF, Graves LM, et al. An outbreak of gastroenteritis and fever due to Listeria monocytogenes in milk. N Engl J Med. Massachusetts Medical Society; 1997;336: 100–5. doi:10.1056/NEJM199701093360204

63. Dinu LD, Delaquis P, Bach S. Nonculturable response of animal enteropathogens in the agricultural environment and implications for food safety. Journal of Food Protection. 2009. pp. 1342–1354.

64. Dreux N, Albagnac C, Federighi M, Carlin F, Morris CE, Nguyen-the C. Viable but non-culturable Listeria monocytogenes on parsley leaves and absence of recovery to a culturable state. J Appl Microbiol. 2007;103: 1272–81. doi:10.1111/j.1365-2672.2007.03351.x

65. Campdepadrós M, Stchigel AM, Romeu M, Quilez J, Solà R. Effectiveness of two sanitation procedures for decreasing the microbial contamination levels (including Listeria monocytogenes) on food contact and non-food contact surfaces in a dessert-processing factory. Food Control. Elsevier Ltd; 2012;23: 26–31. doi:10.1016/j.foodcont.2011.05.017

66. Chavant P, Gaillard-Martinie B, Hébraud M. Antimicrobial effects of sanitizers against planktonic and sessile Listeria monocytogenes cells according to the growth phase. FEMS Microbiol Lett. 2004;236: 241–8. doi:10.1016/j.femsle.2004.05.040

67. Saá Ibusquiza P, Herrera JJR, Cabo ML. Resistance to benzalkonium chloride, peracetic acid and nisin during formation of mature biofilms by Listeria monocytogenes. Food Microbiol. 2011;28: 418–25. doi:10.1016/j.fm.2010.09.014

68. Pan Y, Breidt F, Kathariou S. Resistance of Listeria monocytogenes biofilms to sanitizing agents in a simulated food processing environment. Appl Environ Microbiol. 2006;72: 7711–7. doi:10.1128/AEM.01065-06

69. Mack WN, Mack JP, Ackerson AO. Microbial film development in a trickling filter. Microb Ecol. 1975;2: 215–226. doi:10.1007/BF02010441

70. Donlan RM, Costerton JW. Biofilms: Survival mechanisms of clinically relevant microorganisms. Clinical Microbiology Reviews. American Society for Microbiology; 2002. pp. 167–193. doi:10.1128/CMR.15.2.167-193.2002

71. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. Annual Review of Microbiology. 1995. pp. 711–745.

72. Winkelströter LK, Teixeira FB dos R, Silva EP, Alves VF, De Martinis ECP. Unraveling Microbial Biofilms of Importance for Food Microbiology. Microb Ecol. Springer New York LLC; 2013;68: 35–46. doi:10.1007/s00248-013-0347-4

73. Abdallah M, Benoliel C, Drider D, Dhulster P, Chihib NE. Biofilm formation and persistence on abiotic surfaces in the context of food and medical environments. Arch Microbiol. 2014;196: 453–472. doi:10.1007/s00203-014-0983-1

74. Vlamakis H. The world of biofilms. Virulence. 2011;2: 431–434. doi:10.4161/viru.2.5.17902

75. Watnick P, Kolter R. Biofilm, city of microbes. Journal of Bacteriology. American Society for Microbiology; 2000. pp. 2675–2679. doi:10.1128/JB.182.10.2675-2679.2000

76. Stewart PS, Franklin MJ. Physiological heterogeneity in biofilms. Nat Rev Microbiol. Nature Publishing Group; 2008;6: 199–210. doi:10.1038/nrmicro1838

77. Bjarnsholt T, Alhede M, Alhede M, Eickhardt-Sørensen SR, Moser C, Kühl M, et al. The in vivo biofilm. Trends Microbiol. 2013;21: 466–74. doi:10.1016/j.tim.2013.06.002

78. Hall-Stoodley L, Costerton J, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol. 2004;2: 95–108. doi:10.1038/nrmicro821

79. Petrova OE, Sauer K. Escaping the biofilm in more than one way: Desorption, detachment or dispersion. Current Opinion in Microbiology. 2016. pp. 67–78. doi:10.1016/j.mib.2016.01.004

80. Rutherford ST, Bassler BL. Bacterial quorum sensing: its role in virulence and possibilities for its control. Cold Spring Harb Perspect Med. 2012;2: 1–25. doi:10.1101/cshperspect.a012427

81. Nadell CD, Xavier JB, Levin SA, Foster KR. The evolution of quorum sensing in

bacterial biofilms. Moran NA, editor. PLoS Biol. Public Library of Science; 2008;6: e14. doi:10.1371/journal.pbio.0060014

82. Xavier KB, Bassler BL. Interference with AI-2-mediated bacterial cell-cell communication. Nature. Nature Publishing Group; 2005;437: 750–3. doi:10.1038/nature03960

83. Garmyn D, Gal L, Lemaitre JP, Hartmann A, Piveteau P. Communication and autoinduction in the species Listeria monocytogenes: A central role for the agr system. Communicative and Integrative Biology. 2009. pp. 371–374. doi:10.4161/cib.2.4.8610

84. Van Der Veen S, Abee T. Importance of SigB for listeria monocytogenes static and continuous-flow biofilm formation and disinfectant resistance. Appl Environ Microbiol. 2010;76: 7854–7860. doi:10.1128/AEM.01519-10

85. Dickson JS, Koohmaraie M, Hruska RL. Cell Surface Charge Characteristics and Their Relationship to Bacterial Attachment to Meat Surfaces. Appl Environ Microbiol. 1989;55: 832–836.

86. Palmer J, Flint S, Brooks J. Bacterial cell attachment, the beginning of a biofilm. J Ind Microbiol Biotechnol. 2007;34: 577–588. doi:10.1007/s10295-007-0234-4

87. Davies DG. Physiological events in biofilm formation. In: Allison D, Gilbert P, Lappin-Scott HM, Wilson M, editors. Community structure and co-operation in biofilms. 2000. pp. 37–51.

88. Dunne WM. Bacterial adhesion: Seen any good biofilms lately? Clin Microbiol Rev. American Society for Microbiology; 2002;15: 155–166. doi:10.1128/CMR.15.2.155-166.2002

89. Schwab U, Hu Y, Wiedmann M, Boor KJ. Alternative Sigma Factor σB Listeria monocytogenes Is Not Essential for Surface Attachment. J Food Prot. 2005;68: 311–317.

90. Herald PJ, Zottola EA. Attachment of Listeria monocytogenes to Stainless Steel Surfaces at Various Temperatures and pH Values. J Food Sci. 1988;53: 1549–1562. doi:10.1111/j.1365-2621.1988.tb09321.x

91. Poimenidou S, Belessi CA, Giaouris ED, Gounadaki AS, Nychas GJE, Skandamis PN. Listeria monocytogenes attachment to and detachment from stainless steel surfaces

in a simulated dairy processing environment. Appl Environ Microbiol. 2009;75: 7182–7188. doi:10.1128/AEM.01359-09

92. Briandet R, Meylheuc T, Maher C, Bellon-Fontaine MN. Listeria monocytogenes Scott A: cell surface charge, hydrophobicity, and electron donor and acceptor characteristics under different environmental growth conditions. Appl Environ Microbiol. 1999;65: 5328–5333.

93. Peel M, Donachie W, Shaw A. Temperature-dependent expression of flagella of Listeria monocytogenes studied by electron microscopy, SDS-PAGE and western blotting. J Gen Microbiol. 1988;134: 2171–2178. doi:10.1099/00221287-134-8-2171

94. Mai TL, Conner DE. Effect of temperature and growth media on the attachment of Listeria monocytogenes to stainless steel. Int J Food Microbiol. 2007;120: 282–286. doi:10.1016/j.ijfoodmicro.2007.09.006

95. Camper AK, Hayes JT, Sturman PJ, Jones WL, Cunningham AB. Effects of motility and adsorption rate coefficient on transport of bacteria through saturated porous media. Appl Environ Microbiol. 1993;59: 3455–3462.

96. Kim KY, Frank JF. Effect of Nutrients on Biofilm Formation by Listeria monocytogenes on Stainless Steel. J Food Prot. 1995;58: 24–28.

