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Biofilm disinfection survival and growth dynamics of *Listeria monocytogenes* and *Pseudomonas spp.* from a Norwegian salmon processing facility

by

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Abstract

Norway is the world's leading producer of farmed Atlantic salmon (*Salmo salar*), exporting the majority for further processing in EU countries. With the rise of ready-to-eat salmon products, the contamination and proliferation of the deadly human foodborne pathogen *Listeria monocytogenes* is a major problem for both the industry and health authorities.

L. monocytogenes is primarily transmitted to salmon from contamination of the processing environment. The species can establish persistent colonization in biofilms, surviving cleaning and disinfection routines over several years. There is growing agreement that the relationship between *L. monocytogenes* and the ubiquitous microflora of the food processing environment play an important role in the persistence of this pathogen. Of the non-pathogenic microflora, *Pseudomonas spp.* are of special regard due to surviving disinfection exceptionally well, being strong biofilm producers as well as being linked to the survival of *L. monocytogenes*.

This project has elucidated some of the growth dynamics in biofilm and suspension of a mix of five *Pseudomonas spp.* isolates and one *L. monocytogenes* ST8 isolate sampled from contact surfaces and equipment in a Norwegian salmon processing facility. *Pseudomonas spp.* dominated in both suspension and biofilm. *L. monocytogenes* constituted approximately 2 % of the total population in suspension at 72 h and less than 1 % at 144 h, after a second incubation cycle. The opposite pattern was seen in biofilm, as the *L. monocytogenes* subpopulation increased from between 1 and 2 % at 72 h to 4 % at 144 h.

Biofilm disinfection survival characteristics of the cultures was investigated. Biofilms grown for 72 h on stainless steel coupons were submerged in a 1 % solution of the peracetic acid disinfectant used at the facility for 10 minutes. A 5 log reduction was achieved for all cultures. No *L. monocytogenes* mono-culture biofilm survived the treatment. *L. monocytogenes* did however survive in 10 of 18 parallels when co-cultured with *Pseudomonas spp.* Surviving cultures exhibited an extended lag-phase, not showing visible growth before 2-4 days after treatment. The results from this project demonstrate how *L. monocytogenes* can survive disinfection better when growing in biofilm with *Pseudomonas spp.* compared to monoculture and shows the importance of frequent and thorough disinfection to supress the growth and spread of *L. monocytogenes*.

Sammendrag

Norge er verdens største produsent av Atlantisk laks (*Salmo salar*) og eksporterer majoriteten til videre prosessering i EU-land. Spiseklare produkter av laks blir en stadig viktigere del av markedet, men innebærer risiko for spredning av den matbårne patogene bakteriearten *Listeria monocytogenes*, noe som er problematisk for både industrien og helsemyndigheter.

L. monocytogenes blir hovedsakelig overført til lakseprodukter via kontaminering av produksjonsmiljøet. Arten kan etablere vedvarende kolonisering av matproduksjonsanlegg ved å vokse i biofilm, og kan overleve rengjørings- og desinfeksjonsbehandlinger i flere år. Forholdet mellom *L. monocytogenes* og den ikke-patogene mikrofloraen i slike miljøer har blitt utpekt som en viktig faktor for hvorvidt *L. monocytogenes* kan etablere en vedvarende kolonisering. I mikrofloraen er *Pseudomonas spp.* i særstilling grunnet stor grad av desinfeksjonsoverlevelse, er en god biofilmdanner, og er ofte knyttet til overlevelse av *L. monocytogenes*.

Dette prosjektet undersøkte vekstdynamikken i biofilm og suspensjon for en blanding av fem *Pseudomonas spp.* isolater og et *L. monocytogenes* ST8 isolat isolert fra kontaktpunkter og produksjonsutstyr på et norsk lakseslakteri. *Pseudomonas spp.* dominerte vekst i både suspensjon og biofilm. *L. monocytogenes* utgjorde omtrent 2% av den totale populasjonen i suspensjon ved 72 t og under 1% ved 144 t, etter andre inkubasjonssyklus. Andre tendenser ble observert i biofilm, hvor *L. monocytogenes* økte fra 1-2 % ved 72 t til 4 % ved 144 t.

