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Listeria monocytogenes biofilms produced under nutrient scarcity and cold stress: disinfectant susceptibility of persistent strains collected from the meat industry in Spain

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Abstract

The contamination of food products with *Listeria monocytogenes* has been related to the presence of biofilms in the production lines, since biofilms can protect the cells from the action of sanitizers. The main goal of this work was to compare the susceptibility of biofilms of persistent strains of *L. monocytogenes* to the compounds benzalkonium chloride (BAC) and peracetic acid (PAA) and to a hydrogen peroxide/peracetic acid commercial disinfectant (P3-Oxonia).

Twelve strains of *L. monocytogenes*, including nine BAC resistant and BAC sensitive persistent strains collected from the meat industry in Spain and three laboratory strains used as controls for BAC resistance, were used to compare the biofilm-forming ability and the disinfectant susceptibility of the strains. The assays tried to mimic food industry conditions. Consequently, biofilms were produced during 48 h at 25 ºC or during seven days at a refrigeration temperature (11 ºC), on stainless steel coupons (SSC), in dirty and clean biofilm-forming conditions, respectively in tryptone soy yeast extract broth (TSB-YE) and in nutrient-limiting, 1/10 diluted TSB-YE. Biofilms were evaluated by enumeration of viable cells on SSC. Concentration and contact time of the disinfectants were raised as needed to achieve the 4 Log reduction threshold in accordance with the European Standard EN 13697 2001.

The results indicate that, in general, nutrient growth conditions and temperature had no significant effect ($p>0.05$) on biofilm formation. Biofilm growth conditions in TSB-YE/10 at 25 ºC and in TSB-YE at 11 ºC showed to be the ones where disinfection treatment with BAC, and particularly with P3, was more effective. Moreover, biofilms formed under nutritional stress (clean condition) tend to differentiate more their response to BAC, than biofilms grown in rich nutrient conditions (dirty condition).

The concentrations and contact times of BAC required to achieve the 4 Log reduction threshold achieved 5120 mg/L for 5 min. Among the biofilms that showed the highest resistance to BAC, strains classified in planktonic as BAC resistant (MIC = 20 mg/L) and BAC sensitive (MIC = 1.25-5 mg/L) were included.

Concentrations and contact times of PAA required to achieve the same 4 Log reduction threshold reached 2000 mg/L for 5 min, while all strains showed the same susceptibility to PAA in the planktonic state (MIC = 200 mg/L or 1600 mg/L depending on the method used). Concentrations and contact times of P3-Oxonia to achieve the 4 Log reduction threshold achieved the 2% (v/v) for 10 min.

The results presented here emphasize the need for an efficient cleaning of equipment and utensils in the food industry especially those maintained at room temperature. The considerable difference between the referenced MICs of BAC and the concentrations required to achieve the 4 Log reduction in the biofilm form suggests that BAC is less effective than PAA in eliminating biofilm cells. PAA mode of action and its small molecular size constitute possible advantages for its efficiency. Overall, the resistant or sensitive character of planktonic *L. monocytogenes* cells to BAC did not dictate their response in biofilm form.

Keywords: *Listeria monocytogenes* biofilms; persistence; benzalkonium chloride (BAC); peracetic acid (PAA); commercial disinfectant P3-Oxonia.

Resumo alargado

Listeria monocytogenes é uma bactéria gram-positiva que pertence ao reino Bacteria, filo Firmicutes, classe Bacilli, ordem Bacillales, família Listeriaceae e género *Listeria*. Caracteriza-se por ser anaeróbia facultativa e por possuir baixo conteúdo em G+C (38%) no seu genoma, sendo caracterizada por bastonetes curtos não esporulados com 0,4 μm de diâmetro e com comprimento entre 1 a 1,5 μm.

Esta bactéria é capaz de crescer na presença de concentrações elevadas de sal (10%), bem como em ambientes com grandes variações de pH (pH 4-9). Possui mobilidade entre 10 ºC e 25 ºC e tem a capacidade de crescer entre 1 e 45 ºC, com uma temperatura ótima entre 30 ºC e 37 ºC. Sendo um patogéneo psicrotrófico, consegue crescer a temperaturas de refrigeração (2-4 °C), o que dificulta o seu controlo na indústria alimentar.

O carácter saprófito de *L. monocytogenes* torna este organismo muito versátil, tendo sido isolado de uma grande variedade de ambientes: solo, água, esgoto e fezes de animais, bem como de alimentos crus, processados e de produtos refrigerados prontos a comer (Ready-To-Eat (RTE)). Devido ao facto de os produtos RTE serem consumidos sem a necessidade de aquecimento prévio, estes apresentam um elevado risco para o consumidor. O aumento do consumo de produtos RTE, motivado pelas mudanças de hábitos dos consumidores, e as dificuldades existentes no controlo da temperatura, particularmente, na distribuição alimentar, contribuem para a recorrência deste microrganismo na indústria alimentar. A sua presença nos alimentos tem uma importância considerável, quer pela mortalidade que provoca, principalmente em crianças, idosos, grávidas e imunocomprometidos, quer pelos custos associados a despesas hospitalares, indemnizações e retiradas de produtos do mercado.

Nas indústrias de processamento de alimentos, os equipamentos e utensílios são reconhecidos como os principais locais para a aderência microbiana e a formação de biofilme. A aderência de microrganismos a superfícies que estão em contacto com os alimentos, pode levar a potenciais problemas sanitários e económicos, uma vez que os biofilmes atuam como reservatórios de recontaminação. Praticamente todos os microrganismos são capazes de realizar esta aderência e formação de biofilme, sendo *L. monocytogenes* um dos patogéneos envolvidos em contaminações persistentes nas indústrias alimentares.

Sabendo que a maioria dos microrganismos vive na forma de biofilme, urge identificar as vantagens desta estrutura séssil no processo de desinfeção, quando comparado com a forma planctónica. O principal objetivo deste trabalho foi assim comparar a suscetibilidade de biofilmes de estirpes persistentes de *L. monocytogenes* a dois compostos químicos - cloreto de benzalcónio (BAC) e ácido peracético (PAA) - e a um desinfetante comercial que contém PAA (P3-Oxonia). Foram testadas doze estirpes de *L. monocytogenes*, das quais nove estirpes recolhidas da indústria de carne espanhola e três estirpes laboratoriais usadas como controlos de resistência ao BAC. De forma a simular as condições ambientais nas indústrias de processamento alimentar, os biofilmes foram produzidos a 25 ºC durante 48 h e a uma temperatura de refrigeração (11 ºC), durante sete dias, em cupões de aço inoxidável (SSC). Para a produção dos biofilmes foram usadas condições que pretenderam similar, quer condições de limpeza mais eficaz, quer condições de limpeza menos eficaz, representada pela incubação dos biofilmes em meio rico diluído (TSB-YE/10) e meio rico (TSB-YE), respetivamente. Os biofilmes foram avaliados pela enumeração de células viáveis em cupões de aço inoxidável. Sempre que necessário, as concentrações e tempos de contato com os desinfetantes foram aumentados de forma a atingir o limiar de redução logarítmica de 4 Log (European Standard EN 13697 2001).

Os resultados obtidos mostraram que diferentes condições nutritivas para o crescimento dos biofilmes não se refletiram em diferenças significativas ($p > 0.5$) na formação dos biofilmes, principalmente quando produzidos a 11 ºC. No entanto, os biofilmes incubados em TSB/10 (25 ºC) e em TSB (11 ºC)

mostraram maior suscetibilidade ao BAC e ao P3-Oxonia, sugerindo um processo de desinfeção mais eficiente nestas condições de formação. Estes resultados enfatizam a necessidade de uma limpeza eficiente dos equipamentos e utensílios em uso na indústria alimentar, particularmente aqueles mantidos à temperatura ambiente. Quando comparada a suscetibilidade ao BAC de biofilmes crescidos em condições de riqueza ou de escassez de nutrientes, verificou-se maior diferenciação na resposta ao BAC em biofilmes crescidos sob stress nutritivo, do que em biofilmes crescidos em condições de grande disponibilidade de nutrientes.

A concentração de P3-Oxonia necessária para atingir a redução de 4 Log em todas as estirpes em estudo foi de 2% (v/v) por 10 minutos.

Os resultados dos ensaios de suscetibilidade ao BAC e ao PAA foram comparados com resultados anteriores de suscetibilidade, das mesmas estirpes, na forma planctónica.

No geral, a concentração de BAC e o tempo de exposição dos biofilmes necessários para atingir a redução de 4 Log, foi de 5120 mg/L durante 5 minutos. As estirpes que mostraram maior resistência ao BAC no estado de biofilme incluíram estirpes previamente classificadas no estado planctónico, como resistentes ao BAC (MIC = 20 mg/L) e sensíveis ao BAC (MIC = 1,25-2,5). No caso do PAA, a concentração e o tempo de exposição mais elevados para atingir a redução de 4 Log foi de 2000 mg/L (5 minutos), tendo todas as estirpes a mesma suscetibilidade no estado planctónico (MIC = 200 mg/L).

Os resultados anteriores mostram que, no caso do BAC e do PAA, as concentrações requeridas para atingir a redução de 4 Log no estado de biofilme foram consideravelmente elevadas quando comparadas com a resistência das células na forma planctónica. Isto confirma a ideia de que os biofilmes são dotados de uma maior resistência aos desinfetantes.

A diferença considerável entre os valores de MIC do BAC e as concentrações necessárias para atingir a redução de 4 Log nas células do biofilme sugere que o BAC é um composto menos eficiente na eliminação de biofilmes, quando comparado com o PAA. Esta diferença de eficiência dos dois compostos poderá estar condicionada pelo diferente modo de ação, bem como por distintas respostas bacterianas ao stress. O PAA caracteriza-se pela sua capacidade oxidativa e pela elevada reatividade através da geração de aniões superóxido e radicais hidroxilo que afetam o DNA bacteriano e outros constituintes celulares. A sua baixa massa molecular constitui uma vantagem aquando da penetração no biofilme. Pelo contrário, o BAC é um agente que atua essencialmente na membrana celular e o estado de biofilme surge como uma barreira física à penetração do BAC nas células. Isto ocorre quer pela elevada massa molecular do BAC, quer pela presença do glicocálix, de natureza polianiónica, que neutraliza o BAC retendo-o, e reduzindo assim a sua capacidade de difusão na matriz do biofilme dificultando o alcance das células.

Sete das 12 estirpes usadas neste trabalho possuem marcadores de resistência ao BAC: três possuem o gene *qacH*, enquanto quatro estirpes apresentam o gene *bcrAB*. No entanto, os ensaios de suscetibilidade dos biofilmes mostraram que das cinco estirpes mais resistentes ao BAC, apenas uma delas possui um destes marcadores genéticos. Estes resultados indicam que estirpes com marcadores de resistência como *bcrABC* e *qacH* não apresentam necessariamente menor suscetibilidade ao BAC no estado de biofilme.

No presente trabalho, a ação do desinfetante foi considerada eficaz quando se registou uma redução de células do biofilme de 4 Log. Outros autores têm sugerido que tal eficácia apenas se verifica quando há uma redução de 5 Log. Considerando este requisito, a concentração mais elevada de BAC aplicada, 5120 mg/L (5 minutos), não seria suficiente para eliminar nenhum dos biofilmes das 12 estirpes testadas. Estes resultados sugerem que a persistência de *L. monocytogenes* na indústria alimentar poderá não estar relacionada com fenómenos de resistência das bactérias aos desinfetantes, mas com a aplicação de concentrações dos desinfetantes insuficientes para eliminar biofilmes.

Os resultados aqui apresentados enfatizam a necessidade de trabalhos futuros com vista à clarificação da suscetibilidade de biofilmes a desinfetantes. A escolha do desinfetante, as condições de aplicação que incluem a concentração aplicada e o tempo de exposição deverão ser os adequados à erradicação de biofilmes na indústria alimentar.

Palavras-chave: Biofilmes de *Listeria monocytogenes*; persistência; cloreto de benzalcónio (BAC); ácido peracético (PAA); desinfetante comercial P3-Oxonia.

General Index

Figure and Table indexes

1. Introduction

1.1. *Listeria monocytogenes*: characterization

Listeria monocytogenes was first identified in 1924 by Murray, Webb and Swann as a gram-positive bacillus responsible for epidemic cases of mononucleosis in laboratory rabbits and guinea pigs. Later, it was observed the ability to cause sporadic cases of meningitides in humans prior to its recognition as an opportunistic food-born pathogen for animals and humans (Vazquez-Boland et al. 2001; Cossart & Lebreton 2014).