97. Guilbaud M, Piveteau P, Desvaux M, Brisse S, Briandet R. Exploring the Diversity of Listeria monocytogenes Biofilm Architecture by High-Throughput Confocal Laser Scanning Microscopy and the Predominance of the Honeycomb-Like Morphotype. Appl Environ Microbiol. 2015;81: 1813–1819. doi:10.1128/AEM.03173-14

98. Jaradat ZW, Bhunia AK. Glucose and nutrient concentrations affect the expression of a 104-kilodalton Listeria adhesion protein in Listeria monocytogenes. Appl Environ Microbiol. American Society for Microbiology; 2002;68: 4876–83. doi:10.1128/AEM.68.10.4876-4883.2002

99. Kyoui D, Hirokawa E, Takahashi H, Kuda T, Kimura B. Effect of glucose on *Listeria monocytogenes* biofilm formation, and assessment of the biofilm's sanitation tolerance. Biofouling. Taylor & Francis; 2016;32: 815–826. doi:10.1080/08927014.2016.1198953

100. Borucki MK, Peppin JD, White D, Loge F, Call DR. Variation in biofilm formation among strains of Listeria monocytogenes. Appl Environ Microbiol. 2003;69: 7336–7342. doi:10.1128/AEM.69.12.7336

101. Mosquera-Fernández M, Rodríguez-López P, Cabo ML, Balsa-Canto E. Numerical spatio-temporal characterization of Listeria monocytogenes biofilms. Int J Food Microbiol. Elsevier B.V.; 2014;182–183: 26–36. doi:10.1016/j.ijfoodmicro.2014.05.005

102. Saá P, Cabo ML, Rodríguez JJ. Effects of mussel processing soils on the adherence of Listeria monocytogenes to polypropylene and stainless steel. J Food Prot. 2009;72: 1885–90.

103. Silva S, Teixeira P, Oliveira R, Azeredo J. Adhesion to and viability of Listeria monocytogenes on food contact surfaces. J Food Prot. 2008;71: 1379–1385.

104. Lemon KP, Higgins DE, Kolter R. Flagellar motility is critical for Listeria monocytogenes biofilm formation. J Bacteriol. American Society for Microbiology; 2007;189: 4418–4424. doi:10.1128/JB.01967-06

105. Guerrieri E, de Niederhäusern S, Messi P, Sabia C, Iseppi R, Anacarso I, et al. Use of lactic acid bacteria (LAB) biofilms for the control of Listeria monocytogenes in a small-scale model. Food Control. 2009;20: 861–865. doi:10.1016/j.foodcont.2008.11.001

106. Tresse O, Lebret V, Benezech T, Faille C. Comparative evaluation of adhesion, surface properties, and surface protein composition of Listeria monocytogenes strains after cultivation at constant pH of 5 and 7. J Appl Microbiol. Blackwell Publishing Ltd; 2006;101: 53–62. doi:10.1111/j.1365-2672.2006.02968.x

107. Vatanyoopaisarn S, Nazli A, Dodd CER, Rees CED, Waites WM. Effect of flagella on initial attachment of Listeria monocytogenes to stainless steel. Appl Environ Microbiol. 2000;66: 860–863. doi:10.1128/AEM.66.2.860-863.2000

108. Todhanakasem T, Young GM. Loss of flagellum-based motility by Listeria monocytogenes results in formation of hyperbiofilms. J Bacteriol. 2008;190: 6030–6034. doi:10.1128/JB.00155-08

109. Takhistov P, George B. Linearized kinetic model of Listeria monocytogenes biofilm growth. Bioprocess Biosyst Eng. 2004;26: 259–270. doi:10.1007/s00449-004-0357-1

110. Flemming HC, Neu TR, Wozniak DJ. The EPS matrix: The "House of Biofilm Cells." J Bacteriol. 2007;189: 7945–7947. doi:10.1128/JB.00858-07

111. Stewart PS. Diffusion in biofilms. Journal of Bacteriology. American Society for Microbiology; 2003. pp. 1485–1491. doi:10.1128/JB.185.5.1485-1491.2003

112. Stepanović S, Ćirković I, Ranin L, Švabić-Vlahović M. Biofilm formation by Salmonella spp. and Listeria monocytogenes on plastic surface. Lett Appl Microbiol. 2004;38: 428–432. doi:10.1111/j.1472-765X.2004.01513.x

113. Gilbert P, Allison DG, McBain AJ. Biofilms in vitro and in vivo: do singular mechanisms imply cross-resistance? J Appl Microbiol. Blackwell Science Ltd.; 2002;92: 98S–110S. doi:10.1046/j.1365-2672.92.5s1.5.x

114. Chen LH, Köseoğlu VK, Güvener ZT, Myers-Morales T, Reed JM, D'Orazio SEF, et al. Cyclic di-GMP-dependent Signaling Pathways in the Pathogenic Firmicute Listeria monocytogenes. PLoS Pathog. 2014;10: e1004301. doi:10.1371/journal.ppat.1004301

115. Köseoğlu VK, Heiss C, Azadi P, Topchiy E, Güvener ZT, Lehmann TE, et al. Listeria monocytogenes exopolysaccharide: Origin, structure, biosynthetic machinery and c-di-GMP-dependent regulation. Mol Microbiol. 2015;96: 728–743. doi:10.1111/mmi.12966

116. Combrouse T, Sadovskaya I, Faille C, Kol O, Guérardel Y, Midelet-Bourdin G. Quantification of the extracellular matrix of the Listeria monocytogenes biofilms of different phylogenic lineages with optimization of culture conditions. J Appl Microbiol. 2013;114: 1120–31. doi:10.1111/jam.12127

117. Nguyen UT, Burrows LL. DNase I and proteinase K impair Listeria monocytogenes biofilm formation and induce dispersal of pre-existing biofilms. Int J Food Microbiol. Elsevier B.V.; 2014;187: 26–32. doi:10.1016/j.ijfoodmicro.2014.06.025

118. Harmsen M, Lappann M, Knøchel S, Molin S. Role of extracellular DNA during biofilm formation by Listeria monocytogenes. Appl Environ Microbiol. 2010;76: 2271–9. doi:10.1128/AEM.02361-09

119. Brauge T, Sadovskaya I, Faille C, Benezech T, Maes E, Guerardel Y, et al. Teichoic acid is the major polysaccharide present in the Listeria monocytogenes biofilm matrix. FEMS Microbiol Lett. The Oxford University Press; 2016;363: fnv229. doi:10.1093/femsle/fnv229

120. Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol. Nature Publishing Group; 2010;8: 623–33. doi:10.1038/nrmicro2415

121. Neu TR, Lawrence JR. Lectin-binding analysis in biofilm systems. Methods Enzymol. 1999;310: 145–152. doi:10.1016/S0076-6879(99)10012-0

122. Petrova OE, Sauer K. Sticky situations: key components that control bacterial surface attachment. J Bacteriol. 2012;194: 2413–25. doi:10.1128/JB.00003-12

123. Kaplan JB. Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. J Dent Res. 2010;89: 205–218. doi:10.1177/0022034509359403

124. Rice SA, Koh KS, Queck SY, Labbate M, Lam KW, Kjelleberg S. Biofilm formation and sloughing in Serratia marcescens are controlled by quorum sensing and nutrient cues. J Bacteriol. American Society for Microbiology; 2005;187: 3477–3485. doi:10.1128/JB.187.10.3477-3485.2005

125. Boles BR, Thoendel M, Singh PK. Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. Mol Microbiol. Blackwell Science Ltd; 2005;57: 1210–1223. doi:10.1111/j.1365-2958.2005.04743.x

126. Liu D, Lawrence ML, Ainsworth AJ, Austin FW. Toward an improved laboratory definition of Listeria monocytogenes virulence. International Journal of Food Microbiology. 2007. pp. 101–115. doi:10.1016/j.ijfoodmicro.2007.07.045

127. Davey ME, O'toole GA. Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev. 2000;64: 847–67. doi:10.1128/MMBR.64.4.847-867.2000

128. Kalmokoff ML, Austin JW, Wan XD, Sanders G, Banerjee S, Farber JM. Adsorption, attachment and biofilm formation among isolates of listeria monocytogenes using model conditions. Journal of Applied Microbiology. Blackwell Science Ltd; 2001. pp. 725–734. doi:10.1046/j.1365-2672.2001.01419.x

129. Carpentier B, Chassaing D. Interactions in biofilms between Listeria monocytogenes and resident microorganisms from food industry premises. Int J Food Microbiol. 2004;97: 111–22. doi:10.1016/j.ijfoodmicro.2004.03.031

130. Daneshvar Alavi HE, Truelstrup Hansen L. Kinetics of biofilm formation and desiccation survival of Listeria monocytogenes in single and dual species biofilms with Pseudomonas fluorescens, Serratia proteamaculans or Shewanella baltica on food-grade stainless steel surfaces. Biofouling. 2013;29: 1253–68. doi:10.1080/08927014.2013.835805

131. Rieu A, Briandet R, Habimana O, Garmyn D, Guzzo J, Piveteau P. Listeria

monocytogenes EGD-e biofilms: no mushrooms but a network of knitted chains. Appl Environ Microbiol. 2008;74: 4491–7. doi:10.1128/AEM.00255-08

132. van der Veen S, Abee T. Mixed species biofilms of Listeria monocytogenes and Lactobacillus plantarum show enhanced resistance to benzalkonium chloride and peracetic acid. Int J Food Microbiol. Elsevier B.V.; 2011;144: 421–31. doi:10.1016/j.ijfoodmicro.2010.10.029

133. Leriche V, Carpentier B. Limitation of adhesion and growth of Listeria monocytogenes on stainless steel surfaces by Staphylococcus sciuri biofilms. J Appl Microbiol. 2000;88: 594–605. doi:10.1046/j.1365-2672.2000.01000.x