Overlevelsesegenskaper i biofilm ble undersøkt for de ulike bakteriekulturene. Biofilm dyrket på kuponger av rustfritt stål i 72 t ble dekt av 1 % løsning av pereddiksyrebasert desinfeksjonsmiddel, i bruk ved lakseslakteriet, i 10 min. Målet på 5 log reduksjon ble oppnådd for alle kulturer. *L. monocytogenes* renkultur overlevde ikke denne behandlingen. *L. monocytogenes* overlevde derimot i 10 av 18 paralleller i blandingskultur med *Pseudomonas spp.* Overlevende kulturer hadde utvidet lag-fase, og viste synlig tegn til vekst 2 til 4 døgn etter behandling. Resultatene fra dette prosjektet viser at *L. monocytogenes* kan overleve desinfeksjon bedre i blandingskultur med *Pseudomonas spp.* enn alene og demonstrerer betydningen av grundig og hyppig desinfeksjon for å redusere oppblomstring og spredning av *L. monocytogenes*.

Preface

This thesis represents my final project at the Food Technology bachelors programme at the Norwegian University of Science and Technology, Faculty of Natural Sciences, Department of Biotechnology and Food Science in Trondheim. All laboratory work has been carried out in the microbiological laboratory at Akrinn, Kalvskinnet Campus in Trondheim. Work on the project has taken place from January to May 2021, with experiments conducted between February and April.

I want to sincerely thank my supervisors, associate professor Lisbeth Mehli and doctoral fellow Gunn Merethe Bjørge Thomassen for your relentless support and assistance during the entirety of this project. Thank you for the feedback on my written work and the hours upon hours of laboratory assistance.

I would also like to thank my parents, friends and flat mates for your love and support, especially in these last months. I am also sorry for having subjected too many of you to hours of rambling on the intricacies of microbial biofilms.

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1. Introduction

The Norwegian aquaculture industry, especially the farming of Atlantic salmon (*Salmo salar*), is a cornerstone industry of many regional economies along the coast and is one of the most important export commodities on a national basis (Johansen et al., 2019). Norway produces more than half of all farmed salmon worldwide, exporting the majority fresh for further processing in EU countries (Iversen et al., 2020; Johansen et al., 2019; Oglend & Straume, 2019). Both aquaculture and associated industries have experienced exponential growth over the last decades, a trend which is predicted to continue, with worldwide rising demands for food generally and salmon specifically (Brækkan & Thyholdt, 2014; Costello et al., 2020; FAO, 2020; Garlock et al., 2020). This trend can be partially attributed to the rise of minimally processed foods containing salmon, such as sushi and sashimi, as well as the enduring demand for cold smoked salmon and other Ready-To-Eat (RTE) products (Altintzoglou et al., 2016; Hsin-I Feng, 2012). Requirements for food quality and -safety are greater than ever, with the raw material having to be of utmost quality (Eicher et al., 2020; Lehel et al., 2020; Rørvik, 2000).

1.1 Microbial spoilage of salmon

Raw salmon is an easily perishable commodity whose quality rapidly deteriorates during storage. Microorganisms account for the most significant part this process and are in large part responsible for the changes which render raw fish unfit for consumption (Ghaly et al., 2010; Gram & Huss, 1996). These changes include an unpleasant smell as well as visible slime and discolouration of the surface; characteristics consumers are keenly aware of when evaluating the quality of fresh fish (Brunsø et al., 2009; Gram & Huss, 1996). The smell of spoiling fish comes from volatile metabolites produced by its microflora, especially by the subset called specific spoilage organisms (SSOs), and includes biogenic amines, TMA, esters, ketones, aldehydes and sulphuric compounds (Gram & Huss, 1996). Slime and surface discoloration are a result of general microbiological activity and may come from visible microbial colonies, changes to the substrate (degradation of pigments) or metabolites such as extracellular polymeric substances (EPS) (Gram, 2009; Gram & Huss, 1996).