The species is included in the *Bacteria* Kingdom, *Firmicutes* Division, *Bacilli* Class, *Bacillales* Order, *Listeriaceae* Family and *Listeria* Genus. The genus *Listeria* includes seventeen species. *L. monocytogenes, L. ivanovii, L. welshimeri, L. innocua, L. seeligeri* and *L. grayi* were the first to be identified (Vázquez-Boland et al. 2001). In 2010 two additional species were acknowledged designated *L. marthii* (Graves et al. 2010) and *L. rocourtiae* (Leclercq et al. 2010). *L. weihenstephanensis* (Lang Halter et al. 2013) and *L. fleischmannii* (Bertsch et al. 2013) were the next two species to be discovered followed by five new identifications: *L. floridensis, L. aquatica, L. cornellensis, L. riparia and L. grandensis* (den Bakker et al. 2014). Recent studies published the new *L. booriae* and *L. newyorkensis* (Weller et al. 2015). *L. monocytogenes* and *L. ivanovii* are the only considered pathogenic for animals but only the first constitutes a significant human health threat (Orsi & Wiedmann 2016).

L. monocytogenes is a low G+C (38%), non-spore forming and non-capsid rod-like shape bacteria with rounded ends. Ranging from 1-2 µm length and 0.5 µm in diameter, the cells are commonly grouped in single units but might be clustered in short chains arranged in palisades or in V or Y disposition. Bacterial colonies are minor, smooth, oblate, and exhibit a milky white colour by reflecting light (Low & Donachie 1997). It is an anaerobic facultative bacterium being also characterized as catalase positive and oxidase negative (Ryser & Marth 2007).

As a saprotroph, this pathogen is versatile, widespread in nature and commonly found in several environments as soil, water, sewage, silage and animal faeces (Møretrø & Langsrud 2004; Orsi et al. 2011; Gilmartin et al. 2016). The microorganism can accustom to suboptimal conditions and induce adaptive responses resulting in high tolerance against lethal stresses. It is able to survive up to 730 days in soil and up to 28 days in water (Ferreira et al. 2014; Makariti et al. 2015). *L. monocytogenes* grows in high salt concentrations (10%) and in a wide pH range (pH 4-9) and is motile between 10 ºC and 25 ºC. (Weinmaier et al. 2013; Zoz et al. 2016). It is able to grow in a wide range of temperatures (2 ºC to 45 ºC) with an optimal between 30 ºC and 37 ºC (Møretrø & Langsrud 2004).

L. monocytogenes is defined as a foodborne facultative intracellular pathogen and a causative agent of gastroenteritis in healthy individuals being considered as a potential public health risk by the U.S Food and Drug Administration (FDA) (Lomonaco et al. 2015; Rychli et al. 2016). *L. monocytogenes* is placed among the top five foodborne pathogens (along with *Toxoplasma, Salmonella, Norovirus* and *Campyobacter*) and is responsible for listeriosis, a severe animal and human life threatening disease (Lourenço et al. 2011; Scallan et al. 2011; Lourenço et al. 2013).

This pathogen displays a significant level of heterogeneity among strains and thirteen serovars are acknowledged in the species: 1\2a, 1\2b, 1\2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4 and 7 (Nadon et al. 2001; Doumith, Cazalet, et al. 2004; Hyden et al. 2016). Among the thirteen serovars identified, isolates from 1/2a, 1/2b, 1/2c and 4b are responsible for over 98% of reported human listeriosis cases, in which 4b has the most prominent role (Chambel et al. 2007; Hamdi et al. 2007; Rebuffo-Scheer et al. 2007; Renier et al. 2011).

Due to the high diversity of strains with variable virulence, the ability to precisely track strains or subtypes involved in severe cases of listeriosis is imperative for preventing the manifestation of the disease. Besides serotyping methods, genomic macro restriction based on rare cutting endonucleases *AscI* and *ApaI* followed by pulsed-filed gel electrophoresis (PFGE) constitutes a powerful typing method mainly because of the high discriminatory power of the enzymes used. PFGE types (or pulsotypes) are obtained by combining both restriction enzyme profiles into unique profiles. A PFGE profile is considered unique when one or more bands differ from others PFGE profiles (López et al. 2007; López et al. 2008).

Despite the high discriminatory power of PFGE, this method is been replaced by microbial whole genome sequencing, since this has revealed important aspects as conservation or divergence among plasmids and prophages, SNPs patterns and specific transposons recurrence (TN6188) affecting persistence and/or disinfectant resistance (Orsi et al. 2008; Muller et al. 2013; Schmitz-Esser et al. 2015; Stasiewicz et al. 2015).

1.2. Epidemiology of listeriosis

Although some cases of infection are associated with *L. ivanovii*, most of the infections in humans are caused by *L. monocytogenes* (Nyarko & Donnelly 2015).

L. monocytogenes may cause two forms of listeriosis: invasive and non-invasive gastrointestinal listeriosis. Non-invasive listeriosis is a typical gastroenteritis followed by fever, diarrhoea and vomiting. Mostly all people exposed to the pathogen are suitable for acquiring the disease in which the clinical symptoms typically begin 20 h after ingestion of heavily contaminated food. In contrast, invasive listeriosis is associated with severe symptoms, high fatality rates and much longer incubation periods (between 20 and 30 days) than gastrointestinal listeriosis (Garrido et al. 2010). Invasive listeriosis can also be acquired by the fetus from the infected mother, via placenta, possibly leading to miscarriage (Allerberger & Wagner 2010; de Noordhout et al. 2014; Nyarko & Donnelly 2015).

Outbreaks of listeriosis have being related with the ingestion of ready-to-eat (RTE) food. The constantly rising demand for processed RTE food and also the preference for natural products without preserving procedures are a major concern worldwide (Dhama et al. 2015; Melo et al. 2015). Products as milk, dairy products, fermented sausages and fresh produce as cabbage, seafood and fish have all been associated with listeriosis as they can easily be consumed without heat treatments (Gandhi & Chikindas 2007; Costa et al. 2016).

The first listeriosis outbreak, which conclusively demonstrated foodborne transmission, involved coleslaw and took place in 1981, in Maritime Provinces, Canada. The following outbreaks in Europe and North America were related to refrigerated RTE processed products of animal origin, such as hot dogs, deli meats, other processed speciality meat products, seafood as well as soft cheeses (Garner & Kathariou 2016).

In 2015, 2.206 confirmed human cases of listeriosis were reported. The EU notification rate was 0.46 cases per 100.000 population, which was similar to 2014. There was a statistically significant increasing trend of listeriosis over 2008–2015. Nineteen state-members reported 270 deaths due to listeriosis in 2015, which was the highest annual number of deaths reported since 2008. The EU case fatality was 17.7% among the 1.524 confirmed cases with known outcome. Listeriosis infections were most commonly reported in the elderly population in the age group over 64 years old and particularly in the age group over 84 years (Anonymous 2016).

Portugal did not report listeriosis cases to EFSA between 2007 and 2014 since it was not for mandatory reporting. However, authorities have been notifying the existence of, at least, 46 cases of listeriosis (mortality rate of 43.5%) in Lisboa and Vale do Tejo region, between January 2009 and February 2012. In accordance to DGS (Direcção-Geral de Saúde) listeriosis came to be of mandatory reporting since January 2015.

Data from human disease and food industry surveillance should be gathered and analysed to understand the ecology of *L. monocytogenes* and its routes of transmission. Contamination source and vehicle should be identified, removed from the market and denied for consumers (Allerberger & Wagner 2010; Garrido et al. 2010). Furthermore, adequate prevention strategies along with an efficient quality control must be implemented for listeriosis' avoidance in agricultural, health and environmental systems (Shoukat et al. 2013; Magalhães et al. 2015).

1.3. *Listeria* in food associated environments

Several studies report the occurrence of *L. monocytogenes* in food processing facilities and its enhanced adaptation and surviving ability in extreme conditions when compared to other pathogens (Fox et al. 2011). Such capability makes the eradication of this pathogen a challenge and its long-term survival an issue of concern for hygienic control (Motarjemi et al. 2014).

The obstacle for the pathogen's eradication seems to be its capacity of growing under a wide variety of environmental stresses. Organic residues in industrial facilities constitute a niche for microorganisms to accumulate (Lourenço et al. 2011; Lourenço et al. 2013; Srey et al. 2013).

Thermal processing constitutes one of the most employed procedures to control microbial food deterioration and refrigeration temperatures are used in food chain to control microbial growth and extending food shelf-life. Considering that *L. monocytogenes* can grow at refrigerating temperatures and RTE are usually consumed without previous heating, these products represent a substantial risk to the consumers. In this regard, difficulties in controlling the temperature in trade distribution and current changes in lifestyle with increasing RTE consumption constitute some of the reasons for listeriosis increment in recent years (Garrido et al. 2010).

The World Health Organization (WHO) denoted that food contamination could possibly take place in any stage of food production. Raw products, that initiate the production line, might possibly be already contaminated by *L. monocytogenes*, or the following thermal treatment aiming pathogen elimination might be a failure (Ferreira et al. 2014; Law et al. 2015).

The capability of growing in different substrates and colonizing biotic and abiotic surfaces seems to be related with the easiness of microorganisms to form biofilms. This affects a wide range of food industries as brewing, meat, seafood, dairy and poultry processing and constitutes a frequent source of foodborne infection (Kyoui et al. 2016). Therefore, biofilms are determinant for food industries because surfaces, utensils and equipment are acknowledged as the most prominent for microbial adhesion and consequently biofilm formation (Shi & Zhu 2009; Oliveira et al. 2010; Law et al. 2015). Procedures as slicing at retail facilities are also known to be crucial in the post processing contamination of RTE. Retail-sliced products are 1.7 times more likely to be associated with fatal listeriosis when compared with pre-packaged deli meats (Ferreira et al. 2014). *L. monocytogenes* can adhere and grow on diverse food-contact surfaces such as stainless steel, polystyrene, rubber, wood, PTFE and glass (Bonsaglia et al. 2014; Mosquera-Fernández et al. 2014). In addition, detection of identical isolates from different areas inside establishments and from previously disinfected equipment highlights the risk of growth and establishment of *Listeria* in difficult access areas (Motarjemi et al. 2014; Martínez-Suárez et al. 2016).

1.4. Bacterial biofilms

1.4.1. Concept, composition and organization

Microorganisms have been envisaged as free suspended cells. However, discovery that bacteria are able to attach and to grow on biotic and abiotic surfaces redefined the previous concept of microorganisms (Donlan 2002). In 1970, bacteria are considered and validated in a sessile mode of existence, as well as the fact it represents a major element of bacterial biomass. Ten years later, bacteria were already recognized as being organized in elaborated communities presenting functional heterogeneity (Costerton 1999; Donlan 2002).

Recent advances and developments of analytic tools lead to a more accurate definition of biofilm as a sessile and structured community of bacterial cells that are irreversibly attached to a substratum, interface or to each other, embedded in a matrix of self-produced extracellular polymeric substances (EPS) that accounts for more than 90% of the biofilm dry mass (Chmielewski & Frank 2003; da Silva & de Martinis 2013; Colagiorgi et al. 2016; Azeredo et al. 2017). Matrix contains exopolysaccharides, lipids, glycolipids, DNA, and proteins and bacteria exhibit altered growth rate and gene transcription (Lourenço et al. 2012; Lourenço et al. 2013; Gilmartin et al. 2016; Azeredo et al. 2017). The components of the matrix participate in different functions such as cohesion within the biofilm, aggregation of bacterial cells, surface adhesion, protection against antimicrobial agents, prevention of desiccation, concentration of nutrients, enzymatic activity and intake of inorganic and organic compounds (Garrett et al. 2008; Hingston et al. 2015).

Extracellular and surface proteins were demonstrated to constitute a crucial factor for the initial attachment and for survival under extreme environmental conditions. Proteins and polysaccharides cooperate with extracellular DNA ensuring biofilm structure integrity (Alonso et al. 2014; Colagiorgi et al. 2016).

In community, bacteria are thought to exhibit phenotypic heterogeneity which may be interpreted as specialization and division of functions within the structure, similarly with what happens in multicellular organisms (Srey et al. 2013). This benefit is intensified with the segregation of signalling molecules commonly recognized as auto-inducers that accumulate as cell density increases. Binding of these auto-inducers to specific receptors entails transcriptional regulation of gene expression (Renier et al. 2011). Such molecules are involved in communication mechanisms within bacteria through "quorum sensing" (Bassler 2002). This mechanism requires the presence of a proper threshold cell density which ensures the adequate concentration of signalling molecules (Gandhi & Chikindas 2007). Currently, has been reported that this mechanism is not only associated to cell density but also to other adaptive functions (Garmyn et al. 2011).

The ubiquitousness of the biofilm form probably suggests a wide range of advantages for microorganisms when compared to the planktonic form. The biofilm appears as an anchor sustaining the cells and avoiding detachment by allowing colonization in a beneficial niche. Nutrients are abundant and the development of extracellular polymers contributes even more for glucose accumulation (Barbosa et al. 2013; da Silva & de Martinis 2013). Moreover, the ability to attach to surfaces and to form biofilms is taught to be important for pathogen resistance to internal shear forces generated by the passage of fluids, predation, phagocytosis, extreme temperatures, pH shifts, desiccation, osmotic shock, ultraviolet rays, biocides and increased salt concentrations (Carvalho 2007; Gilmartin et al. 2016). A proper understanding of biofilm formation and susceptibility will certainly aid in the design of effective prevention strategies against *L. monocytogenes* (Kyoui et al. 2016).