134. Giaouris E, Chorianopoulos N, Doulgeraki A, Nychas G-J. Co-culture with Listeria monocytogenes within a dual-species biofilm community strongly increases resistance of Pseudomonas putida to benzalkonium chloride. PLoS One. 2013;8: e77276. doi:10.1371/journal.pone.0077276

135. Kolenbrander PE. Oral microbial communities: biofilms, interactions, and genetic systems. Annual review of microbiology. 2000. pp. 413–437. doi:10.1146/annurev.micro.54.1.413

136. Benítez-Páez A, Belda-Ferre P, Simón-Soro A, Mira A. Microbiota diversity and gene expression dynamics in human oral biofilms. BMC Genomics. 2014;15: 311. doi:10.1186/1471-2164-15-311

137. Hall-Stoodley L, Stoodley P, Kathju S, Høiby N, Moser C, William Costerton J, et al. Towards diagnostic guidelines for biofilm-associated infections. FEMS Immunol Med Microbiol. 2012;65: 127–145. doi:10.1111/j.1574-695X.2012.00968.x

138. Yang L, Liu Y, Wu H, Song Z, H??iby N, Molin S, et al. Combating biofilms. FEMS Immunol Med Microbiol. 2012;65: 146–157. doi:10.1111/j.1574-695X.2011.00858.x

139. Yang L, Liu Y, Wu H, Hóiby N, Molin S, Song Z. Current understanding of multi-species biofilms. Int J Oral Sci. 2011;3: 74–81. doi:10.4248/IJOS11027

140. Chasseignaux E, Gérault P, Toquin MT, Salvat G, Colin P, Ermel G. Ecology of Listeria monocytogenes in the environment of raw poultry meat and raw pork meat processing plants. FEMS Microbiol Lett. 2002;210: 271–275. doi:10.1016/S0378-1097(02)00637-7

141. Bridier A, Sanchez-Vizuete P, Guilbaud M, Piard JC, Naïtali M, Briandet R. Biofilm-

associated persistence of food-borne pathogens. Food Microbiol. Elsevier Ltd; 2015;45: 167–178. doi:10.1016/j.fm.2014.04.015

142. Giaouris E, Heir E, Desvaux M, Hébraud M, Møretrø T, Langsrud S, et al. Intra- and inter-species interactions within biofilms of important foodborne bacterial pathogens. Front Microbiol. 2015;6: 1–26. doi:10.3389/fmicb.2015.00841

143. Røder HL, Raghupathi PK, Herschend J, Brejnrod A, Knøchel S, Sørensen SJ, et al. Interspecies interactions result in enhanced biofilm formation by co-cultures of bacteria isolated from a food processing environment. Food Microbiol. Elsevier Ltd; 2015;51: 18–24. doi:10.1016/j.fm.2015.04.008

144. Puga CH, SanJose C, Orgaz B. Spatial distribution of Listeria monocytogenes and Pseudomonas fluorescens in mixed biofilms. In: Hambrick EC, editor. Listeria monocytogenes: Food Sources, Prevalence and Management Strategies. New York, NY: Nova Publishers; 2014. pp. 115–132.

145. Rodríguez-López P, Carballo-Justo A, Draper LA, Cabo ML. Removal of Listeria monocytogenes dual-species biofilms using enzyme-benzalkonium chloride combined treatments. Biofouling. 2017;33: 45–58. doi:10.1080/08927014.2016.1261847

146. Almeida C, Azevedo NF, Santos S, Keevil CW, Vieira MJ. Discriminating multi-species populations in biofilms with peptide nucleic acid fluorescence in situ hybridization (PNA FISH). PLoS One. 2011;6: e14786. doi:10.1371/journal.pone.0014786

147. Puga CH, Orgaz B, SanJose C. Listeria monocytogenes Impact on Mature or Old Pseudomonas fluorescens Biofilms During Growth at 4 and 20°C. Front Microbiol. 2016;7: 1–9. doi:10.3389/fmicb.2016.00134

148. Puga CH, SanJose C, Orgaz B. Biofilm development at low temperatures enhances Listeria monocytogenes resistance to chitosan. Food Control. 2016;65: 143–151. doi:10.1016/j.foodcont.2016.01.012

149. Sutton S. Accuracy of plate counts. J Valid Technol. 2011;17: 42–46.

150. Liu J, Dazzo FB, Glagoleva O, Yu B, Jain AK. CMEIAS: A Computer-Aided System for the Image Analysis of Bacterial Morphotypes in Microbial Communities. Microb Ecol. 2001;41: 173–194. doi:10.1007/s002480000004

151. Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersboll BK, et al. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology. 2000;146: 2395–2407.

152. Yang X, Beyenal H, Harkin G, Lewandowski Z. Quantifying biofilm structure using image analysis. J Microbiol Methods. 2000;39: 109–119. doi:10.1016/S0167-7012(99)00097-4

153. Beyenal H, Lewandowski Z, Harkin G. Quantifying biofilm structure: facts and fiction. Biofouling. 2004;20: 1–23. doi:10.1080/0892701042000191628

154. de Carvalho C, da Fonseca MM. Assessment of three-dimensional biofilm structure using an optical microscope. Biotechniques. 2007;42: 616–620. doi:10.2144/000112403

155. Bridier a, Dubois-Brissonnet F, Boubetra a, Thomas V, Briandet R. The biofilm architecture of sixty opportunistic pathogens deciphered using a high throughput CLSM method. J Microbiol Methods. Elsevier B.V.; 2010;82: 64–70. doi:10.1016/j.mimet.2010.04.006

156. Dusane DH, Rajput JK, Kumar AR, Nancharaiah Y V, Venugopalan VP, Zinjarde SS. Disruption of fungal and bacterial biofilms by lauroyl glucose. Lett Appl Microbiol. 2008;47: 374–9. doi:10.1111/j.1472-765X.2008.02440.x

157. Jackson G, Beyenal H, Rees WM, Lewandowski Z. Growing reproducible biofilms with respect to structure and viable cell counts. J Microbiol Methods. 2001;47: 1–10. doi:10.1016/S0167-7012(01)00280-9

158. Lewandowski Z. Structure and function of biofilms. In: Evans L V, editor. Biofilms: Recent advances in their study and control. Harwood Academic Publishers; 2000. pp. 1–17.

159. Lewandowski Z, Webb D, Hamilton M, Harkin G. Quantifying biofilm structure. Water Sci Technol. 1999;39: 71–76. doi:10.1016/S0273-1223(99)00152-3

160. Christensen BE, Ertesvag H, Beyenal H, Lewandowski Z. Resistance of biofilms containing alginate-producing bacteria to disintegration by an alginate degrading enzyme (Algl). Biofouling. 2001;17: 203–210. doi:10.1080/08927010109378479

161. Fox E, Hunt K, O'Brien M, Jordan K. Listeria monocytogenes in Irish Farmhouse cheese processing environments. Int J Food Microbiol. Elsevier B.V.; 2011;145

Suppl: S39-45. doi:10.1016/j.ijfoodmicro.2010.10.012

162. Tompkin RB. Control of Listeria monocytogenes in the food-processing environment. J Food Prot. 2002;65: 709–725.

163. Vongkamjan K, Roof S, Stasiewicz MJ, Wiedmann M. Persistent Listeria monocytogenes subtypes isolated from a smoked fish processing facility included both phage susceptible and resistant isolates. Food Microbiol. Elsevier Ltd; 2013;35: 38–48. doi:10.1016/j.fm.2013.02.012

164. Wulff G, Gram L, Ahrens P, Vogel BF. One group of genetically similar Listeria monocytogenes strains frequently dominates and persists in several fish slaughter- and smokehouses. Appl Environ Microbiol. 2006;72: 4313–22. doi:10.1128/AEM.02288-05

165. Unnerstad H, Bannerman E, Bille J, Danielsson-Tham ML, Waak E, Tham W. Prolonged contamination of a dairy with Listeria monocytogenes. Netherlands Milk Dairy J. 1996;50: 493–499.

166. Morganti M, Scaltriti E, Cozzolino P, Bolzoni L, Casadei G, Pierantoni M, et al. Processing-dependent and clonal contamination patterns of Listeria monocytogenes in the cured ham food chain revealed by genetic analysis. Appl Environ Microbiol. 2015;82: 822–831. doi:10.1128/AEM.03103-15

167. Stasiewicz MJ, Oliver HF, Wiedmann M, den Bakker HC. Whole-genome sequencing allows for improved identification of persistent Listeria monocytogenes in food-associated environments. Appl Environ Microbiol. 2015;81: 6024–6037. doi:10.1128/AEM.01049-15

168. Kornacki JL, Gurtler JB. Incidence and Control of Listeria in Food Processing Facilities. In: Ryser ET, Marth EH, editors. Listeria, Listeriosis and Food Safety. 3rd ed. CRC Press, Taylor and Francis Group, Boca Raton, FL; 2007.