Both onset and severity of microbial spoilage in fresh salmon depends on a number of variables, such as the conditions at which the fish is stored and the makeup of the microflora. The storage temperature can be regarded as the most important determinant for shelf life, as

fish stored at room temperature will spoil in a matter of hours and superchilled fish can be acceptable after several weeks (Erikson, Misimi & Gallart-Jornet, 2011; Ghaly et al., 2010). Another major determinant is the atmosphere in which the product is stored. This has the effect of inhibiting some undesirable chemical reactions, mostly related to discolouration and oxidation, and selects which microorganisms can grow (Gram & Huss, 1996; Lerfall, Thomassen & Jakobsen, 2018). It is not only growth during storage, but the quantitative and qualitative makeup of the microflora at the beginning of this period that influence how quickly spoilage proceeds. Live fish have areas of high microbial activity, notably the skin, gills and gut, juxtaposed to virtually sterile muscle (Gram & Dalgaard, 2002; Minniti et al., 2017; Rudi et al., 2018). The processing environment and equipment also serves as a reservoir for undesirable microorganisms which may be transferred onto the salmon (Gram, 2009; Larsen et al., 2014). It is possible to limit the amount and kind of microbes contaminating the product during processing if good hygienic practices (GHP) and sufficient cleaning and disinfection (CD) routines are followed (Ashie, Smith, Simpson & Haard, 1996; Fagerlund et al., 2017, Larsen et al., 2014).

Raw, chilled salmon is an environment which supports the growth of a wide range of microorganisms, but some of these are especially relevant for further study. One group which attracts special attention are the SSOs. For marine fish from cold climates stored at low temperatures, the most important spoilage organisms are bacteria in the genera *Pseudomonas*, *Shewanella* and *Photobacterium* (Boziaris & Parlapani, 2017; Macé et al., 2012). *Pseudomonas* and *Shewanella* dominate in aerobic conditions, while *Photobacterium* is the most important SSO when oxygen is limited (Boziaris & Parlapani, 2017; Tryfinopoulou, Tsakalidou & Nychas, 2002). These bacteria produce the abovementioned metabolites which are tied to the sensory experience of spoiling fish (Gram, 2009; Gram & Huss, 1996). *Pseudomonas spp.* are involved in the production of esters and biogenic amines, important in the characteristic fruity aromas at the onset of microbial spoilage, while Shewanella and Photobacterium reduce TMAO to TMA and produce sulphuric compounds linked to the later stages of spoilage (Castell & Greenough, 1957; Geornaras, Dykes & von Holy 1995; Gram & Huss, 1996; Miller et al., 1973; Parlapani et al., 2015; Rezaei et al., 2007). Whereas all these spoilage organisms are natural parts of the salmon microbiome, *Pseudomonas spp.* are of special note due to their ability to colonize the processing environment and survive CD routines (Fagerlund et al., 2017; Gram & Dalgaard, 2002; Minniti et al., 2017; Møretrø & Langsrud, 2017; Rudi et al., 2018). Another important group is human pathogens, of which

Listeria monocytogenes is significant for its ability to proliferate during cold storage (Eicher et al., 2020).

1.2 *Pseudomonas*

Pseudomonas is a diverse and widely distributes genus of Gram-negative bacteria of major importance in salmon processing. *Pseudomonas spp.* are obligate aerobes (although some may grow anaerobically using nitrate), grow quickly at refrigeration temperatures and have few specific nutritional requirements (Palleroni, 2015; Xie et al., 2018). The genus is divided into two phylogenetic groups, the *P. aeruginosa*- and *P. fluorescens* lineage, of which the latter is relevant in the current context (García-Valdés & Lalucat, 2016).

A wide range of behaviours and lifestyles are observed for the members of this diverse genus. The most common lifestyle for *Pseudomonas spp.* is saprophytically, breaking down organic matter in the environment, but some species have shown the ability to live pathogenically in plants, animals and, in a few cases, humans (Palleroni, 2015; Peix, Ramírez-Bahena & Velázquez, 2018). The most famous example of human pathogenicity is that of *P. aeruginosa* in sufferers of cystic fibrosis, but some of the species which are found on salmon and in salmon processing plants (*P. fluorescens* and *P. putida*) have also been implied in pathogenic behaviour towards severely immunocompromised individuals (Iglewski, 1996; Sadikot et al., 2005; Von Graevenitz & Winstein, 1971). There are also examples of fish pathogens and documentation of these targeting salmon (Wiklund & Bylund, 1990). Their main role in the context of salmon processing is however in aerobic spoilage of chilled fish and as colonizers of the processing environment.