1.4.2. Biofilm formation in the food industry

Initially, organic molecules are deposited on the equipment's surfaces attracting biological active microorganisms. Some microbial cells remain even after sanitizing and initiate growth with quorum sensing and the expression of specific genes, supporting the formation of larger biofilms (Shi & Zhu 2009). Biofilm formation is a multistage process including five successive steps which consists of initial attachment, irreversible attachment, early development of biofilm architecture (micro colony formation), maturation and dispersion (Fig. 1.1) (Srey et al. 2013; Azeredo et al. 2017).
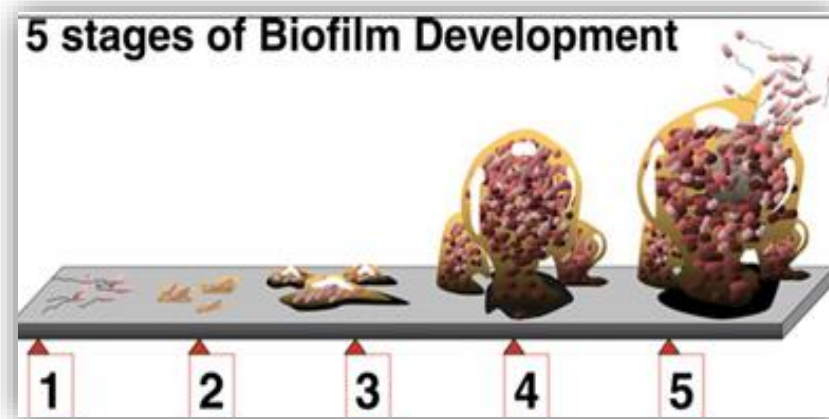
Figure 1. 1. - Diagram showing the five stages of biofilm development
1) initial attachment; 2) irreversible attachment; 3) early development of biofilm architecture; 4) maturation ; 5) dispersion (Srey *et al.,* 2013).

In the first phase of the initial attachment, adhesion is reversible. Attached bacteria are not yet committed to undergo the morphological changes necessary for biofilm formation and can easily detach from the surface exhibiting planktonic lifestyle. Such detachment only occurs because adherent cells that form biofilm at the surface have a negligible amount of EPS beyond a pilus mediated motility enabling independent movement (Srey et al. 2013). Reversible attachment involves van der Waals, electrostatic forces and hydrophobic interactions which makes bacteria easily removed by mild shear forces (Chmielewski & Frank 2003; Mata et al. 2015).

The most prominent feature of the second step, irreversible attachment (Fig. 1. 1), is the switch from a reasonable weak interaction of bacteria with the surface to a stronger and permanent bonding with the anchoring of appendages and EPS production (da Silva & de Martinis 2013). Bonding between substratum and appendages as flagella, pili and adhesion proteins involves short range forces such as dipole-dipole interactions, hydrogen bonds, hydrophobic and ionic covalent bonding (Chmielewski & Frank 2003). To destroy such chemical bonding and to remove biofilms, compounds as enzymes, disinfectants, surfactants and detergents are crucial (Srey et al. 2013).

The continuous accumulation of bacteria and EPS production lead to micro colony formation. EPS helps to fortify the connection between the surface and the bacteria, to protect the micro colony from environmental stresses as well as to recruit planktonic cells from the medium (Srey et al. 2013). Quorum sensing has been demonstrated to account for cell attachment and detachment but also for the growth and development of the micro colony (Donlan 2002; Chmielewski & Frank 2003).

The next step in biofilm formation is maturation (Fig. 1.1) which is defined as the continuous development of the initial biofilm into an organized structure. It may present the shape of a mushroom

or simply be flat, depending on the available nutrient source. Such complexity allows growth and/or survival on extreme environments (Chmielewski & Frank 2003; Shi & Zhu 2009).

A mature biofilm may be considered as a source of contamination due to detachment of bacterial cells from its surface (Kyoui et al. 2016). In fact, any external perturbation such as enzymatic degradation, increased fluid shear and release of EPS or surface-binding proteins can easily cause detachment of biofilm cells and revert them into the planktonic type. Furthermore, dispersion may also result by shedding of daughter cells from intense growth as a result of quorum sensing, or starvation (Donlan 2002; Harvey et al. 2007). This last step of biofilm formation, also represented in Figure 1.1, is responsible for new colonization and establishment of new bacterial niches.

1.4.3. Factors influencing biofilm formation

Several factors account for biofilm formation as properties of substratum and cell surfaces, hydrodynamics, surrounding environmental factors, nutrient availability, composition of microbial community, species interaction as well as genetic regulation (Srey et al. 2013; Mata et al. 2015).

Bacterial adhesion is related to the physicochemical characteristics of the cell surface (Mata et al. 2015). Despite the fact that the majority of bacteria are negatively charged, they have surface constituents as flagella, lipopolysaccharide and fimbriae with hydrophobic amino acid residues allowing the reduction of the repulsive forces (Donlan 2002; Shi & Zhu 2009).

The physical characteristics of the solid surfaces as critical surface tension, free energy, and humidity also influence the initial cell attachment. Wet surfaces promote bacterial adhesion as well as hydrophilic surfaces, like stainless steel or glass. These materials typically enable not only an increased bacterial attachment but also more biofilm formation than hydrophobic surfaces as nylon, Teflon, rubber and fluorinated polymers (Shi & Zhu 2009). Nevertheless, other researchers argue that differences in surface are not necessarily of practical significance (Chmielewski & Frank 2003).

Environmental factors such as temperature, nutrient composition and pH values can determine the phenotypic alteration between sessile (attached) and planktonic (free) forms (Chmielewski & Frank 2003). This switch entails a deep physiological transformation as a consequence of regulation of gene expression (Renier et al. 2011). However, the molecular mechanisms that regulate the microorganism's ability to attach are still unnoticed (Mata et al. 2015).

Optimal adherence is thought to coincide with higher metabolic activity which takes place at pH of 7 (Chmielewski & Frank 2003). Whereas, despite not being unanimously acceptable, is thought that higher temperatures increase adherence capability (Di Bonaventura et al. 2008). Despite high capacities for adhesion seem to be related with long term survival of *L. monocytogenes*, there is still uncertainty in the relation between biofilm formation and adherence. However, Carpentier (2011) observed that conditions promoting bacterial growth decreased cells adhesion (Renier et al. 2011).

The influence of nutrients in biofilm formation is not yet fully understood. Most of the research suggest that nutrient abundance can induce biofilm formation (Stepanovic et al. 2004). Kyoui (2016) has even referred that although high concentration of glucose decreased the number of viable cells in the biofilm structure, it augments the production of extracellular polymeric substances. Such idea is rebutted by others, arguing that adhesion of bacterial cells to abiotic surfaces is more intense in starvation conditions, with decreased metabolic activity or excessive secretion of extracellular molecules (Norwood & Gilmour 1999; Królasik et al. 2010). No correlation between nutrient content and surviving ability has been shown (Pan et al. 2006).

1.5. Persistence

Persistence is commonly described as the long-term survival of a microorganism in a simple or defined matrix but also in a complex natural or human made environment. Pathogen survival in food industries is potentially harmful and may compromise public health (Pricope et al. 2013; Ferreira et al. 2014).

Some authors state that persistence in the food processing industry might possibly rely on different factors as the infrastructures, physical and microbial natural habitat, transmission routes and genetic determinants (Larsen et al. 2014). Indeed, the reasons for persistence and survival are poorly understood and are not consensual (Wang et al. 2015; Rychli et al. 2016).

Greater adherence and stronger biofilms have been associated to the persistence of certain strains of *L. monocytogenes* (Norwood & Gilmour 1999; Borucki et al. 2003; Nakamura et al. 2013). Nevertheless, others argue that biofilms formed by persistent strains tend to be thicker or stronger than others formed by sporadic isolates (Ochiai et al. 2014; Colagiorgi et al. 2016). However, other authors did not find any correlation between those conditions and state that persistent bacteria do not necessarily produce stronger biofilms, neither survive longer in the presence of disinfectants, desiccation or in the absence of nutrients (Holch et al. 2013; Wang et al. 2015; Costa et al. 2016). Some studies have even showed that persistent *L. monocytogenes* isolates ended up producing less biofilms than sporadic strains (Ortiz et al. 2014).

Occasionally, harbourage sites were proposed to justify persistence. It was demonstrated that certain strains of *L. monocytogenes* can easily be established in specific facilities or equipments along with the resident microbiota, as niche-adapted bacteria even at low temperatures (Carpentier & Cerf 2011; Cabrita et al. 2013). The possible correlation between the psychrotropic character of *L. monocytogenes* and its persistence has also been considered (Cabrita et al. 2013; Cabrita et al. 2015). Scarcity of nutrients and disinfectant tolerance, allowing growth under sub lethal concentrations of the disinfectant, are also factors suggested to explain persistence (Wang et al. 2015; Colagiorgi et al. 2016).

Lastly, the phylogeny of the isolates has been compared with the biofilm forming ability and no correlation was found, possibly indicating that the serovar of the strain is not determinant in biofilm formation (Lourenço et al. 2011; Colagiorgi et al. 2016). Nevertheless, other results indicate that differential transcription may lead to persistence (Carpentier & Cerf 2011; Fox et al. 2011).

1.6. Susceptibility and resistance

In general, biocides are chemical agents with a wide spectrum applied to inactivate microorganisms on surfaces. They are used in the food industry as part of the sanitizer protocol, a combined cleaning and disinfection programme (Holah et al. 2002; Pfuntner 2011; Kakurinov 2014).

Cleaning is a requirement for a proper and efficient disinfection. The purpose of the following disinfection process is to reduce the remaining microbial population and to sustain microbial growth, on surfaces, before production restart. This is accomplished by destroying or irreversibly inactivating contaminant microorganisms. Such measures guarantee high quality specifications of the food products by reducing the risk of foodborne illness and enhancing food shelf life (Wirtanen & Salo 2003; Pricope et al. 2013). One of the strategies used for sanitation in food processing plants is the Clean-In-Place programme (CIP). This automatic approach is responsible for cleaning the interior surface of pipes, vessels, processing equipment, filters and associated fittings, without the need for disassembly. This is a faster, less labour-intensive and more repeatable process (Motarjemi et al. 2014).

The ideal disinfectant should break down waste materials, maintain them in suspension, be efficient in low concentrations, as well as in the presence of organic matter. It should not be hazardous for handling,

be easily dissolved in water, should cause no corrosion and should be rinsed freely from the surfaces. Moreover, should have low surface tension to allow penetration in all crevices (Kakurinov 2014).

The chemicals used by the food industry for sanitation are pooled in seven distinct groups: halogen-releasing agents, quaternary ammonium compounds, peroxygens, alcohols, aldehydes, bisphenols and biguanides. Peroxygens (HP) and quaternary ammonium compounds (QAC) are the most usually applied (Asselt & Giffel 2005; Aarnisalo et al. 2007; Ceragioli et al. 2010). Nevertheless, the effect of QAC has been widely studied due to the occurrence of resistance phenomena in industrial scenario.

Resistance may be defined as the capability of an organism, as well as its progeny, to multiply or to stay viable under certain conditions that would inhibit or constrain other members of the species. Indeed, bacteria might be considered resistant when they are more able to survive to repeated and standardized disinfection programmes than others (Holah et al. 2002; Carpentier & Cerf 2011).

Isolates of *L. monocytogenes* have been collected from food processing environments even after disinfection procedure. In fact, different studies demonstrated that adherent microorganisms are far more resistant than free living cells (Wirtanen & Salo 2003; Pan et al. 2006; Kostaki et al. 2012; Rodríguez-López et al. 2017). Biofilm cells can be up to 1000 times more resistant to biocides than cells in suspension (Królasik et al. 2010; Bae et al. 2012). In addition, the resistance of biofilms and the inefficiency of the disinfection agent used have not only been linked to the mechanical protection provided by biofilm exopolysaccharides and surrounding nutrients (Martínez-Suárez et al. 2016), but also to their intrinsic stress adaptive capabilities (Belessi et al. 2011; Ibusquiza et al. 2011).

Several factors influence the antimicrobial activity of the disinfectants such as concentration, chemical composition, contact time and temperature, pH, water hardness, and the eventual presence of organic matter related with insufficient cleaning (Bisbiroulas et al. 2011). All these factors must be considered to avoid the exposure of contaminant microorganisms to sub-lethal conditions and consequently to the emergence of persistent strains which are far more challenging to eradicate (Ibusquiza et al. 2011). Some authors even admit that long-time exposure to such sub-lethal concentrations could potentially lead to adaptation and resistance. Nevertheless, identification of strains with high, acquired resistance to disinfectants at in-use concentrations are rarely reported (Kastbjerg & Gram 2012; Pricope et al. 2013).