169. Muhterem-Uyar M, Dalmasso M, Bolocan AS, Hernandez M, Kapetanakou AE, Kuchta T, et al. Environmental sampling for Listeria monocytogenes control in food processing facilities reveals three contamination scenarios. Food Control. 2015;51: 94–107. doi:10.1016/j.foodcont.2014.10.042

170. Miettinen MK, Björkroth KJ, Korkeala HJ. Characterization of Listeria monocytogenes from an ice cream plant by serotyping and pulsed-field gel electrophoresis. Int J Food Microbiol. 1999;46: 187–192. doi:10.1016/S0168-

1605(98)00185-8

171. Autio T, Hielm S, Miettinen M, Aarnisalo K, Björkroth J, Mattila-Sandholm T, et al. Sources of Listeria monocytogenes Contamination in a Cold-Smoked Rainbow Trout Processing Plant Detected by Pulsed-Field Gel Electrophoresis Typing Sources of Listeria monocytogenes Contamination in a Cold-Smoked Rainbow Trout Processing Plant Detected by. Appl Environ Microbiol. 1999;65: 150–155.

172. Peran i Sala RM, de Balabarca VC, Etoundi JM, Odame-Darkwah J, Oppong-Otoo J, Hinson DCT, et al. Establishment of good hygiene practice-based microbiological criteria in food industries: Guidelines using an example for meat preparations. Food Control. 2015;58: 7–11. doi:10.1016/j.foodcont.2014.10.041

173. Rocourt J, Cossart P. Listeria monocytogenes. In: Doyle MP, Beuchat LR, Montville TJ, editors. Food Microbiology Fundamentals and Frontiers. Washington, DC: American Society for Microbiology; 1997. pp. 337–352.

174. Bonnet M, Montville TJ. Acid-tolerant Listeria monocytogenes persist in a model food system fermented with nisin-producing bacteria. Lett Appl Microbiol. Blackwell Science Ltd; 2005;40: 237–242. doi:10.1111/j.1472-765X.2005.01661.x

175. Cordeiro AL, Werner C. Enzymes for Antifouling Strategies. J Adhes Sci Technol. 2011;25: 2317–2344. doi:10.1163/016942411X574961

176. Lequette Y, Boels G, Clarisse M, Faille C. Using enzymes to remove biofilms of bacterial isolates sampled in the food-industry. Biofouling. 2010;26: 421–31. doi:10.1080/08927011003699535

177. Johansen C, Falholt P, Gram L. Enzymatic removal and disinfection of bacterial biofilms. Appl Environ Microbiol. 1997;63: 3724–3728.

178. Orgaz B, Kives J, Pedregosa AM, Monistrol IF, Laborda F, SanJose C. Bacterial biofilm removal using fungal enzymes. Enzyme Microb Technol. 2006;40: 51–56. doi:10.1016/j.enzmictec.2005.10.037

179. Kaplan JB. Biofilm matrix-degrading enzymes. Methods Mol Biol. Humana Press Inc.; 2014;1147: 203–13. doi:10.1007/978-1-4939-0467-9_14

180. Giaouris E, Heir E, Hébraud M, Chorianopoulos N, Langsrud S, Møretrø T, et al. Attachment and biofilm formation by foodborne bacteria in meat processing environments: Causes, implications, role of bacterial interactions and control by

alternative novel methods. Meat Sci. Elsevier Ltd; 2014;97: 289–309. doi:10.1016/j.meatsci.2013.05.023

181. Meireles A, Borges A, Giaouris E, Simões M. The current knowledge on the application of anti-biofilm enzymes in the food industry. Food Res Int. 2016;86: 140–146. doi:10.1016/j.foodres.2016.06.006

182. Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils – A review. Food Chem Toxicol. 2008;46: 446–475. doi:10.1016/j.fct.2007.09.106

183. Burt S. Essential oils: their antibacterial properties and potential applications in foods - a review. Int J Food Microbiol. 2004;94: 223–253.

184. Perricone M, Arace E, Corbo MR, Sinigaglia M, Bevilacqua A. Bioactivity of essential oils: A review on their interaction with food components. Frontiers in Microbiology. Frontiers Media SA; 2015. p. 76. doi:10.3389/fmicb.2015.00076

185. Viuda-Martos M, Ruiz Navajas Y, Sánchez Zapata E, Fernández-López J, Pérez-Álvarez JA. Antioxidant activity of essential oils of fi ve spice plants widely used in a Mediterranean diet. Flavour Fragr J. 2010;25: 13–19. doi:10.1002/ffj .1951

186. Hyldgaard M, Mygind T, Meyer RL. Essential oils in food preservation: Mode of action, synergies, and interactions with food matrix components. Front Microbiol. Frontiers; 2012;3: 12. doi:10.3389/fmicb.2012.00012

187. Cotter PD, Ross RP, Hill C. Bacteriocins - a viable alternative to antibiotics? Nat Rev Microbiol. 2013;11: 95–105. doi:10.1038/nrmicro2937

188. Bolocan AS, Pennone V, Connor PMO, Coffey A, Jordan K, Food T. Inhibition of Listeria monocytogenes biofilms by bacteriocin producing bacteria isolated from mushroom compost Corresponding author : J Appl Microbiol. 2016; 1–18. doi:10.1111/jam.13337

189. Pérez-Ibarreche M, Castellano P, Leclercq A, Vignolo G. Control of Listeria monocytogenes biofilms on industrial surfaces by the bacteriocin-producing Lactobacillus sakei CRL1862. FEMS Microbiology Letters. 2016. pp. 1–6. doi:10.1093/femsle/fnw118

190. Camargo AC, de Paula OAL, Todorov SD, Nero LA. In Vitro Evaluation of Bacteriocins Activity Against Listeria monocytogenes Biofilm Formation. Appl Biochem Biotechnol. 2016;178: 1239–1251. doi:10.1007/s12010-015-1941-3

191. Loc-Carrillo C, Abedon ST. Pros and cons of phage therapy. Bacteriophage. 2011;1: 111–114. doi:10.4161/bact.1.2.14590

192. Garcia P, Martinez B, Obeso JM, Rodriguez A. Bacteriophages and their application in food safety. Lett Appl Microbiol. 2008;47: 479–485. doi:10.1111/j.1472-765X.2008.02458.x

193. Sillankorva SM, Oliveira H, Azeredo J. Bacteriophages and their role in food safety. Int J Microbiol. 2012; 863945. doi:10.1155/2012/863945

194. Motlagh AM, Bhattacharjee AS, Goel R. Biofilm control with natural and genetically-modified phages. World J Microbiol Biotechnol. Springer Netherlands; 2016;32: 1–10. doi:10.1007/s11274-016-2009-4

195. Hagens S, Loessner MJ. Phages of Listeria offer novel tools for diagnostics and biocontrol. Front Microbiol. 2014;5: 159. doi:10.3389/fmicb.2014.00159

196. Klumpp J, Loessner MJ. Listeria phages: Genomes, evolution, and application. Bacteriophage. Taylor & Francis; 2013;3: e26861. doi:10.4161/bact.26861

197. Soni K a, Nannapaneni R. Removal of Listeria monocytogenes biofilms with bacteriophage P100. J Food Prot. 2010;73: 1519–1524.

198. Montañez-Izquierdo VY, Salas-Vázquez DI, Rodríguez-Jerez JJ. Use of epifluorescence microscopy to assess the effectiveness of phage P100 in controlling Listeria monocytogenes biofilms on stainless steel surfaces. Food Control. Elsevier Ltd; 2012;23: 470–477. doi:10.1016/j.foodcont.2011.08.016

199. EFSA. The use and mode of action of bacteriophages in food production -- Scientific Opinion of the Panel on Biological Hazards. EFSA J. 2009;10: 1–9. doi:10.2903/j.efsa.2015.3991

200. Marriott N, Gravani RB. Principles of food sanitation. 5th ed. New York, NY: Springer; 2006.

201. Mcdonnell G, Russell AD. Antiseptics and disinfectants: Activity, action, and resistance. Clin Microbiol Rev. 1999;12: 147–179. doi:10.1007/s13398-014-0173-7.2

202. Gerba CP. Quaternary Ammonium Biocides: Efficacy in Application. Appl Environ Microbiol. 2015;81: 464–469. doi:10.1128/AEM.02633-14

203. Tezel U, Pavlostathis SG. Quaternary ammonium disinfectants: microbial

adaptation, degradation and ecology. Curr Opin Biotechnol. Elsevier Ltd; 2015;33: 296–304. doi:10.1016/j.copbio.2015.03.018

204. Nakamura H, Takakura K-I, Sone Y, Itano Y, Nishikawa Y. Biofilm formation and resistance to benzalkonium chloride in Listeria monocytogenes isolated from a fish processing plant. J Food Prot. 2013;76: 1179–86. doi:10.4315/0362-028X.JFP-12-225