1.2.1 *Pseudomonas* in salmon and salmon processing

Pseudomonas spp. enjoy many competitive advantages compared to other species of the microflora in salmon and the salmon processing environment. They are naturally present as part of the salmon microbiome and grow very well on the protein-rich substrate in the temperatures at which fresh fish are stored (Fogarty et al., 2019; Gram et al., 2002). Their competitive advantage is a result of rapid growth, the production of toxic metabolites, antibacterial and -fungal compounds and siderophore-mediated sequestration of iron (Gram et al., 2002). *Pseudomonas spp.* therefore play an important role in shaping the composition of the spoilage microflora (Eissa et al., 2014; Gram, 1993; Gram & Melchiorsen, 1996). In the

processing environment, rapid growth and production of large amounts of extracellular polymeric substances (EPS) are important differentiating factors (Møretrø & Langsrud, 2017; Palleroni, 2015). Cells encapsulated in EPS are protected from both active threats (disinfectants) and passive threats (desiccation, nutrient starvation) and may explain why *Pseudomonas spp.* are the most commonly isolated microbes after CD routines (Bagge-Ravn et al., 2003; Langsrud et al., 2016; Møretrø et al., 2016; Møretrø & Langsrud, 2017; Odeyemi et al., 2020). Fagerlund et al. (2017) showed that *P. putida, P. fluorescens* and *L. monocytogenes* survive CD routines better than other representative isolates from the food processing environment.

1.3 *Listeria monocytogenes*

The pathogen that is most widely associated with foodborne disease from RTE-salmon products is the Gram-positive bacterial species *Listeria monocytogenes* (Gimenez & Dalgaard, 2004; Jinneman, Wekell & Eklund, 2007; Rørvik, 2000). *L. monocytogenes* is microaerophilic and facultatively anaerobic, can grow from the freezing point to body temperature and has a high tolerance for environmental stresses like low pH, desiccation and high salt concentration (Bucur et al., 2018; Farber & Peterkin, 1991; Junttiila et al., 1988; McLauchlin & Rees, 2015; Rocourt & Buchrieser, 2007; Walker et al., 1990). The disease caused by *L. monocytogenes*, listeriosis, accounts for a small amount of foodborne illness incidents, but a highly disproportionate number of fatalities, especially in high-risk groups (BIOHAZ, 2018; Painter & Slutsker, 2007). A major outbreak of listeriosis in 2018, claiming several lives across a number of EU member states, was linked to consumption of RTE salmon products made from fish farmed in Norway (EFSA, 2018).

L. monocytogenes is, unlike most other pathogenic bacteria, capable of living both saprophytically and pathogenically (Weis & Seeliger, 1975). This explains its tolerance for a broad range of temperatures, atmospheric conditions and other environmental conditions. *L. monocytogenes* is able to regulate gene expression based on environmental cues, exhibiting saprophyte- or pathogen-phenotype based on e.g., temperature or available carbohydrates (Freitag, Port & Miner, 2009). In the environment, the bacterium lives on decaying organic matter and is common in low levels in soil (Vivant, Garmyn & Piveteau, 2013). It is especially linked to agricultural activity, particularly ruminant farms (Weis & Seeliger, 1975; Welshimer & Donker-Voet, 1971). It can infect the ruminants through feed, growing as a

pathogen and dispersing back to the environment (Nightingale et al., 2004). Such farms and their downstream ecosystems, like rivers and coastal areas, are *L. monocytogenes* hotspots (Ivanek, Gröhn & Wiedmann, 2006; Lyautey et al., 2007; Linke et al., 2014; Rodas-Suárez et al., 2006). Areas close to human activity are also linked to an increased occurrence of *L. monocytogenes*, with approximately 1 % of the general population, and a higher proportion of those regularly exposed, being asymptomatic carriers and reservoirs for the bacterium (Ivanek, Gröhn & Wiedmann, 2006; Schoder & Wagner, 2012).