Another possibility to explain resistance is the acquisition and dissemination of resistance genes by plasmids and transposons, the occurrence of mutations and the functioning of efflux pumps which decrease the intracellular concentration of the disinfectants diminishing its effect (Kakurinov 2014; Martínez-Suárez et al. 2016).

In planktonic bacteria, several studies have not found correlation between "resistance" and "persistence". Persistent strains did not show higher resistance to disinfectants, desiccation or to acidic stress, than non-persistent (Lourenço et al. 2009; Carpentier & Cerf 2011; Ortiz et al. 2014).

Considering that one of the main advantages of *L. monocytogenes* in the food industry is the ability to form biofilms, disinfection is required to prevent serious health problems as well as economical losses (Lourenço et al. 2009; Lourenço et al. 2012).

1.6.1. Benzalkonium chloride

Benzalkonium chloride (BAC) is an active compound belonging to the group of QACs. It is effective against bacteria and fungi and commonly used in the food processing environment, household or for personal use (Elhanafi et al. 2010; van der Veen & Abee 2011; Giaouris et al. 2013; Poimenidou et al. 2016).

BAC is a cationic molecule that penetrates the cell wall to disrupt the negatively charged cytoplasmic membrane. The structural integrity of the cell is altered by cytosolic leakage and nucleic acid and protein degradation (Seymour S.Block 1983a; Bridier et al. 2011; Rodríguez-López et al. 2017). BAC surface tension allows a good intrusion in the materials, BAC is non-corrosive, non-tainting, non-toxic and presents residual antimicrobial activity if not rinsed. Nevertheless, decreases its efficiency by most of the detergents, by low pH, by the presence of organic matter and at low temperatures (0-22 °C). Moreover, BAC showed lack of efficiency against Gram negative bacteria (Seymour S.Block 1983a; Chaitiemwong et al. 2014; Kakurinov 2014).

Quaternary ammonium compounds appeared very effective in the elimination of pathogens, but resistance to these compounds has been observed. The causes for BAC resistance vary. Some authors state that *L. monocytogenes* adaptation to BAC might reflect lipid changes which decrease membrane fluidity, but also modifications of the physicochemical properties of the cell surface, altering bacterial biofilms (Chavant 2004; Bisbiroulas et al. 2011; Pricope et al. 2013). Others studies also showed a possible correlation between resistance to BAC and the presence of some energy-dependent efflux systems such as MdrL (multidrug resistance *Listeria*) or Lde (*Listeria* drug efflux) (Romanova et al. 2006; Houari & Di Martino 2007; Kakurinov 2014). Acquisition of genetic markers as *bcrABC* resistance cassette or the *qacH* gene of the *Tn6188* transposon are also possible explanations of BAC resistance since QacH, BcrB, and BcrC are transporters belonging to the small multidrug resistance (SMR) protein family (Ferreira et al. 2014; Larsen et al. 2014; Møretrø et al. 2016; Ortiz et al. 2016).

QAC have low biodegradability which means that the contact with bacteria is prolonged and consequently microbial communities might be exposed to sub-inhibitory concentrations (Ortiz et al. 2014; Liu et al. 2016). Martínez-Suárez and colleagues (2016) observed that sub-inhibitory concentrations may cause expression of genes related with stress response causing reduction in cell permeability. Membrane fatty acids and phospholipids suffer modifications augmenting the anionic and hydrophobic character of the cell surface, making more difficult QACs penetration (Pricope et al. 2013).

Scarce experiences on biofilm resistance to BAC have been made. Biofilms showed to exhibit higher resistance to BAC when compared to planktonic cells, both in Gram positive as in Gram negative microorganisms (van der Veen & Abee 2011; Rodríguez-López et al. 2017). In addition, Ortiz and colleagues (2014) observed that the effect of subminimal inhibitory concentrations of BAC on biofilm formation by *L. monocytogenes* might differ between strains with different MICs and even between resistant strains with similar MICs, but with different genetic determinants of BAC resistance.

Since resistance to QAC and, particularly, to BAC occurs, understanding microbial response mechanisms on planktonic and particularly on biofilms, constitutes a way to improve the effectiveness of such compounds (Bisbiroulas et al. 2011; Kakurinov 2014; Ortiz et al. 2014).

1.6.2. Peracetic Acid and P3-Oxonia active

Peracetic acid (PAA) is an oxidant associated with the generation of superoxide anions and a burst of free hydroxyl radicals (OH⁻) which damage bacterial DNA and cause cell death (Seymour S.Block 1983b; Belessi et al. 2011). It was already reported that PAA presents higher efficiency in removing adherent cells, compared with hydrogen peroxide (Chmielewski & Frank 2003; Marques et al. 2007; Souza et al. 2014). PAA is unstable and does not exist in a pure state. It is available as an aqueous solution mixed with acetic acid and hydrogen peroxide (Lee et al. 2016). In contrast to QACs, PAA is known to be active at low temperatures (0-25 °C) and decompose into safe environmental friendly residues (Ölmez & Kretzschmar 2009; Lee et al. 2016). PAA solutions, similarly do QACs, can be

attenuated by organic load but PAA begin to lose activity as the pH approaches neutrality (Chmielewski & Frank 2003; Pfuntner 2011)

Hydrogen peroxide (HP) is commonly used in the food industry due to its strong oxidant ability to damage bacterial proteins, DNA and cellular membranes (Seymour S.Block 1983b; Yun et al. 2012). Hydrogen peroxide's effect is intracellular, broad-spectrum and depends on the production of free radicals, constituting effective permeabilizers (Wirtanen & Salo 2003; Caixeta et al. 2012). Its high oxidizing capacity, high decomposition rate and low molecular size entail an advantage on its penetration in the biofilm matrix (Ibusquiza et al. 2011).

P3-Oxonia is a commercial disinfectant belonging to the group of peroxygens with HP and PAA as active compounds. These chemicals are frequently combined as they both function effectively under low temperatures (4 ºC). Peroxygen compounds have a large antimicrobial spectrum and are efficient against adherent microorganisms. Nevertheless, they might cause corrosion of some metals, may have low efficiency against yeast and molds and may be easily inactivated by organic matter (Pfuntner 2011; Kakurinov 2014).

1.7. Background and objectives of the work

The work developed previously within our research group was searching for the possible relations between persistence of *L. monocytogenes* and biofilm-forming ability. Regarding *Listeria* growing temperature, Cabrita et al. (2015) found differences in the expression of cold stress related genes between one persistent and one sporadic strain of *L. monocytogenes*, both collected from the dairy industry, suggesting gene expression networks differently adjusted, in the two strains, to the low-temperature environment from where they were collected. These authors recommended that strain response to low temperature should be investigated in other sporadic and persistent strains.

The present work was performed under the scope of Proyecto RTA2014-00045-C03-00 "Survival of *L. monocytogenes* on food contact surfaces: a multidisciplinary approach to a complex problem". The overall objective of the project is to investigate the influence of disinfectants on the potential for biofilm eradication. The work presented here focused on the characterization of biofilms of a set of nine persistent strains, collected in Spain, from the pork and poultry industries, in order to investigate the reasons for the persistence of these strains. Three strains were isolated from one chicken meat product company and six strains were isolated from pork sausage companies. Three strains were additionally used as controls. The assays tried to mimic food industry conditions. Consequently, biofilms were produced during 48 h at 25 ºC or during seven days at a refrigeration temperature (11 ºC), on stainless steel coupons (SSC), in dirty and in clean biofilm-forming conditions, respectively in tryptone soy yeast extract broth (TSB-YE) and in nutrient-limiting, 1/10 diluted TSB-YE. Biofilms produced under these conditions were exposed to two active compounds (benzalkonium chloride - BAC and peracetic acid - PAA) and to one hydrogen peroxide/PAA based commercial disinfectant (P3-Oxonia). These biocides are commonly used in the food industry.

These results will complement other ongoing research on the persistent strains used and will contribute with additional information related to their eradication using BAC and PAA based disinfectants.

2. Materials and Methods

2.1. Bacterial isolates

The 12 bacterial strains used in this study are listed in Table 2.1. Six were isolated from the pork industry, three were from the chicken industry and the other three were control strains.

Strains withdrawn from -80 ℃ stock collection were streaked onto tryptone soy yeast extract agar (TSA-YE). After overnight growth at 37 ℃, from isolated colonies a work collection in semi-solid TSA-YE in cryogenic microtubes was prepared. After overnight incubation at 37 ℃, the work collection was maintained at 4 ℃, until use.

Table 2.1. - *Listeria monocytogenes* strains used in this study.

Original Reference*	CBISA Reference	Source	Sensibility to BAC	Reference
R6	4471	chicken	harbors *bcrABC* gene	López et al., 2013
S1®	4472	pork	multidrug resistance mutant	Ortiz et al., 2014; Ortiz et al., 2016
S2BAC	4473	pork	multidrug resistance mutant	Ortiz et al., 2014
EGD-e	4474	control	sensitivity control	Ortiz et al., 2014; Ortiz et al., 2016
S2-1	4475	pork	sensitive to BAC	Ortiz et al., 2014
S1(S)	4476	pork	sensitive to BAC	Ortiz et al., 2014; Ortiz et al., 2016
4423	4477	control	Resistance control (*qacH*)	Ortiz et al., 2016
S10-1	4478	pork	harbors *qacH* gene	Ortiz et al., 2014; Ortiz et al., 2016
S2-2	4479	pork	harbors *qacH* gene	Ortiz et al., 2016
CDL69	4480	control	resistance control (*bcrABC*)	Ortiz et al., 2016
A7	4481	chicken	harbors *bcrABC* gene	López et al., 2008
P12	4482	chicken	harbors *bcrABC* gene	López et al., 2007

CBISA – Coleção de Bactérias do Instituto Superior de Agronomia.

* Except for controls, the first letter of the strain designation refers to its origin: A- abattoir; P- processing plant; R- retail; S- swine (pig abattoir and pork processing plants).

The first number identifies the AscI profile being followed by a hyphen and another number identifying the different ApaI profiles.

2.2. Disinfectants and neutralizer solution used

Two active substances - benzalkonium chloride (Sigma-Aldrich, St. Louis, USA) and peracetic acid (Sigma-Aldrich, St. Louis, USA) - and one industrial disinfectant - P3-Oxonia active (Ecolab, Saint Paul, Minnesota, EUA) – were used to assess their efficacy on *L. monocytogenes* biofilms. The active ingredients of P3-Oxonia are H_2O_2, acetic acid and peracetic acid.

P3-Oxonia was diluted in hard water (magnesium chloride, calcium chloride and sodium bicarbonate, pH 7 ± 0.2, prepared according to EN 13697) to achieve the concentrations indicated by the manufacturer.

Dey-Engley neutralizing broth solution (D/E) (Difco Laboratories, New Jersey, USA) was used after each contact time to neutralize the disinfectants used.

2.3. Stainless steel coupons (SSC)

Stainless steel coupons (1 x 1 cm) type 316 finish 4b (University of Georgia instrument shop, Athens) were used. Before use, coupons were cleaned in acetone to remove grease, rinsed in distilled water, and consecutively immersed into a phosphoric-acid-based cleaner (CIP 200; Steris Corp.) for 20 min. The coupons were rinsed again and sterilized by autoclaving in test tubes.

2.4. Evaluation of biofilm forming ability on stainless steel by cell enumeration

The method used to evaluate the ability of the bacterial strains to form biofilms on SSC was adapted from the work developed by Costa and colleagues (2016).

Each strain from the work collection was streaked onto TSA-YE and incubated overnight at 37 °C. From each culture, one isolated bacterial colony from the TSA-YE plate was suspended in 10 mL of tryptone soy yeast extract broth (TSB-YE) and another isolated colony was suspended in 10 mL of nutrient-limiting, 1/10 diluted TSB-YE, trying to mimic, respectively, dirty and clean biofilm-forming conditions in the food processing industry. Coupons were immersed in 1.5 mL of the respective suspensions and incubated, without agitation, for 48 h at 25 °C or for seven days at 11 °C in Parafilm sealed 24-well microplates (Orange Scientific). In each microplate, both clean and dirty conditions were assessed for each strain.

After the incubation period, each coupon was rinsed by pipetting 1 mL of Ringer's solution on both surfaces, to remove the planktonic cells, and placed in a new 24-well microplate already containing 20 glass beads (Ø = 3 mm) per well. On the top of each coupon additional 30 glass beads and 1 mL of Ringer's solution were added. The 24-well microplate was sealed with Parafilm and vortexed (Tittertek DSG, Flowlabs, Germany) for 1 min at maximum speed to detach biofilm cells. For each well, the resulting suspension was decimal diluted, inoculated onto TSA-YE plates and incubated overnight at 37 °C for colony forming units (CFU) counting. At least, two biological replicates were performed, with two technical replicates, each.

2.5. Evaluation of the listericidal activity of the disinfectants

The procedure for cell enumeration on SSC was performed as previously described in biofilms produced under nutrient-limiting and nutrient-rich conditions. After rinsing both surfaces with 1 mL of Ringer's solution, coupons were immersed in 1 mL of the tested disinfectant in selected concentration and contact time. For all strains at both temperatures and nutrient conditions concentration and contact time of all sanitizers were raised as needed to achieve the 4 Log reduction threshold (European Standard EN 13697 2001).