205. Houari A, Di Martino P. Effect of chlorhexidine and benzalkonium chloride on bacterial biofilm formation. Lett Appl Microbiol. 2007;45: 652–6. doi:10.1111/j.1472-765X.2007.02249.x

206. Machado I, Lopes SP, Sousa AM, Pereira MO. Adaptive response of single and binary Pseudomonas aeruginosa and Escherichia coli biofilms to benzalkonium chloride. J Basic Microbiol. 2012;52: 43–52. doi:10.1002/jobm.201100137

207. Phillips CA. Bacterial biofilms in food processing environments: a review of recent developments in chemical and biological control. Int J Food Sci Technol. 2016;51: 1731–1743. doi:10.1111/ijfs.13159

208. Stiefel P, Mauerhofer S, Schneider J, Maniura-Weber K, Rosenberg U, Ren Q. Enzymes enhance biofilm removal efficiency of cleaners. Antimicrob Agents Chemother. 2016;60: 3647–3652. doi:10.1128/AAC.00400-16

209. Vickery K, Pajkos A, Cossart Y. Removal of biofilm from endoscopes: Evaluation of detergent efficiency. Am J Infect Control. 2004;32: 170–176. doi:10.1016/j.ajic.2003.10.009

210. Parkar SG, Flint SH, Brooks JD. Evaluation of the effect of cleaning regimes on biofilms of thermophilic bacilli on stainless steel. J Appl Microbiol. 2004;96: 110–116. doi:10.1046/j.1365-2672.2003.02136.x

211. Kaplan JB. Therapeutic potential of biofilm-dispersing enzymes. Int J Artif Organs. 2009;32: 545–554.

212. Denyer SP, Stewart GSAB. Mechanisms of action of disinfectants. Int Biodeterior Biodegrad. 1998;41: 261–268. doi:10.1016/S0964-8305(98)00023-7

213. Maillard JY. Bacterial target sites for biocide action. J Appl Microbiol Symp Suppl. 2002;92: 16S–27S.

214. Rahman S, Khan I, Oh DH. Electrolyzed Water as a Novel Sanitizer in the Food

Industry: Current Trends and Future Perspectives. Compr Rev Food Sci Food Saf. 2016;15: 471–490. doi:10.1111/1541-4337.12200

215. Ayebah B, Hung Y-C, Kim C, Frank JF. Efficacy of electrolyzed water in the inactivation of planktonic and biofilm Listeria monocytogenes in the presence of organic matter. J Food Prot. 2006;69: 2143–2150.

216. Luo K, Oh DH. Inactivation kinetics of Listeria monocytogenes and Salmonella enterica serovar Typhimurium on fresh-cut bell pepper treated with slightly acidic electrolyzed water combined with ultrasound and mild heat. Food Microbiol. 2016;53: 165–171. doi:10.1016/j.fm.2015.09.014

217. Du S, Zhang Z, Xiao L, Lou Y, Pan Y, Zhao Y. Acidic electrolyzed water as a novel transmitting medium for high hydrostatic pressure reduction of bacterial loads on shelled fresh shrimp. Front Microbiol. 2016;7: 305. doi:10.3389/fmicb.2016.00305

218. Wirtanen G, Salo S. Disinfection in food processing – efficacy testing of disinfectants. Rev Environ Sci Biotechnol. 2003;2: 293–306. doi:10.1023/B:RESB.0000040471.15700.03

219. Romanova NA, Wolffs PFG, Brovko LY. Role of Efflux Pumps in Adaptation and Resistance of Listeria monocytogenes to Benzalkonium Chloride Role of Efflux Pumps in Adaptation and Resistance of Listeria monocytogenes to Benzalkonium Chloride. Appl Environ Microbiol. 2006;72: 3498–3503. doi:10.1128/AEM.72.5.3498-3503.2006

220. Kovacevic J, Ziegler J, Walecka-Zacharska E, Reimer A, Kitts DD, Gilmour MW. Tolerance of Listeria monocytogenes to quaternary ammonium sanitizers is mediated by a novel efflux pump encoded by emrE. Appl Environ Microbiol. 2015;82: 939–953. doi:10.1128/AEM.03741-15

221. Jiang X, Yu T, Liang Y, Ji S, Guo X, Ma J, et al. Efflux pump-mediated benzalkonium chloride resistance in Listeria monocytogenes isolated from retail food. Int J Food Microbiol. 2016;217: 141–145. doi:10.1016/j.ijfoodmicro.2015.10.022

222. Martinez-Suarez J V., Ortiz S, López-Alonso V. Potential impact of the resistance to quaternary ammonium disinfectants on the persistence of Listeria monocytogenes in food processing environments. Front Microbiol. Frontiers Media SA; 2016;7: 1–8. doi:10.3389/fmicb.2016.00638

223. To MS, Favrin S, Romanova N, Griffiths MW. Postadaptational resistance to

benzalkonium chloride and subsequent physicochemical modifications of Listeria monocytogenes. Appl Environ Microbiol. 2002;68: 5258–5264. doi:10.1128/AEM.68.11.5258-5264.2002

224. Møretrø T, Schirmer BCT, Heir E, Fagerlund A, Hjemli P, Langsrud S. Tolerance to quaternary ammonium compound disinfectants may enhance growth of Listeria monocytogenes in the food industry. Int J Food Microbiol. 2017;241: 215–224. doi:10.1016/j.ijfoodmicro.2016.10.025

225. Costerton JW, Stewart PS, Geenberg EP. Bacterial biofilms: a common cause of persistent infections. Science (80-). 1999;284: 1318–1322.

226. Fatemi P, Frank JF. Inactivation of Listeria monocytogenes/Pseudomonas biofilms by peracid sanitizers. J Food Prot. International Association for Food Protection; 1999;62: 761–5.

227. Campanac C, Pineau L, Payard A, Baziard-Mouysset G, Roques C. Interactions between biocide cationic agents and bacterial biofilms. Antimicrob Agents Chemother. 2002;46: 1469–1474. doi:10.1128/AAC.46.5.1469-1474.2002

228. Mah T-FC, O'Toole G a. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 2001;9: 34–39. doi:10.1016/S0966-842X(00)01913-2

229. Corona F, Martinez J. Phenotypic Resistance to Antibiotics. Antibiotics. 2013;2: 237–255. doi:10.3390/antibiotics2020237

230. Saá Ibusquiza P, Herrera JJR, Vázquez-Sánchez D, Cabo ML. Adherence kinetics, resistance to benzalkonium chloride and microscopic analysis of mixed biofilms formed by Listeria monocytogenes and Pseudomonas putida. Food Control. 2012;25: 202–210. doi:10.1016/j.foodcont.2011.10.002

231. Kostaki M, Chorianopoulos N, Braxou E, Nychas G-J, Giaouris E. Differential biofilm formation and chemical disinfection resistance of sessile cells of listeria monocytogenes strains under monospecies and dual-species (with Salmonella enterica) conditions. Appl Environ Microbiol. 2012;78: 2586–95. doi:10.1128/AEM.07099-11

232. Fox EM, Leonard N, Jordan K. Molecular Diversity of Listeria monocytogenes Isolated from Irish Dairy Farms. 2011;8.

233. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification

for phylogenetic study. J Bacteriol. 1991;173: 697–703.

234. Van Belkum A, Kluytmans J, Van Leeuwen W, Bax R, Quint W, Fluit A, et al. Multicenter evaluation of arbitrarily primed PCR for typing of Staphylococcus aureus strains . Multicenter Evaluation of Arbitrarily Primed PCR for Typing of Staphylococcus aureus Strains. J Clin Microbiol. 1995;33: 1537–1547.

235. Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey S V. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 1990;18: 6531–6535. doi:10.1093/nar/18.22.6531

236. Halpin JL, Garrett NM, Ribot EM, Graves LM, Cooper KL. Re-evaluation, optimization, and multilaboratory validation of the PulseNet-standardized pulsed-field gel electrophoresis protocol for Listeria monocytogenes. Foodborne Pathog Dis. 2010;7: 293–298. doi:10.1089/fpd.2009.0394

237. Vázquez-Sánchez D, López-Cabo M, Saá-Ibusquiza P, Rodríguez-Herrera JJ. Incidence and characterization of Staphylococcus aureus in fishery products marketed in Galicia (Northwest Spain). Int J Food Microbiol. Elsevier B.V.; 2012;157: 286–296. doi:10.1016/j.ijfoodmicro.2012.05.021

238. Hunter PR, Gaston MA. Numerical Index of the Discriminatory Ability of Typing Systems : an Application of Simpson â€™ s Index of Diversity. 1988;26: 2465–2466.