1.3.1 Routes of transmission for *L. monocytogenes*

There are a number of ways *L. monocytogenes* can be transmitted onto salmon products, with some routes being more relevant than others (figure 1). Norwegian aquaculture sites are generally located on the coast and in fjords, adjacent to agricultural activity and ruminant farms (Fiskeridirektoratet, 2021). This may result in the spread of *L. monocytogenes* from farm runoff through water to salmon in nearby sea pens. Waterborne transmission of parasites and fish pathogens between aquaculture sites in fjord systems is a documented phenomenon, but *L. monocytogenes* does not exhibit the same behaviour of growing in and spreading between fish as the relevant organisms (Alaliyat, Osen & Kvile, 2013; Stene et al., 2013). Embarek et al. (1997) could not find *Listeria* in salmon taken directly from Norwegian sea pens, but too few studies of this kind have been conducted to confidently say that this route is irrelevant. It is also a possibility that living fish may be contaminated in other ways, like through feed. The most important source of contamination is however believed to be processing; slaughter, bleeding, filleting and in certain cases smoking and slicing of the fish (Embarek, 1994; Holch et al., 2013; Larsen et al., 2014; Rørvik, Caugant & Yndestad, 1995). Surveys of salmon processing plants have found both sporadic and persistent colonization of *L. monocytogenes* on processing equipment and in the facilities at large (Langsrud et al., 2016; Løvdal et al., 2017; Møretrø et al., 2016; Rørvik, Caugant & Yndestad, 1995). The MLST 8 subtype is of special note, as it has been documented to both establish persistent colonization of food processing environments and has been implied in several outbreaks, including the abovementioned 2018 outbreak (EFSA, 2018; Fagerlund et al., 2016)