BAC was used in four different conditions: 1280 mg/L for 5 min, 2560 mg/L for 5 and 10 min and 5120 mg/L for 5 min. Peracetic acid was used in 500 mg/L, 1000 mg/L and 2000 mg/L for 5 min. P3 was used in three different conditions: 0.5% (v/v) for 5 min, 1% (v/v) for 10 min and 2% (v/v) for 10 min of exposure.

After the respective exposure period, each coupon was rinsed with 1 mL of Ringer's solution and transferred to another 24-well microplate already provided with a 20-glass bead layer, per well. On the top of the coupons 30 glass beads and 1 mL of D/E were added for 5 min of contact.

After this neutralization period, the 24-well microplate was vortexed for 1 min at maximum speed to remove biofilm cells from the surface of the coupons. Consecutively, 0.1 mL of the suspension was

directly inoculated onto TSA-YE plates, whereas the remaining suspension was decimal diluted and 100 µL aliquots were spread onto TSA-YE plates.

The treatment was considered effective if a 4 Log reduction (difference between Log of CFU/cm^2 SSC not exposed and exposed to disinfectant) was observed. Two biological replicates were performed with two technical replicates each.

2.6. Minimal inhibitory concentration (MIC)

From the initial set of twelve strains, three strains (EGD-e, S2-1 and CDL69) were selected for BAC and PAA MIC determination. MIC assessment was performed according to the microdilution broth method described by Lourenço and colleagues (2009) in 96-well microplates (Orange Scientific, Braine-l' Alleud, Belgium) during 24 h incubation at 25 ºC.

Strains were streaked onto TSA-YE plates and incubated overnight at 37 ºC. Bacterial cells taken from single isolated colonies were used to inoculate 20 mL of Mueller Hinton Broth (Biokar Diagnostics, Pantin, France) supplemented with 1% (w/v) glucose (Copam, Loures, Portugal) (MHG) and incubated for 18 h at 37 ºC.

Briefly, after producing serial twofold dilutions of the disinfectant stock solutions in each microplate, 100 µL of the inoculum was added to obtain a final bacterial concentration of 5×10^5 CFU/ mL on each well in a total volume of 200 µL. Cells that were not exposed⁻ to disinfectants at any time and MHG that was not inoculated at any time constituted controls for viability of the inoculum and sterility of the culture medium. Wells were finally sealed with 50 µL of sterile paraffin (Vencilab, Vila Nova de Gaia, Portugal) to prevent evaporation.

The MIC was defined as the lowest concentration of the disinfectant agent that prevents visible growth of a selected isolate after an incubation period of 24 h (Lourenço et al. 2009). The determination of the MIC values was performed in triplicate, at least in two independent biological assays.

2.7. Data analysis

The values of Log CFU/cm^2 were tested for ANOVA assumptions. By using MiniTab17 (Minitab, Inc., Pennsylvania, USA), conformance to normality was determined using the Anderson-Darling test and conformance to homogeneity was determined with the Leven's test.

When normality and homogeneity of variances were confirmed, a one-way factor ANOVA with the Tukey test was performed to evaluate statistical differences between average values. When the data did not comply with the normality or the homogeneous distribution of the variance, the non-parametric Kruskal–Wallis median test was applied. The biofilm forming ability of the strains, as well as their susceptibility to BAC and P3 were treated through Principal Component Analysis (PCA) and "Cluster analysis". For this statistical analysis, the software Statistica version 7.0 (Statsoft Inc., Tulsa, USA) was used.

For all tests, the confidence level was 95% ($p < 0.05$).

3. Results and Discussion

3.1. Biofilm forming ability on SSC

Biofilm formation is a phenomenon occurring wherever microorganisms and surfaces exist in close proximity (Giaouris et al. 2013). The evaluation of biofilm forming ability on SSC was performed trying to simulate food industry conditions, where cell deposition is made on stainless steel surfaces and microorganisms may face different nutritional availability as well as different growth temperatures.

In fact, cleaning procedures may not be effective and biofilms may be formed under nutrient abundance. On the contrary, an efficient cleaning procedure assures a scarce nutrient availability for biofilm growth. Besides, biofilm formation in food industry also occurs both at room temperature as at refrigeration temperatures, depending on the processing stage.

Therefore, in this work, biofilms were grown at 25 ℃ for 48 h and at 11 ℃ for 7 days. The seven days incubation time was used based on previous results obtained within the research group. As mentioned before, to simulate poor and good cleaning procedures, strains were grown both in TSB-YE medium as well as in 1:10 diluted TSB-YE medium, respectively (Fig. 3.1).
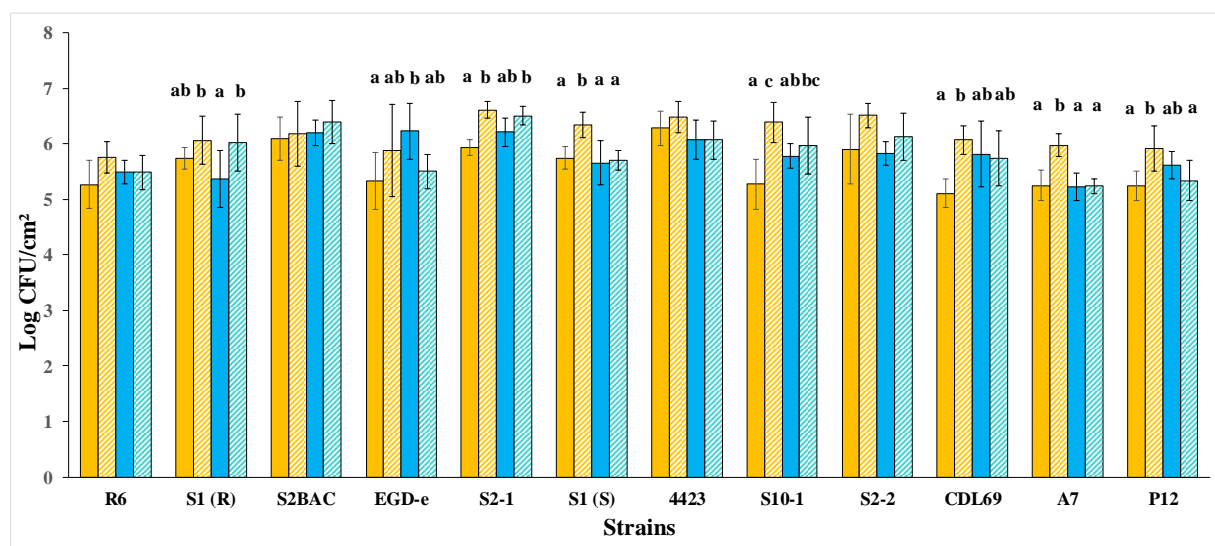
Figure 3.1. - Biofilm forming ability of the strains.

Biofilms were grown in TSB-YE (25 ℃) (■), TSB-YE/10 (25 ℃) (⬤), TSB-YE (11 ℃)(■) and in TSB-YE/10 (11 ℃)(⬤). Error bars represent standard deviation. Different letters in the columns indicate significant differences (p < 0.05) between average values. Results of rich media are from Vera Maia (Thesis Project, 2017).

Previous research from Stepanovic and colleagues (2004), Harvey and colleagues (2007) and Poimenidou and colleagues (2016) showed that at 25 ℃, biofilm production in rich nutritive conditions was more efficient than biofilm production in diluted medium.

Such findings do not corroborate our results since, at 25 ℃, half of the strains did not show differences between media and the other half showed higher biofilm-forming ability (p < 0.05) in diluted medium. (Fig. 3.1). The six strains with the higher biofilm production were both control and persistent strains collected from the food industry.

At 11 ºC, except for strain S(R), the strains did not show significant differences (p > 0.05) in biofilm production, between rich and diluted medium, suggesting that at low temperatures, biofilm growth tends to be more homogenous within the species.

When the biofilm production of the strains was compared in TSB-YE, at both temperatures, except for one strain (EDG-e), this homogeneity was also verified.

When biofilms were produced in diluted medium, at different temperatures, nine out of 12 strains showed no significant differences between temperatures (p > 0.05).

3.2. Biofilm susceptibility to disinfectants

Since most microorganisms live in the form of biofilm, there is a need to discern the benefits for the pathogen of this sessile structure, particularly in food industries, as it constitutes a continuous source of contamination (Romling & Balsalobre 2012; Abdallah et al. 2014).

To investigate biofilm susceptibility, biofilms were exposed to different concentrations of BAC, PAA and P3-Oxonia (see chapter 2.5.) to achieve a 4 Log reduction threshold (European Standard EN 13697 2001).

The time of exposure did not surpass 10 min since ideal disinfection procedures should allow high concentrations for short times (5-10 min) (Aarnisalo et al. 2007)

3.2.1. BAC

When comparing the susceptibility of the biofilms, grown in different media at 25 ºC, to 1280 mg/L of BAC (5 min of exposure) (Fig. 3.2A), six strains showed higher susceptibility in diluted medium, two showed lower susceptibility in the diluted medium and four strains did not show significant differences between grow media (p > 0.05). This diversity of response was less evident at 11 ºC when comparing the two media: eight out of the 12 strains showed no differences in susceptibility (p > 0.05). When the incubation medium was TSB-YE, half of the strains showed higher susceptibility (p < 0.05) at 11 ºC, compared with 25 ºC. When incubation medium was TSB-YE/10, eight strains did not show significant differences at 11 ºC compared with 25 ºC.

When 2560 mg/L of BAC with 5 min of exposure was used, a considerable uniformity in the results was observed since less significant differences (p < 0.05) were detected (Fig. 3.2B). When comparing the susceptibility of biofilms formed in both media at 25 ºC, six out of eight strains that did not reach the 4 Log reduction in the previous condition (1280 mg/L), did not show significant differences between growth media (p > 0.05). The same homogeneity was verified in the comparison of both growth media, at 11 ºC, since no significant differences (p > 0.05) were noticed. In rich nutrient conditions, six out of nine strains did not show significant differences (p > 0.05) between susceptibility of biofilms grown at 25 ºC and 11 ºC. The same lack of significant differences (p > 0.05) was also shown, at scarce nutrient conditions, for nine out of ten strains.

Five strains (S1(R), S2[BAC], EGD-e, S2-1 and CDL69) did not reach the 4 Log reduction with 5 min of exposure to 2560 mg/L and were subjected to 2560 mg/L for 10 min (Fig. 3.2C). Only one strain (CDL69) grown in rich conditions did not reach the 4 Log reduction and underwent a 5 min exposure to 5120 mg/L of BAC (Fig. 3.2C). Interestingly, among these five less susceptible biofilms to BAC, CDL69, S1(R) and S2[BAC] are referenced as BAC resistant strains in the planktonic form while EGD-e and S2-1 are acknowledge as BAC sensitive strains.

Figure 3.2. – *L. monocytogenes* **biofilm Log reductions after exposure to BAC.**

A - exposure to 1280 mg/L for 5 min; **B** - exposure to 2560 mg/L for 5 min; **C** - exposure to 2560mg/L for 10 min and to 5120 mg/L for 5 min.

Biofilms were grown in TSB-YE (25 ºC) (▮), TSB-YE/10 (25 ºC) (▱), TSB-YE (11 ºC)(▮) and in TSB-YE/10 (11 ºC)(▱). Error bars represent standard deviation. Different letters in the columns indicate significant differences (p < 0.05) in average values.

3.2.2. PAA

Biofilms of the 12 strains grown in TSB-YE at 25 ºC were exposed to PAA (Fig. 3.3). Concentration of PAA was raised as needed to achieve the 4 Log reduction threshold. All strains were exposed to 500 mg/L of PAA for 5 min but only one strain (S(1)) reached the required 4 Log reduction. The remaining strains were exposed to 1000 mg/L of the disinfectant. Strains CDL69 and P12 showed the highest resistance to PAA, having only achieved the 4 Log reduction threshold after a 5-minute exposure to 2000 mg/L of PAA.

Figure 3.3. - *L. monocytogenes* biofilm Log reductions after exposure to different concentrations of PAA for 5 min.
Biofilms were grown in TSB-YE at 25 ºC. Error bars represent standard deviation. Different letters in the columns indicate significant differences ($p < 0.05$) between average values.

3.2.3. P3-Oxonia

When comparing the susceptibility of the biofilms to 0.5% of P3 (5 min of exposure) in both media at 25 ºC (Fig. 3.4A), six out of 12 strains showed higher susceptibility (p < 0.05) in the diluted medium. At 11 ºC, nine out of twelve strains showed higher susceptibility (p < 0.05) in rich conditions. When comparing the susceptibility of the biofilms formed in rich nutrient conditions at 25 ºC and 11 ºC, seven out of 12 strains did not show significant differences (p > 0.05) between growth temperatures. In scarce nutrient conditions, eight out of 12 strains showed higher susceptibility (p < 0.05) at 25 ºC. If all strains were considered as replicas (data not shown), the statistical comparison corroborates the conclusion of individual comparisons: susceptibility to 0.5% of P3 is higher in TSB-YE/10 at 25ºC and in TSB-YE at 11ºC (p < 0.05).