239. Tenover FC, Arbeit RD, Goering R V, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed- field gel electrophoresis: Criteria for bacterial strain typing. J Clin Microbiol. 1995;33: 2233–2239. doi:0095-1137/

240. Bagge-Ravn D, Ng Y, Hjelm M, Christiansen JN, Johansen C, Gram L. The microbial ecology of processing equipment in different fish industries—analysis of the microflora during processing and following cleaning and disinfection. Int J Food Microbiol. 2003;87: 239–250. doi:10.1016/S0168-1605(03)00067-9

241. Gram L, Dalgaard P. Fish spoilage bacteria – problems and solutions. Curr Opin Biotechnol. 2002;13: 262–266. doi:10.1016/S0958-1669(02)00309-9

242. Ercolini D, Russo F, Nasi A, Ferranti P, Villani F. Mesophilic and Psychrotrophic Bacteria from Meat and Their Spoilage Potential In Vitro and in Beef. Appl Environ Microbiol. 2009;75: 1990–2001. doi:10.1128/AEM.02762-08

243. Furet J-P, Firmesse O, Gourmelon M, Bridonneau C, Tap J, Mondot S, et al. Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR. FEMS Microbiol Ecol. 2009;68: 351–362. doi:10.1111/j.1574-6941.2009.00671.x

244. Walk ST, Alm EW, Calhoun LM, Mladonicky JM, Whittam TS. Genetic diversity and population structure of Escherichia coli isolated from freshwater beaches. Environ Microbiol. 2007;9: 2274–2288. doi:10.1111/j.1462-2920.2007.01341.x

245. Costa RA. Escherichia coli in seafood : A brief overview. 2013;2013: 450–454.

246. Gómez D, Ariño A, Carramiñana JJ, Rota C, Yangüela J. Comparison of sampling procedures for recovery of Listeria monocytogenes from stainless steel food contact surfaces. J Food Prot. 2012;75: 1077–82. doi:10.4315/0362-028X.JFP-11-421

247. Gudmundsdóttir S, Gudbjörnsdóttir B, Lauzon HL, Einarsson H, Kristinsson KG, Kristjánsson M. Tracing Listeria monocytogenes isolates from cold-smoked salmon and its processing environment in Iceland using pulsed-field gel electrophoresis. Int J Food Microbiol. 2005;101: 41–51. doi:10.1016/j.ijfoodmicro.2004.08.023

248. Chitlapilly Dass S, Abu-Ghannam N, Antony-Babu S, J. Cummins E. Ecology and molecular typing of L. monocytogenes in a processing plant for cold-smoked salmon in the Republic of Ireland. Food Res Int. Elsevier Ltd; 2010;43: 1529–1536. doi:10.1016/j.foodres.2010.04.030

249. Rivoal K, Fablet A, Courtillon C, Bougeard S, Chemaly M, Protais J. Detection of Listeria spp. in liquid egg products and in the egg breaking plants environment and tracking of Listeria monocytogenes by PFGE. Int J Food Microbiol. 2013;166: 109–116. doi:10.1016/j.ijfoodmicro.2013.06.014

250. Thévenot D, Delignette-Muller ML, Christieans S, Vernozy-Rozand C. Prevalence of Listeria monocytogenes in 13 dried sausage processing plants and their products. Int J Food Microbiol. 2005;102: 85–94. doi:10.1016/j.ijfoodmicro.2004.12.008

251. Kovačević J, McIntyre LF, Henderson SB, Kosatsky T. Occurrence and distribution of listeria species in facilities producing ready-to-eat foods in British Columbia, Canada. J Food Prot. 2012;75: 216–24. doi:10.4315/0362-028X.JFP-11-300

252. Serraino a, Giacometti F, Piva S, Florio D, Pizzamiglio V, Zanoni RG. Isolation of glucosidase and phospholipase positive Bacillus circulans on ALOA medium. Lett Appl Microbiol. 2011;53: 244–6. doi:10.1111/j.1472-765X.2011.03084.x

253. Valderrama WB, Cutter CN. An ecological perspective of Listeria monocytogenes biofilms in food processing facilities. Crit Rev Food Sci Nutr. 2013;53: 801–17. doi:10.1080/10408398.2011.561378

254. Gianfranceschi M, Gattuso A, Tartaro S, Aureli P. Incidence of Listeria monocytogenes in food and environmental samples in Italy between 1990 and 1999: Serotype distribution in food, environmental and clinical samples. Eur J Epidemiol. 2003;18: 1001–1006. doi:10.1023/A:1025849532417

255. Lambertz ST, Ivarsson S, Lopez-Valladares G, Sidstedt M, Lindqvist R. Subtyping of Listeria monocytogenes isolates recovered from retail ready-to-eat foods, processing plants and listeriosis patients in Sweden 2010. Int J Food Microbiol. 2013;166: 186–192. doi:10.1016/j.ijfoodmicro.2013.06.008

256. Nucera D, Lomonaco S, Bianchi DM, Decastelli L, Grassi MA, Bottero MT, et al. A five year surveillance report on PFGE types of Listeria monocytogenes isolated in Italy from food and food related environments. Int J Food Microbiol. 2010;140: 271–276. doi:10.1016/j.ijfoodmicro.2010.04.016

257. Fox E, Leonard N, Jordan K. Physiological and transcriptional characterization of persistent and nonpersistent Listeria monocytogenes isolates. Appl Environ Microbiol. 2011;77: 6559–69. doi:10.1128/AEM.05529-11

258. Verghese B, Lok M, Wen J, Alessandria V, Chen Y, Kathariou S, et al. comK prophage junction fragments as markers for Listeria monocytogenes genotypes unique to individual meat and poultry processing plants and a model for rapid niche-specific adaptation, biofilm formation, and persistence. Appl Environ Microbiol. 2011;77: 3279–92. doi:10.1128/AEM.00546-11

259. Gram L, Ravn L, Rasch M, Bruhn JB, Christensen AB, Givskov M. Food spoilage—interactions between food spoilage bacteria. Int J Food Microbiol. 2002;78: 79–97. doi:10.1016/S0168-1605(02)00233-7

260. Talon R, Lebert I, Lebert a, Leroy S, Garriga M, Aymerich T, et al. Traditional dry fermented sausages produced in small-scale processing units in Mediterranean countries and Slovakia. 1: Microbial ecosystems of processing environments. Meat Sci. 2007;77: 570–9. doi:10.1016/j.meatsci.2007.05.006

261. Schirmer BCT, Heir E, Møretrø T, Skaar I, Langsrud S. Microbial background flora in small-scale cheese production facilities does not inhibit growth and surface

attachment of Listeria monocytogenes. J Dairy Sci. 2013;96: 6161–6171. doi:10.3168/jds.2012-6395

262. Campos C a., Mazzotta AS, Montville TJ. Inhibition of Listeria Monocytogenes By Carnobacterium Piscicola in Vacuum-Packaged Cooked Chicken At Refrigeration Temperatures. J Food Saf. 1997;17: 151–160. doi:10.1111/j.1745-4565.1997.tb00184.x

263. Brillet a, Pilet M-F, Prevost H, Bouttefroy a, Leroi F. Biodiversity of Listeria monocytogenes sensitivity to bacteriocin-producing Carnobacterium strains and application in sterile cold-smoked salmon. J Appl Microbiol. 2004;97: 1029–37. doi:10.1111/j.1365-2672.2004.02383.x

264. Lourenço A, Machado H, Brito L. Biofilms of Listeria monocytogenes produced at 12 °C either in pure culture or in co-culture with Pseudomonas aeruginosa showed reduced susceptibility to sanitizers. J Food Sci. 2011;76: M143-8. doi:10.1111/j.1750-3841.2010.02011.x

265. EFSA. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. EFSA J 2015. 2015;13: 3991.

266. Giaouris E, Chorianopoulos N, Nychas G-J. Impact of acid adaptation on attachment of Listeria monocytogenes to stainless steel during long-term incubation under low or moderate temperature conditions and on subsequent recalcitrance of attached cells to lethal acid treatments. Int J Food Microbiol. 2014;171: 1–7. doi:10.1016/j.ijfoodmicro.2013.11.013

267. Norwood DE, Gilmour A. The growth and resistance to sodium hypochlorite of Listeria monocytogenes in a steady-state multispecies biofilm. J Appl Microbiol. 2000;88: 512–520. doi:10.1046/j.1365-2672.2000.00990.x

268. Allison DG, Ruiz B, Sanjose C, Jaspe A, Gilbert P. Extracellular products as mediators of the formation and detachment of Pseudomonas fluorescens biofilms. FEMS Microbiol Lett. 1998;167: 179–184. doi:10.1016/S0378-1097(98)00386-3

269. Fortineau N, Trieu-Cuot P, Gaillot O, Pellegrini E, Berche P, Gaillard J-L. Optimization of green fluorescent protein expression vectors for in vitro and in vivo detection of Listeria monocytogenes. Res Microbiol. 2000;151: 353–360. doi:10.1016/S0923-2508(00)00158-3

270. Lauer P, Yin M, Chow N, Loessner MJ, Portnoy A, Calendar R, et al. Construction ,

Characterization , and Use of Two Listeria monocytogenes Site-Specific Phage Integration Vectors Construction , Characterization , and Use of Two Listeria monocytogenes Site-Specific Phage Integration Vectors. J Bacteriol. 2002;184: 4177–4186. doi:10.1128/JB.184.15.4177