When comparing the susceptibility of biofilms grown at 25 ºC, in both media, and exposed for 10 min to 1% P3 (Fig. 3.4B), five out of nine strains demonstrated higher susceptibility (p < 0.05) in diluted medium. At 11 ºC, seven out of 12 strains did not show significant differences (p > 0.05) between growth media. This higher homogeneity of response was also observed in the comparison between 25 ºC and 11 ºC in both rich and diluted media. In rich medium, seven out of 10 strains did not show significant differences (p > 0.05) whereas in scarce nutrient conditions eight out of nine strains showed no significant differences (p > 0.05).

The five strains that did not reach the 4 Log reduction with 1% P3 were exposed to 2% P3 for 5 min. Strain EGD-e showed a higher susceptibility in diluted medium, at 25 ºC (Fig. 3.4C).

Altogether, the results suggest that susceptibility to P3 is higher when biofilms are produced with low concentration of nutrients at room temperature, regardless of the tested concentration.

In general, strain susceptibility to 0.5% of P3 for 5 min is very low. Such findings are consistent with the ones of Królasik and colleagues (2010) in which strains of *L. monocytogenes* were exposed to a mixture of peracetic acid and hydrogen peroxide. Concentrations of 0.2 and 0.5% of the disinfectant were not successful in achieving the 4 Log reduction threshold, after an exposure of 10 min. Consecutively, the researchers increased the concentration of the disinfectant to 1%, for 30 min of exposure, and the strains achieved a 5 Log reduction. In the present work, 1% of P3 showed effectiveness for 10 min of exposure for most of the strains and conditions used.

The more resistant strains to P3 were R6, S2[BAC], EGD-e, S1(S) and P12. From those five, only one (P12) was also one of the most resistant to PAA. Considering the obtained results, no conclusion can be inferred about the preponderance of PAA in the commercial disinfectant P3.

The action of hydrogen peroxide and peracetic acid based disinfectants, such as P3, on biofilms of *L. monocytogenes* has been previously studied (Królasik et al. 2010; Costa et al. 2016; Poimenidou et al. 2016). These compounds are two of the most efficient and currently applied disinfectants in the food industry. Previous research with different active compounds present in diverse disinfectants demonstrated that only chlorine dioxide, acidified sodium chlorite and mixtures of hydrogen peroxide and peracetic acid based products (out of twenty-one sanitizers tested) allowed to reach a 5 Log reduction in biofilm CFU (Cruz & Fletcher 2012).

Cleaning and sanitizing are among the harshest stresses that bacteria may experience in a typical food processing environment. Several sequenced steps are included in an usual sanitation procedure, being rising, cleaning, rising, and sanitizing, in this order (Pan et al. 2006). Biofilm growth conditions in TSB-YE/10 at 25 ºC and in TSB-YE at 11 ºC showed to be the ones where disinfection treatment with BAC, and particularly with P3, was more effective. In fact, the results showed at 25 ºC a higher susceptibility to BAC and to P3 of biofilms formed under poor nutrient conditions. These results emphasize the need

for an efficient cleaning of equipment and utensils in the food industry especially those maintained at room temperatures.
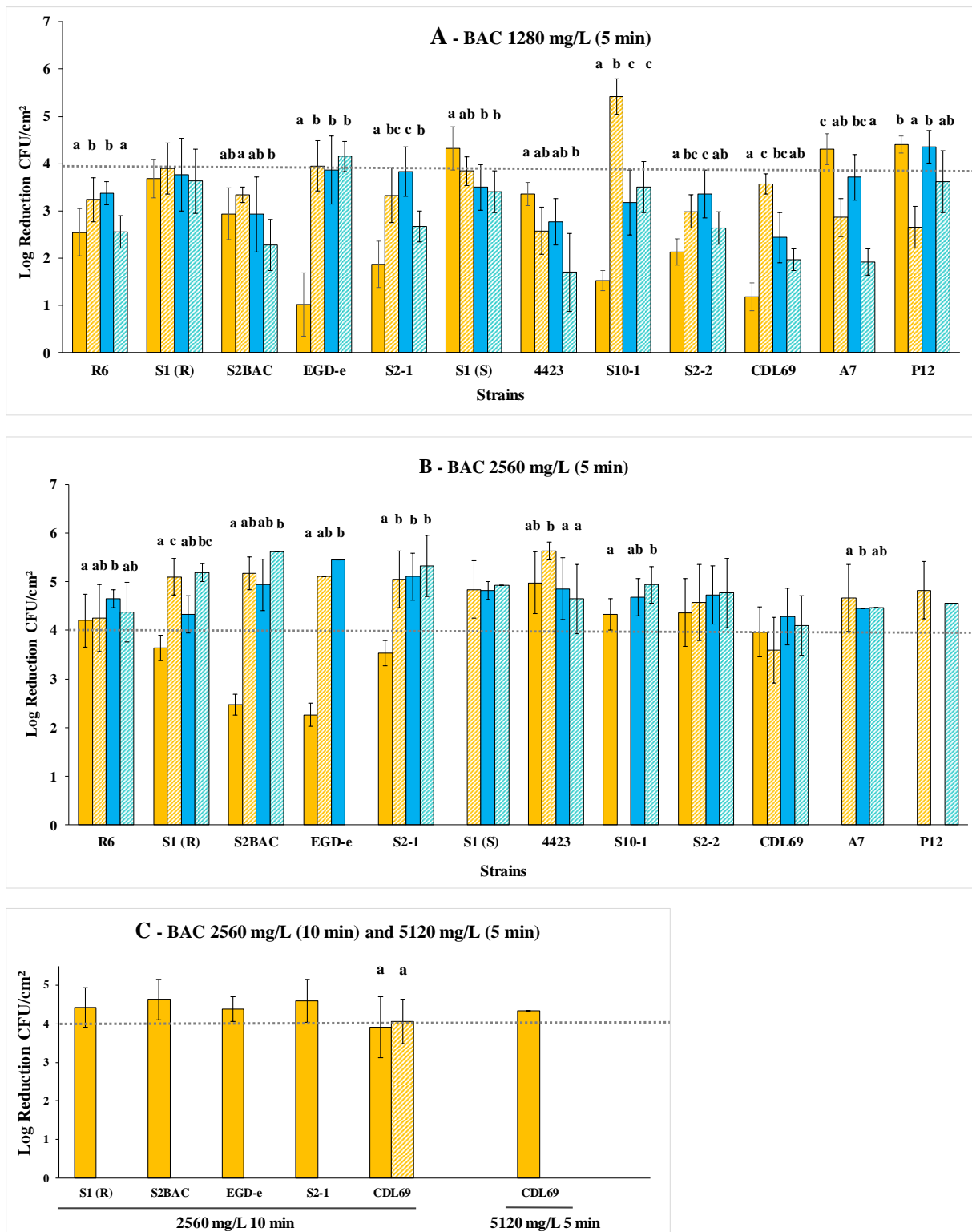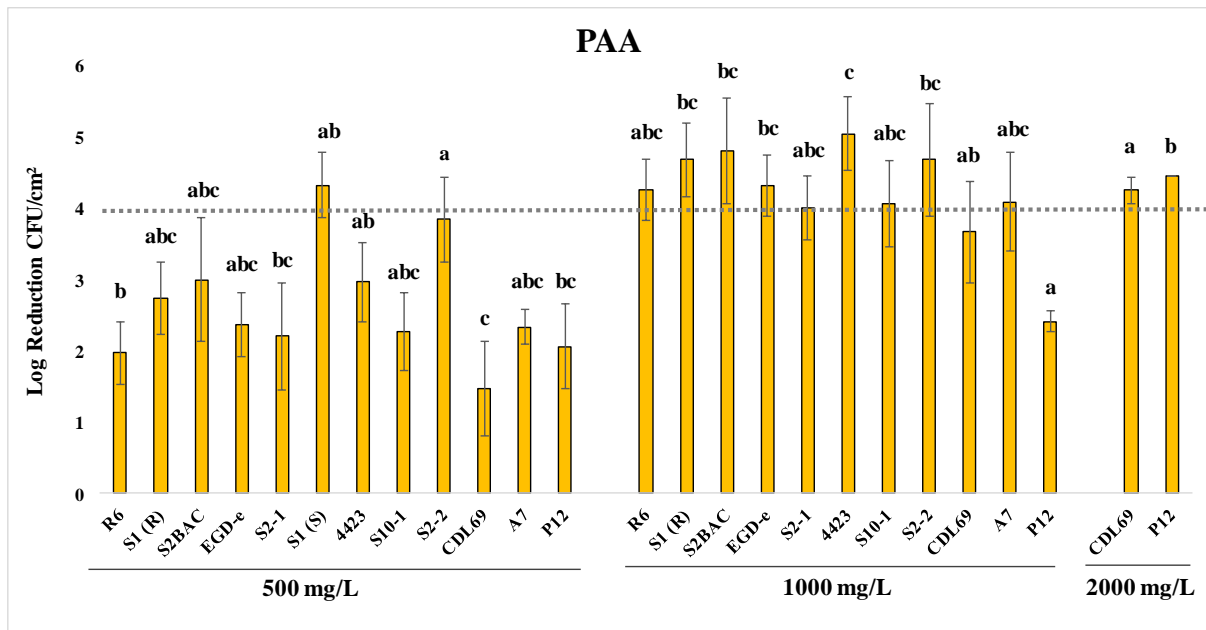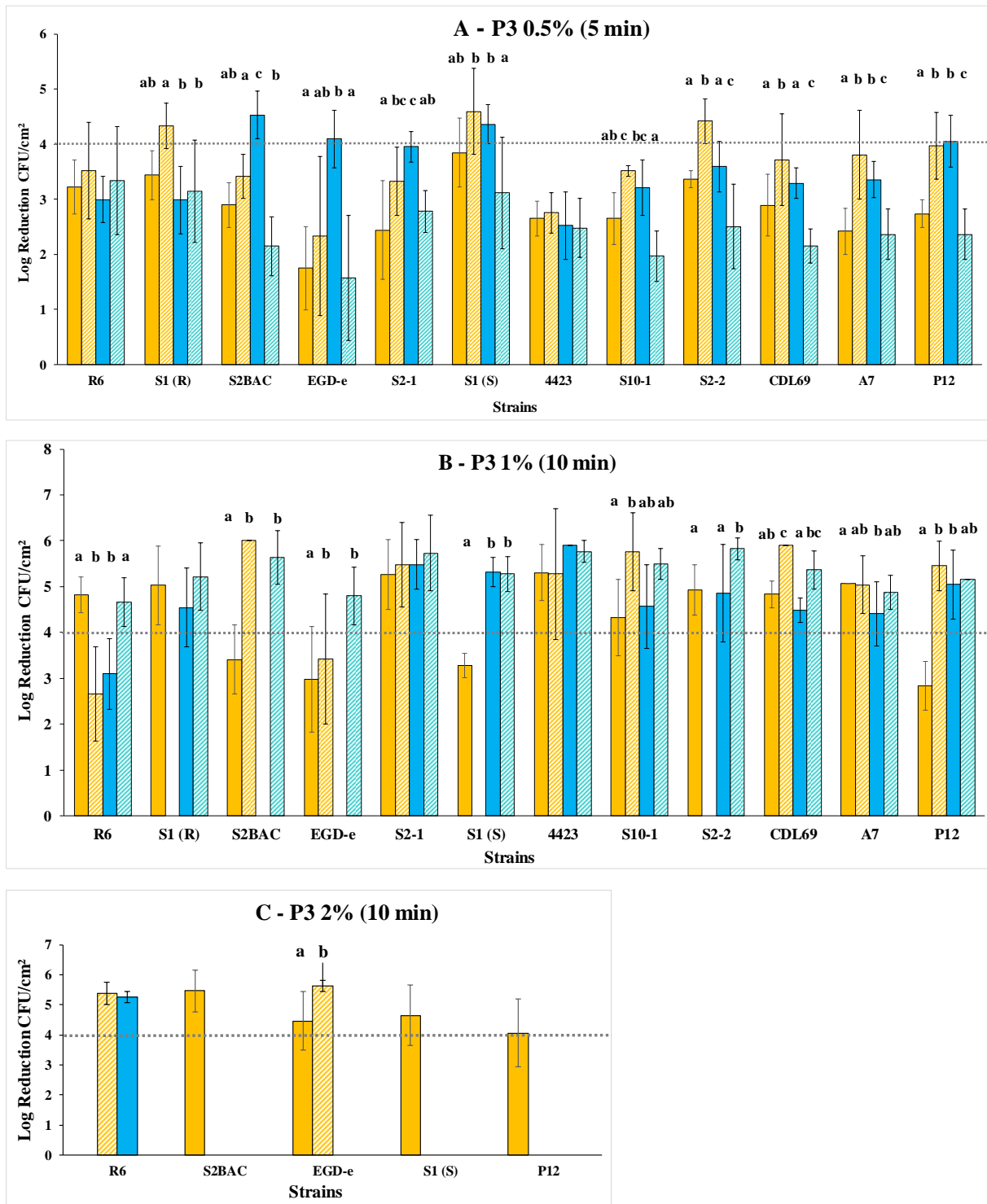

Figure 3.4. – *L. monocytogenes* biofilms Log reductions after exposure to P3.

A - exposure to 0.5% for 5 min; **B** - exposure to 1% for 10 min; **C** - exposure to 2% for 10 min.

Biofilms were grown in TSB-YE (25 ºC) (■), TSB-YE/10 (25 ºC) (▨), TSB-YE (11 ºC) (■) and TSB-YE/10 (11 ºC) (▨). Error bars represent standard deviation. Different letters in the columns indicate significant differences ($p < 0.05$) in average values. Results of rich media are from Vera Maia (Thesis Project, 2017).

3.3. Disinfectant susceptibility of planktonic cells

The twelve strains used in this work were previously characterized concerning their Minimum Inhibitory Concentrations (MIC) of BAC and PAA (López et al. 2007; López et al. 2008; López et al. 2013; Ortiz et al. 2014; Ortiz et al. 2016). For BAC, MIC ranged between 1.25 and 20 mg/L. Strains EGD-e, S2-1 and S1(S) are the most sensitive to the disinfectant with MIC values of 1.25, 2.5 and 2.5 mg/L respectively. For PAA, all strains were reported with the same MIC of 200 mg/L.

In the present work, the susceptibility to BAC and to PAA of strains EGD-e, S2-1 and CDL69 was determined by the "Microdilution broth method". The obtained MIC values of BAC were 5 mg/L for EGD-e and S2-1 and 20 mg/L for CDL69. For PAA, all strains had the same MIC value (1600mg/L) (Table 3.1.).

Table 3.1. - Minimum Inhibitor Concentration of BAC and PAA determined by the "Agar dilution assay on Mueller Hinton agar plates" and by the "Microdilution method".

	Disinfectants			
	Benzalkonium chloride		**Peracetic acid**	
Strains	**MIC (mg/L)** (This work)	**MIC (mg/L)** (Ortiz et al., 2014; Ortiz et al., 2016)	**MIC (mg/L)** (This work)	**MIC (mg/L)** (Ortiz et al., 2014; Ortiz et al., 2016)
EGD-e	5	1.25	1600	200
S2-1	5	2.5	1600	200
CDL69	20	20	1600	200

Comparing the MICs of BAC with the previously obtained with the "Agar dilution assay on Mueller Hinton agar plates", strain CDL69 had the same value, regardless the method used, and strains S2-1 and EGD-e have slight different values (Table 3.1). This can be explained by the differences in both procedures for MIC determination. Nevertheless, with both methods, the MICs values of PAA for the three strains analysed were the same (Table 3.1), suggesting the same PAA susceptibility among strains.

According to the biofilm susceptibility to BAC (Fig. 3.2), strain CDL69 showed the highest resistance followed by a group of four strains (S1(R), S2[BAC], EGD-e and S2-1). Interestingly, strains EGD-e and S2-1 had the lowest MIC values of BAC, in the planktonic state (Table 3.1).

The 12 strains were previously characterized regarding the MIC of PAA for planktonic cells, and all displayed identical MICs. By contrast, the evaluation of the listericidal activity of PAA in the biofilm state showed different susceptibility among strains, since three different PAA concentrations were used to achieve the 4 Log reduction threshold (Fig. 3.3).

These results indicate that the response of *L. monocytogenes* in the sessile form to disinfectants is different from the one in the planktonic form.

3.4. Combined results

A Principal Component Analysis (PCA) of the data concerning the values of SSC enumeration in the four growth conditions and of susceptibility to BAC (1280 mg /L) of biofilms grown in TSB-YE at 11 ºC and 25 ºC was performed (Fig. 3.5).

By PCA, the initial six variables could be reduced to a plane F1F2 defined by the two first principal components. This plane accounts for about 76% of the variance explained by the original data (Fig. 3.5). The correlations between the original variables and the first two principal components are presented in Fig. 3.5A.

Values of biofilm cell enumeration are in quadrants 2 and 3 presenting negative correlation values with F1, decreasing along this axis. Values of BAC susceptibility are located in quadrant 1 presenting simultaneously positive correlation with F1 and F2, increasing along both axis.

The projection of the twelve strains in the plane F1F2 is presented in Fig. 3.5B. Strains in quadrant 1 (S1(S), S1(R), P12 e A7) are characterized by a higher susceptibility when compared with the rest of strains. The grouping profile is mainly determined by BAC susceptibility since strains present higher heterogeneity in biofilm forming ability.

Cluster analysis (Fig. 3.6) confirmed the presence of two clusters of strains suggested by PCA. In fact, when a linkage distance of about 1.3 is used, the different strains could be grouped in two clusters (C1 and C2).

Within cluster C2, corresponding to the most BAC susceptible biofilms, there are strains referenced as sensitive and resistant to BAC, based on MIC values (data not shown). The same heterogeneity in MIC values was observed for cluster C1, which corresponds to the less susceptible strains.

Figure 3.5. - Principal Component Analysis.
Loadings of SSC enumeration at four different conditions (25.T, 25.T/10, 11.T, 11.T/10) and BAC susceptibility values of biofilms grown **in rich nutrient conditions** (BAC.25.T and BAC.11.T) on the first and second principal components **(Fig. 3.5A)**; plot of the strains on the plane defined by first and second principal components **(Fig. 3.5B).**

Biofilm growth conditions: 25.T and 25.T/10 - biofilms grown at 25 ºC in TSB-YE and in TSB-YE/10, respectively; 11.T and 11.T/10 - biofilms grown at 11 ºC in TSB-YE and in TSB-YE/10, respectively.
BAC treatment: 1280 mg/L, 5 min.

Figure 3.6. - Dendrogram for *L. monocytogenes* strains.
Loadings of SSC enumeration at four different conditions (25.T, 25.T/10, 11.T, 11.T/10) and BAC susceptibility values of biofilms grown **in rich nutrient conditions** (BAC.25.T and BAC.11.T).

Biofilm growth conditions: 25.T and 25.T/10 - biofilms grown at 25 ºC in TSB-YE and in TSB-YE/10, respectively; 11.T and 11.T/10 - biofilms grown at 11 ºC in TSB-YE and in TSB-YE/10, respectively.
BAC treatment: 1280 mg/L, 5 min.

Similarly, a PCA of the data concerning the values of SSC enumeration in the four growth conditions and of susceptibility to 1280 mg /L BAC of biofilms grown in TSB-YE/10, at 11 ºC and 25 ºC was also performed (Fig. 3.7).

By PCA, the initial six variables could be reduced to a plane F1F2 defined by the two first principal components. This plane accounts for about 75% of the variance explained by the original data (Fig. 3.7). The correlations between the original variables and the first two principal components are presented in Fig. 3.7A.

Values of biofilm enumeration are in the quadrants 2 and 3 (Fig. 3.7A), presenting negative correlation values with F1, decreasing along this axis. Values of BAC susceptibility are located in the quadrant 4, presenting simultaneously positive values of correlation coefficient with F1 and negative values of correlation with F2, increasing along the F1 axis and decreasing along F2 axis.

The projection of the different isolates in the plane F1F2 is presented in Fig. 3.7B. Strains in quadrant 2 and 3 (C1) are characterized by a higher biofilm ability when compared with the strains present in quadrants 1 and 4 (C2, C3 and C4). Strain S10-1 (C4) is the most susceptible to 1280 mg/L of BAC followed by strains S1(S), S1(R) and EGD-e (C3). The grouping profile is determined equally by SSC enumeration and BAC susceptibility since strains present high heterogeneity in both types of variables. Once more, BAC resistance in biofilm form did not corroborate BAC MICs previously referenced (López et al. 2007; López et al. 2008; López et al. 2013; Ortiz et al. 2014; Ortiz et al. 2016), since BAC resistant strains in the planktonic state are the most susceptible in the biofilm form.

Cluster analysis (Fig. 3.8) confirmed the presence of the four clusters of strains suggested by PCA. In fact, when a linkage distance of about 1.25 is used, the different strains can be grouped in four clusters (C1 C2, C3 and C4).

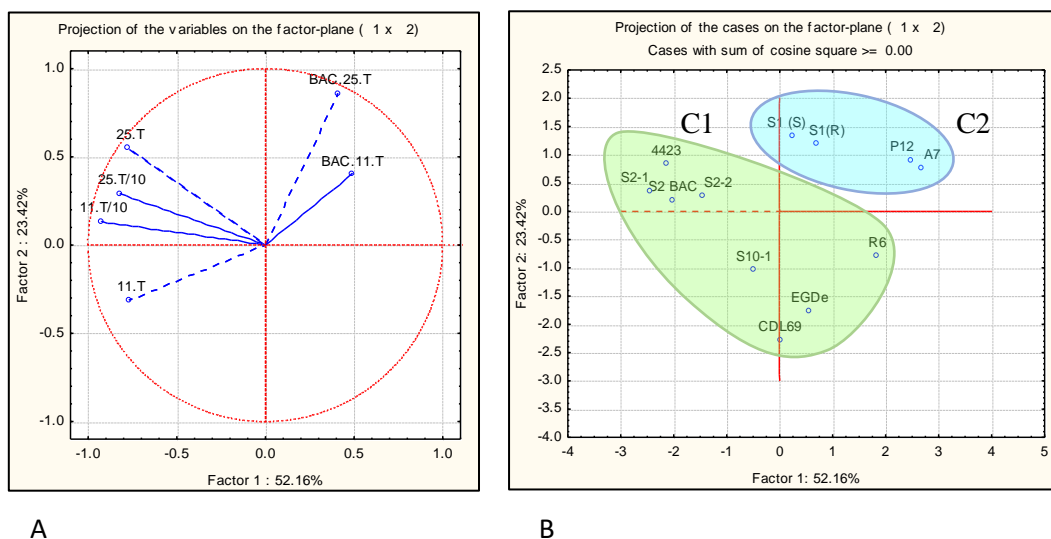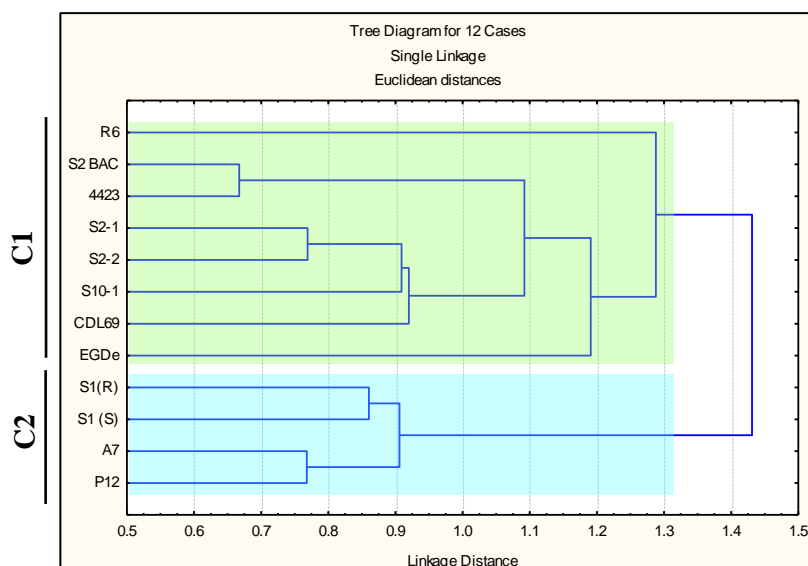A   B
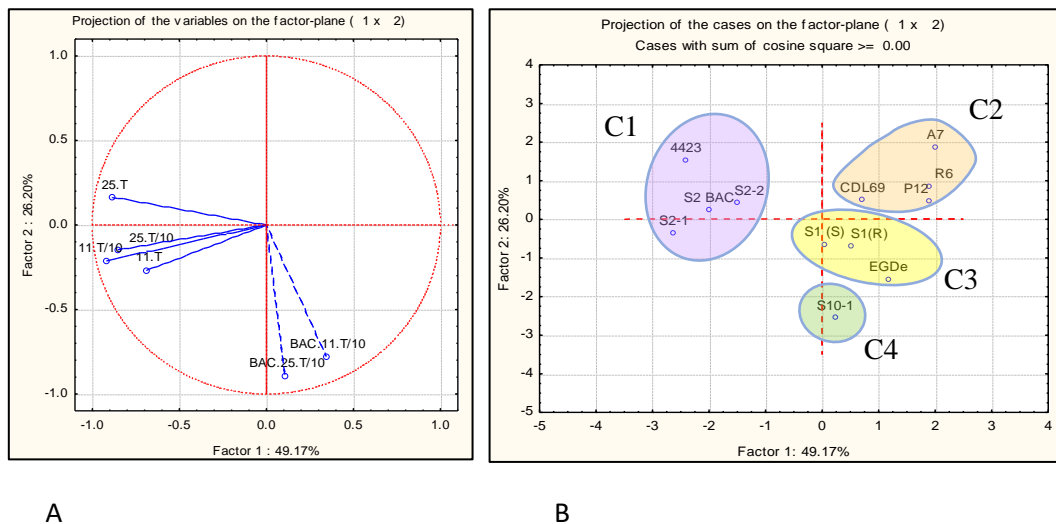
Figure 3.7. - Principal Component Analysis.
Loadings of SSC enumeration at four different conditions (25.T, 25.T/10, 11.T, 11.T/10) and BAC susceptibility values of biofilms grown **under nutritional stress** (BAC.25.T/10 and BAC.11.T/10) on the first and second principal components **(Fig. 3.7A)**; plot of the strains on the plane defined by first and second principal components **(Fig. 3.7B).**