271. Monk IR, Gahan CGM, Hill C. Tools for functional postgenomic analysis of listeria monocytogenes. Appl Environ Microbiol. 2008;74: 3921–34. doi:10.1128/AEM.00314-08

272. Bron PA, Monk IR, Corr SC, Hill C, Gahan CGM. Novel luciferase reporter system for in vitro and organ-specific monitoring of differential gene expression in Listeria monocytogenes. Appl Environ Microbiol. 2006;72: 2876–84. doi:10.1128/AEM.72.4.2876-2884.2006

273. Hillyar CRT. Genetic recombination in bacteriophage lambda. Biosci Horizons. 2012;5: 1–7.

274. Serra-Moreno R, Acosta S, Hernalsteens JP, Jofre J, Muniesa M. Use of the lambda Red recombinase system to produce recombinant prophages carrying antibiotic resistance genes. BMC Mol Biol. 2006;7: 31. doi:10.1186/1471-2199-7-31

275. Gonzales MF, Brooks T, Pukatzki SU, Provenzano D. Rapid protocol for preparation of electrocompetent Escherichia coli and Vibrio cholerae. J Vis Exp. 2013;50684: e50684. doi:10.3791/50684

276. Chaveroche MK, Ghigo JM, d'Enfert C. A rapid method for efficient gene replacement in the filamentous fungus Aspergillus nidulans. Nucleic Acids Res. 2000;28: E97.

277. Sutcliffe JG. Nucleotide sequence of the ampicillin resistance gene of Escherichia coli plasmid pBR322. Proc Natl Acad Sci. 1978;75: 3737–3741. doi:10.1073/pnas.75.8.3737

278. Lagendijk EL, Validov S, Lamers GEM, de Weert S, Bloemberg G V. Genetic tools for tagging Gram-negative bacteria with mCherry for visualization in vitro and in natural habitats, biofilm and pathogenicity studies. FEMS Microbiol Lett. 2010;305: 81–90. doi:10.1111/j.1574-6968.2010.01916.x

279. Cabo ML, Murado MA, Gonzalez MP, Pastoriza L. A method for bacteriocin quantification. J Appl Microbiol. 1999;87: 907–914. doi:10.1046/j.1365-2672.1999.00942.x

280. Knight KP, McKellar RC. Influence of cinnamon and clove essential oils on the D- and z-values of Escherichia coli O157:H7 in apple cider. J Food Prot. 2007;70: 2089–94.

281. Murado MA, Vázquez JA. The notion of hormesis and the dose-response theory: A unified approach. J Theor Biol. 2007;244: 489–499. doi:10.1016/j.jtbi.2006.09.002

282. Murado MA, González MP, Vázquez JA. Dose–response relationships: an overview, a generative model and its application to the verification of descriptive models. Enzyme Microb Technol. 2002;31: 439–455. doi:10.1016/S0141-0229(02)00109-6

283. Azevedo AS, Almeida C, Melo LF, Azevedo NF. Interaction between atypical microorganisms and E. coli in catheter-associated urinary tract biofilms. Biofouling. Taylor & Francis; 2014;30: 893–902. doi:10.1080/08927014.2014.944173

284. Gião MS, Keevil CW. Listeria monocytogenes can form biofilms in tap water and enter into the viable but non-cultivable state. Microb Ecol. 2014;67: 603–11. doi:10.1007/s00248-013-0364-3

285. Lowder M, Unge A, Maraha N, Jansson JK, Swiggett J, Oliver JD. Effect of Starvation and the Viable-but-Nonculturable State on Green Fluorescent Protein (GFP) Fluorescence in GFP-Tagged Pseudomonas fluorescens A506. Appl Environ Microbiol. 2000;66: 3160–3165. doi:10.1128/AEM.66.8.3160-3165.2000

286. Cho J-C, Kim S-J. Viable, but non-culturable, state of a green fluorescence protein-tagged environmental isolate of Salmonella typhi in groundwater and pond water. FEMS Microbiol Lett. The Oxford University Press; 1999;170: 257–264. doi:10.1111/j.1574-6968.1999.tb13382.x

287. Thallinger B, Prasetyo EN, Nyanhongo GS, Guebitz GM. Antimicrobial enzymes: an emerging strategy to fight microbes and microbial biofilms. Biotechnol J. 2013;8: 97–109. doi:10.1002/biot.201200313

288. Vilain S, Pretorius JM, Theron J, Brözel VS. DNA as an adhesin: Bacillus cereus requires extracellular DNA to form biofilms. Appl Environ Microbiol. 2009;75: 2861–8. doi:10.1128/AEM.01317-08

289. Qin Z, Ou Y, Yang L, Zhu Y, Tolker-Nielsen T, Molin S, et al. Role of autolysin-mediated DNA release in biofilm formation of Staphylococcus epidermidis. Microbiology. 2007;153: 2083–2092. doi:10.1099/mic.0.2007/006031-0

290. Izano EA, Amarante MA, Kher WB, Kaplan JB. Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in Staphylococcus aureus and Staphylococcus epidermidis biofilms. Appl Environ Microbiol. 2008;74: 470–476. doi:10.1128/AEM.02073-07

291. Sanchez-Vizuete P, Orgaz B, Aymerich S, Le Coq D, Briandet R. Pathogens protection against the action of disinfectants in multispecies biofilms. Front Microbiol. 2015;6: 705. doi:10.3389/fmicb.2015.00705

292. Sutherland IW. Microbial polysaccharides from Gram-negative bacteria. Int Dairy J. 2001;11: 663–674. doi:10.1016/S0958-6946(01)00112-1

293. Todd ECD, Greig JD, Bartleson CA, Michaels BS. Outbreaks Where Food Workers Have Been Implicated in the Spread of Foodborne Disease. Part 6. Survival of Pathogens in the Food Processing and Preparation environment. J Food Prot. 2009;72: 202–219.

294. Kusumaningrum HD, Riboldi G, Hazeleger WC, Beumer RR. Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. Int J Food Microbiol. 2003;85: 227–236. doi:10.1016/S0168-1605(02)00540-8

295. Augustin M, Ali-Vehmas T, Atroshi F. Assessment of enzymatic cleaning agents and disinfectants against bacterial biofilms. J Pharm Pharm Sci. 2004;7: 55–64.

296. Miajlovic H, Smith SG. Bacterial self-defence: How Escherichia coli evades serum killing. FEMS Microbiology Letters. 2014. pp. 1–9. doi:10.1111/1574-6968.12419

297. Allison DG. The biofilm matrix. Biofouling. 2003;19: 139–50. doi:10.1080/0892701031000072190

298. Longhi C, Scoarughi GL, Poggiali F, Cellini A, Carpentieri A, Seganti L, et al. Protease treatment affects both invasion ability and biofilm formation in Listeria monocytogenes. Microb Pathog. 2008;45: 45–52. doi:10.1016/j.micpath.2008.01.007

299. Leite P, Rodrigues R, Ferreira M, Ribeiro G, Jacquet C, Martin P, et al. Comparative characterization of Listeria monocytogenes isolated from Portuguese farmhouse ewe's cheese and from humans. Int J Food Microbiol. 2006;106: 111–21. doi:10.1016/j.ijfoodmicro.2005.05.017

300. Synder LR, Kirkland JJ, Doland JW. Method Validation. Introduction to Modern

Liquid Chromatography, Third edition. 2010. pp. 531–567. doi:10.1002/9780470508183.ch12

301. Mark Green J. A practical guide to analytical method validation. Anal Chem. 1996;68.

302. Box GEP. Multi-Factor Designs of First Order. Biometrika. 1952;39: 49–57. doi:10.1093/biomet/39.1-2.49

303. Box GEP, Hunter WG, Hunter JS. Diseños factoriales y transformación de datos. Estadística para investigadores Diseño, innovación y descubrimiento (2nd Edition). 2nd ed. Ed. Reverté; 2008.