Biofilm growth conditions: 25.T and 25.T/10 - biofilms grown at 25 ºC in TSB-YE and in TSB-YE/10, respectively; 11.T and 11.T/10 - biofilms grown at 11 ºC in TSB-YE and in TSB-YE/10, respectively.
BAC treatment: 1280 mg/L, 5 min.
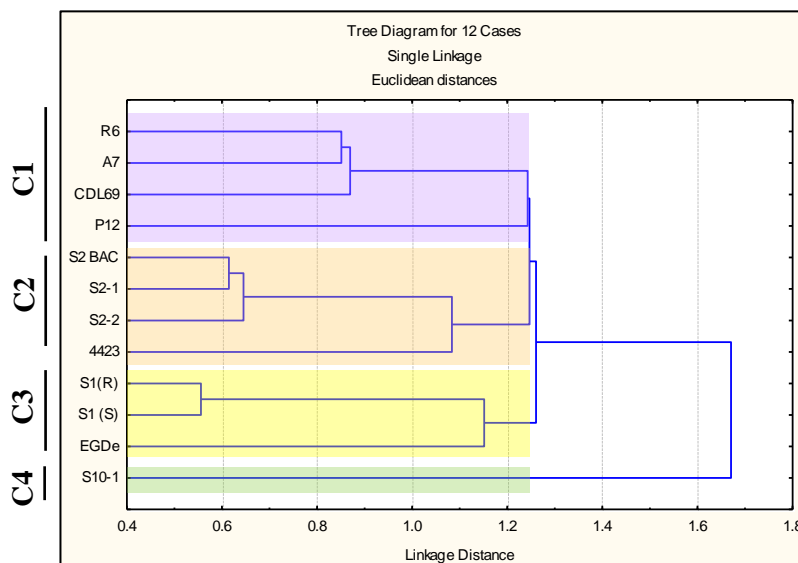
Figure 3.8. - Dendrogram for *L. monocytogenes* strains.
Loading of SSC enumeration at four different conditions (25.T, 25.T/10, 11.T, 11.T/10) and BAC susceptibility values of biofilms grown **under nutritional stress** (BAC.25.T/10 and BAC.11.T/10).

Biofilm growth conditions: 25.T and 25.T/10 - biofilms grown at 25 ºC in TSB-YE and in TSB-YE/10, respectively; 11.T and 11.T/10 - biofilms grown at 11 ºC in TSB-YE and in TSB-YE/10, respectively.
BAC treatment: 1280 mg/L, 5 min.

23

When comparing both cluster analysis (Fig. 3.6 and Fig. 3.8), a higher heterogeneity was observed when BAC susceptibility was tested in biofilms grown under nutritional stress (in TSB-YE/10). This probably suggests that under nutritional stress, the response of the strains tend to differentiate more. In fact, Cabrita and colleagues (2013) showed that the levels of similarity of the polypeptide profiles of *L. monocytogenes* strains decreased with the decreasing growth temperature, suggesting an increasing differentiation of strain response with low temperatures.

3.5. General discussion

In both BAC and PAA listericidal assays, the concentration required to achieve the 4 Log reduction threshold in the biofilm form suggests a considerable increased resistance when compared with the free-living form. In fact, biofilms have been shown to be more resistant to environmental stresses than planktonic counterparts (Wirtanen & Salo 2003; Harvey et al. 2007; van der Veen & Abee 2011; Rodríguez-López et al. 2017).

In the case of BAC, the difference between the MIC values and the concentrations required to achieve the 4 Log reduction threshold in biofilm cells is 1.25-20 mg/L and 1280-5120 mg /L. Whereas, for PAA is 200 mg/L and 500-2000 mg/L.

The concentrations used for the biofilm susceptibility assays coincide with current recommended concentration for both disinfectant agents. PAA recommended concentration range is 800 - 2500 mg/L (4 °C–20 °C, for 5–30 min) while recommended QACs concentrations are referenced as 2000–5000 mg/L (room temperature, for 20–120 min) by Poimenidou and colleagues (2016) or as 200-1000 mg/L by Møretrø and colleagues (2016).

The effectiveness of PAA in removing bacterial biofilms has long been acknowledged justifying the recurrent use in the food industry (Pan et al. 2006; Ceragioli et al. 2010). Marques and colleagues (2007), found that PAA had higher efficiency in removing adhered cells of *Staphylococcus aureus* compared with hydrogen peroxide and sodium dichloroisocyanurate (Chmielewski & Frank 2003; Lee et al. 2016).

The remarkable variation between biofilm susceptibility and the correspondent MICs of BAC and the higher efficiency of PAA on removing biofilms can be explained by the mode of action and the intrinsic characteristics of the disinfectants or even by some microbial stress response to their action. Disinfectants have multiple cellular targets such as the cell wall, the cytoplasmic membrane, DNA, RNA, functional and structural proteins, and other cytosolic components. The two active compounds have different modes of action. BAC, a quaternary ammonium compound, is a membrane-active agent and PAA acts essentially as an oxidising agent (Bridier et al. 2011).

In fact, PAA generates superoxide anions and a burst of free hydroxyl radicals (OH) which can damage bacterial DNA probably leading to cell death (Belessi et al. 2011; van der Veen & Abee 2011). It's high reactivity and oxidizing ability, together with a considerably strong decomposition rate, facilitate PAA entry into the biofilm matrix. Its low molecular size also greatly increases its ability to penetrate the biofilm network (Ibusquiza et al. 2011). Interestingly, previous studies performed with ozone, another molecule that also has high reactivity and small size, showed to be highly effective on *S. aureus* biofilms (when compared to planktonic cells) (Cabo et al. 2009).

In contrast, the structure of the biofilm presented a significant barrier to BAC entry, possibly reducing its ability to diffuse into the matrix and to reach the cells (Ibusquiza et al. 2011; Van Acker et al. 2014). Since the bacterial surface is hydrophilic and negatively charged, BAC, a hydrophilic cationic molecule, can penetrate the cell wall to disrupt the cytoplasmic membrane of the cells. But biofilms act as a ''shield'' decreasing the accessibility to the cells, particularly those present at the bottom of the biofilm.

In addition, BAC is confronted with glycocalyx development. This polyanionic barrier functions as an ion-exchange resin capable of binding a very large number of molecules, hampering the access of the disinfectants to the cell membrane (Chavant 2004; Van Acker et al. 2014).

Results from growth and survival studies also indicated that *L. monocytogenes* harbouring *qacH* or *bcrABC* genes may show increased fitness in situations where cells are exposed to BAC inhibitory concentrations but not to lethal concentrations. In addition, strains adapted to BAC overexpress the chromosomally encoded multidrug efflux pump MdrL (Mereghetti et al. 2000; Romanova et al. 2006; Møretrø et al. 2016). Seven out of the twelve strains used in this study present resistance markers to BAC: strains 4423, S10-1 and S2-2 harbour *qacH* gene, while strains CDL69, A7, P12 and R6 harbour *bcrABC* gene. However, biofilm susceptibility assays showed that only one strain (CDL69) out of the five more resistant to BAC (S1(R), S2^{BAC}, EGD-e, S2-1 and CDL69) harboured one *bcrABC* gene. These results suggest that strains with resistance markers to BAC, such as *bcrABC* and *qacH* genes, might not necessarily show higher resistance in the biofilm state.

In fact, among *L. monocytogenes* with these resistant genes, tolerance levels have been found to be 30-40 mg/L QAC (agar-based MIC assays), while in use concentrations of QAC range between 200-5000 mg/L (Møretrø et al. 2016; Poimenidou et al. 2016). The question raised is whether this tolerance level has any practical relevance in the food industry (Kastbjerg & Gram 2012; Gerba 2015). Recent results indicate that there are conditions where *L. monocytogenes* with *qacH*/*bcrABC* genes may present a growth advantage in the food industry, namely when QAC containing disinfectants are not properly rinsed off after sanitation (Møretrø et al. 2016). Contrary, other authors concluded that the resistance of biofilms may be due to the attributes of the EPS of biofilm structure, and not to genetic attributes of the cells (Yun et al. 2012)

The results presented here emphasize the need for further research on biofilm susceptibility. The choice of the disinfectant, its concentrations and exposure times should be adequate for biofilm eradication from equipment and utensils in the food industry. It is also of utmost importance the understanding of the ecological and genetic characteristics of the strains resistant to QAC, since it constitutes on of the most commonly applied disinfectants.

4. Conclusions

The study of foodborne pathogens concerning their biofilm-forming ability has been crucial for food safety and, consequently, for consumer health. Besides improper cleaning, inadequate disinfection procedures are responsible for the inefficient elimination of microorganisms from surfaces. The efficacy of disinfectants is usually determined by using microbial suspensions, not mimicking the real biofilm growth on surfaces. Disinfectant agents are required to inactivate microorganisms, but often the possible protection of biofilm structures to disinfection is not considered (Wirtanen & Salo 2003).

The results from BAC and PAA *L. monocytogenes* biofilm susceptibility tests showed that concentrations of the disinfectants required to achieve the 4 Log reduction threshold were considerably higher than the respective MIC values of the twelve strains used. These results indicate that biofilm cells are far more resistant than free-living cells and that, in the sanitizing process, the concentrations and exposure times of the disinfectants, must be optimized to effectively eliminate sessile cells. In addition, strains whose biofilms were eliminated with the highest concentration of the disinfectants were not necessarily the ones previously characterized with the highest MICs. This indicates that the response to disinfectants of *L. monocytogenes* in the biofilm state is different from the one in the planktonic form.

Our findings also showed that PAA had a more powerful effect than BAC in eliminating *L. monocytogenes* biofilms, corroborating previous results from different authors (Ceragioli et al. 2010; Bridier et al. 2011; Ibusquiza et al. 2011). The oxidizing ability of PAA together with its low molecular size probability account for its effectiveness.

Seven out of the twelve strains used in this study present BAC resistance markers: three strains harbour the *qacH* gene while four strains harbour the *bcrABC* gene. Our results showed that only one strain (CDL69) out of the five more resistant to BAC in biofilm state (S1(R), S2^{BAC}, EGD-e, S2-1 and CDL69), harboured one of these genetic markers (*bcrABC*), contradicting Moretro and colleagues (2016) stating that strains with *bcrABC* and *qacH* genes might present survival advantage in the food industry.

In the present work, a disinfectant was considered effective when a 4 Log reduction was reached (European Standard EN 13697 2001). However, other researchers used a 5 Log reduction threshold to consider that a properly disinfection process was carried on (Królasik et al. 2010; Cruz & Fletcher 2012). Considering this requirement of a 5 Log reduction, the BAC concentration of 5120 mg/L would not be enough to effectively eliminate any of the twelve strains used in this study. In a twofold dilution assay, probably 10240 mg/L of BAC would result in the required reduction. This suggests that the persistence of *L. monocytogenes* in the food industry environment might not be related with BAC resistance or with other bacterial features but with the use of a low concentration of disinfectant to eliminate biofilm cells.

Biofilm formation assays showed that neither different nutrient conditions nor different temperatures had significant effect ($p > 0.05$) on biofilm formation. By contrast, biofilms incubated in TSB-YE/10 (25 ℃) and in TSB-YE (11 ℃) showed to be more susceptible to BAC and to P3-Oxonia. Moreover, biofilms formed under nutritional stress (TSB-YE/10) tend to differentiate more their response to BAC, than biofilms grown in rich nutrient conditions (TSB-YE). These findings emphasize the need for an efficient cleaning of equipment and utensils in the food industry, especially those maintained at room temperature.

In the future, more research on biofilm's susceptibility should be developed. This include assays trying to mimic the food industry environment, with biofilms in co-culture, exposed to new disinfectants, eventually, to natural active compounds. In order to identify ideal sanitizers, the standardization of the CFU reduction threshold would be important for proper comparison of results between research groups worldwide.

This would certainly contribute to guarantee food safety and consumer welfare. In addition, the reputation of the companies and its products can be protected, customer loyalty retained and profits preserved.

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