304. Bayles KW. The biological role of death and lysis in biofilm development. Nat Rev Microbiol. 2007;5: 721–6. doi:10.1038/nrmicro1743

305. Narahashi Y. Pronase. Methods Enzymol. 1970;19: 651–664. doi:10.1016/0076-6879(70)19052-5

306. Kumar ABV, Gowda LR, Tharanathan RN. Non-specific depolymerization of chitosan by pronase and characterization of the resultant products. Eur J Biochem. 2004;271: 713–723. doi:10.1111/j.1432-1033.2003.03975.x

307. Inoue T, Shingaki R, Sogawa N, Sogawa CA, Asaumi J, Kokeguchi S, et al. Biofilm formation by a fimbriae-deficient mutant of Actinobacillus actinomycetemcomitans. Microbiol Immunol. 2003;47: 877–881. doi:10.1111/j.1348-0421.2003.tb03454.x

308. Orgaz B, Neufeld RJ, SanJose C. Single-step biofilm removal with delayed release encapsulated Pronase mixed with soluble enzymes. Enzyme Microb Technol. 2007;40: 1045–1051. doi:10.1016/j.enzmictec.2006.08.003

309. Buffet-Bataillon S, Tattevin P, Bonnaure-Mallet M, Jolivet-Gougeon A. Emergence of resistance to antibacterial agents: The role of quaternary ammonium compounds - A critical review. International Journal of Antimicrobial Agents. 2012. pp. 381–389. doi:10.1016/j.ijantimicag.2012.01.011

310. Loeffler JM, Nelson D, Fischetti VA. Rapid killing of Streptococcus pneumoniae with a bacteriophage cell wall hydrolase. Science (80-). American Association for the Advancement of Science; 2001;294: 2170–2172. doi:10.1126/science.1066869

311. Oliver JD. The viable but nonculturable state in bacteria. J Microbiol. 2005;43: 93–100.

312. Li L, Mendis N, Trigui H, Oliver JD, Faucher SP. The importance of the viable but non-culturable state in human bacterial pathogens. Front Microbiol. 2014;5: 1–1. doi:10.3389/fmicb.2014.00258

313. Besnard V, Federighi M, Declerq E, Jugiau F, Cappelier JM. Environmental and physico-chemical factors induce VBNC state in Listeria monocytogenes. Vet Res. 2002;33: 359–370.

314. Shen Y, Huang C, Monroy GL, Janjaroen D, Derlon N, Lin J, et al. Response of Simulated Drinking Water Biofilm Mechanical and Structural Properties to Long-Term Disinfectant Exposure. Environ Sci Technol. 2016;50: 1779–1787. doi:10.1021/acs.est.5b04653

315. Selan L, Berlutti F, Passariello C, Berlutii F, Comodi-ballanti MR, Thaller MC. Proteolytic enzymes : a new treatment strategy for prosthetic infections ? Proteolytic Enzymes : a New Treatment Strategy for Prosthetic Infections ? Antimicrob Agents Chemother. 1993;37: 2618–2621. doi:10.1128/AAC.37.12.2618.Updated

316. Saá Ibusquiza P, Herrera JJR, Vázquez-Sánchez D, Parada A, Cabo ML. A new and efficient method to obtain benzalkonium chloride adapted cells of Listeria monocytogenes. J Microbiol Methods. Elsevier B.V.; 2012;91: 57–61. doi:10.1016/j.mimet.2012.07.009

317. Moen B, Rudi K, Bore E, Langsrud S. Subminimal inhibitory concentrations of the disinfectant benzalkonium chloride select for a tolerant subpopulation of Escherichia coli with inheritable characteristics. Int J Mol Sci. 2012;13: 4101–4123. doi:10.3390/ijms13044101

318. Bore E, Hébraud M, Chafsey I, Chambon C, Skjæret C, Moen B, et al. Adapted tolerance to benzalkonium chloride in Escherichia coli K-12 studied by transcriptome and proteome analyses. Microbiology. 2007;153: 935–946. doi:10.1099/mic.0.29288-0

319. Malone M, Goeres DM, Gosbell I, Vickery K, Jensen S, Stoodley P. Approaches to biofilm-associated infections: the need for standardized and relevant biofilm methods for clinical applications. Expert Rev Anti Infect Ther. 2016; doi:10.1080/14787210.2017.1262257

320. Wesche AM, Gurtler JB, Marks BP, Ryser ET. Stress, sublethal injury, resuscitation, and virulence of bacterial foodborne pathogens. J Food Prot. 2009;72: 1121–1138.

321. Wu VCH. A review of microbial injury and recovery methods in food. Food Microbiology. 2008. pp. 735–744. doi:10.1016/j.fm.2008.04.011

322. Simões M, Simões LC, Machado I, Pereira MO, Vieira MJ. Control of Flow-Generated Biofilms with Surfactants. Food Bioprod Process. 2006;84: 338–345. doi:10.1205/fbp06022

323. Aase B, Sundheim G, Langsrud S, Rørvik LM. Occurrence of and a possible mechanism for resistance to a quaternary ammonium compound in Listeria monocytogenes. Int J Food Microbiol. 2000;62: 57–63. doi:10.1016/S0168-1605(00)00357-3

324. Bisbiroulas P, Psylou M, Iliopoulou I, Diakogiannis I, Berberi A, Mastronicolis SK. Adaptational changes in cellular phospholipids and fatty acid composition of the food pathogen Listeria monocytogenes as a stress response to disinfectant sanitizer benzalkonium chloride. Lett Appl Microbiol. 2011;52: 275–280. doi:10.1111/j.1472-765X.2010.02995.x

325. Langsrud S, Sundheim G, Holck AL. Cross-resistance to antibiotics of Escherichia coli adapted to benzalkonium chloride or exposed to stress-inducers. J Appl Microbiol. 2004;96: 201–208. doi:10.1046/j.1365-2672.2003.02140.x

326. Donnelly CW. Detection and isolation of Listeria monocytogenes from food samples: Implications of sublethal injury. J AOAC Int. 2002;85: 495–500.

327. Tamburro M, Ripabelli G, Vitullo M, Dallman TJ, Pontello M, Amar CFL, et al. Gene expression in Listeria monocytogenes exposed to sublethal concentration of benzalkonium chloride. Comp Immunol Microbiol Infect Dis. 2015;40: 31–39. doi:10.1016/j.cimid.2015.03.004

328. Rakic-Martinez M, Drevets DA, Dutta V, Katic V, Kathariou S. Listeria monocytogenes strains selected on ciprofloxacin or the disinfectant benzalkonium chloride exhibit reduced susceptibility to ciprofloxacin, gentamicin, benzalkonium chloride, and other toxic compounds. Appl Environ Microbiol. 2011;77: 8714–8721. doi:10.1128/AEM.05941-11

329. Langsrud S, Sundheim G. Factors contributing to the survival of poultry associated Pseudomonas spp. exposed to a quaternary ammonium compound. J Appl Microbiol. Blackwell Science Ltd; 1997;82: 705–712. doi:10.1046/j.1365-2672.1997.00186.x

330. Sutherland IW. Biofilm exopolysaccharides: A strong and sticky framework. Microbiology. 2001;147: 3–9. doi:10.1051/lait:2001108

331. Hanna A, Berg M, Stout V, Razatos A. Role of capsular colanic acid in adhesion of uropathogenic Escherichia coli. Appl Environ Microbiol. 2003;69: 4474–4481. doi:10.1128/AEM.69.8.4474-4481.2003

332. Gaulin C, Lê M, Shum M, Fong D. Disinfectants and sanitizers for use on food contact surfaces. Natl Collab Cent Environ Heal. 2011; 1–15.

333. Soumet C, Méheust D, Pissavin C, Le Grandois P, Frémaux B, Feurer C, et al. Reduced susceptibilities to biocides and resistance to antibiotics in food-associated bacteria following exposure to quaternary ammonium compounds. J Appl Microbiol. 2016;121: 1275–1281. doi:10.1111/jam.13247

334. Lundén J, Autio T, Markkula A, Hellström S, Korkeala H. Adaptive and cross-adaptive responses of persistent and non-persistent Listeria monocytogenes strains to disinfectants. Int J Food Microbiol. 2003;82: 265–272. doi:10.1016/S0168-1605(02)00312-4

List of original publications

The present thesis is based on the following articles:

Chapter 2

Rodríguez-López P, Saá-Ibusquiza P, Mosquera-Fernández M, López-Cabo M. Listeria monocytogenes-carrying consortia in food industry. Composition, subtyping and numerical characterisation of mono-species biofilms dynamics on stainless steel. Int. J. Food Micro. 2015; 206:84-95 doi:10.1016/j.ijfoodmicro.2015.05.003

Chapter 3

Rodríguez-López P, Carballo-Justo A, Draper L A, Cabo M L. Removal of Listeria monocytogenes dual-species biofilms using enzyme-benzalkonium chloride combined treatments. Biofouling. 2017; 33(1):45-58 doi:10.1080/08927014.2016.1261847

Chapter 4

Rodríguez-López P, Puga C H, Orgaz B, Cabo M L. Quantifying the combined effects of pronase and benzalkonium chloride to remove late-stage Listeria monocytogenes dual-species biofilms. Submitted to Biofouling.

Chapter 5

Rodríguez-López P, Cabo M L. Assessment of tolerance development in Listeria monocytogenes-Escherichia coli mixed biofilms after pronase-benzalkonium chloride exposure. Submitted to Food Microbiology.

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A mi abuela. Un ejemplo a seguir.

A mi padre. Tú que me diste todos los recursos necesarios para llegar donde estoy hoy espero que, estés donde estés, puedas ver el resultado.

A mi madre. Gran parte de este trabajo es mérito tuyo, por proporcionarme siempre la ayuda y los consejos necesarios en el momento justo, incluso en las épocas en las que los ánimos no acompañaban. Por tu fortaleza y sabiduría, siempre vas a ser un referente en mi vida.

A tod@s nuevamente, gracias.

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