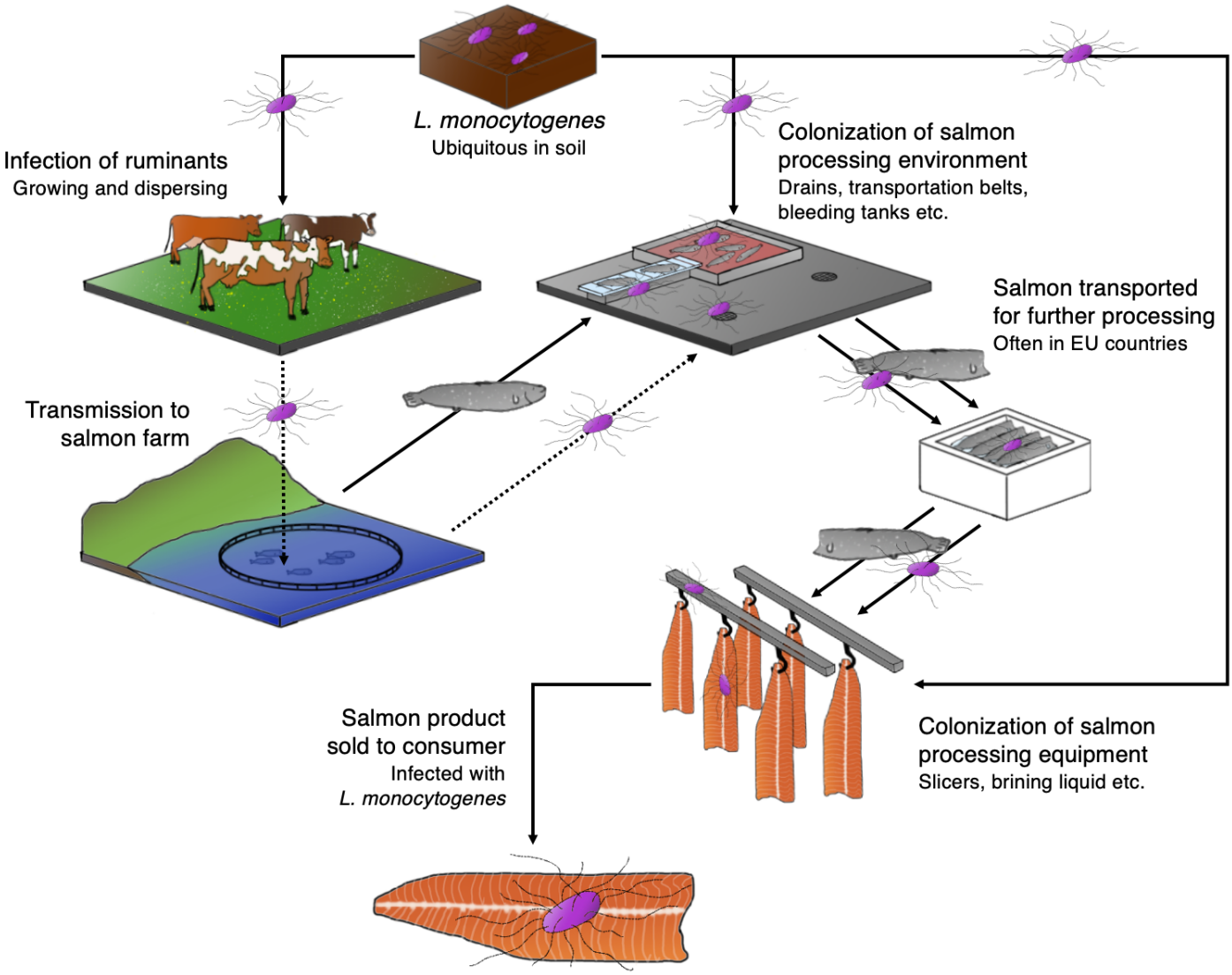

Fig. 1: *Some possible routes of transmission for* L. monocytogenes *to salmon products. "Speculative" connections are marked with dotted lines. Common in soil, the bacteria can infect ruminants, grow, and spread to downstream ecosystems. Salmon farms are often located close to ruminant activity but transmission between these is not documented.* L. monocytogenes *can colonize salmon processing facilities and spread to the product from contact surfaces. Complex supply chains and many actors can be involved in the production and distribution process. (Disclaimer: bacterium not to scale)*

A longitudinal study of several meat processing plants found the same persistent strains over a period of several years, indicating a remarkable ability for certain strains to survive consecutive cycles of cleaning, disinfection and desiccation (Fagerlund, Langsrud & Møretrø, 2020). There is no specific parameter that can explain how or why *L. monocytogenes* colonizes food processing environments like observed, and a multitude of factors are believed to be at play. Salmon products originating from the same producer have been shown to contain significantly similar levels of *L. monocytogenes* compared to products from other producers (Acciari et al., 2017). This points to the importance of proper GHP and CD procedures, as well as an unbroken cold chain, to control *L. monocytogenes*.

1.4 Biofilm in food processing

L. monocytogenes and other microorganisms can survive otherwise uninhabitable conditions like those in salmon processing plants (frequent exposure to disinfectants, washing and rinsing of surfaces, desiccation, etc.) when growing as biofilms (Chmielewski & Frank, 2003). This is the most common way for microorganisms to live in natural environments, as the sessile biofilm lifestyle (compared to planktonic growth) confers a range of advantages. These include adhesion onto surfaces, protection from active threats like predation and harmful chemicals as well as storage of nutrients and water (Flemming & Wingender, 2010).

The biofilm lifecycle is divided into 5 stages (figure 2). The first step is attachment of planktonic cells to a suitable surface, and is dependent on the inherent properties of the surface (such as electrostatic charge, hydrophobicity, micro-topography and pre-conditioning) as well as the microorganism (size, fimbriae, etc.) (Armbrustner & Parsek, 2018; Palmer, Flint & Brooks, 2007). When conditions are favourable, cells will begin to attach irreversibly to the surface (stage 2) by secretion of adhesive polymers (Hinsa et al., 2003). If the concentration of cells reaches a certain threshold, phenotypic changes are initiated through cell-cell communication by a system called «quorum-sensing» (Miller & Bassler, 2001). In the early stages of biofilm production, genes for production of extracellular polymeric substances (EPS) are generally upregulated. EPSs are the building blocks for the biofilm matrix, consisting of proteins, polysaccharides, nucleic acids (eDNA) and lipids, with composition and structure varying considerably between species, strains and environmental conditions (Flemming & Wingender, 2010). As the community matures, a simple array of microcolonies or a thin layer of cells encapsulated in matrix cover the surface (stage 3) (Tolker-Nielsen, 2015). In the next stage of development (stage 4), structural differentiation of the matrix is initiated, being characterized by water channels and spatial organization of cells and/or matrix components (Haagensen et al., 2015; Ude et al., 2006; Watnick & Kolter, 2000). Mature *Pseudomonas* biofilms often form mushroom-shaped structures, which show a large degree of structural heterogeneity (Ghafoor, Hay & Rehm, 2011; Mann & Wozniak, 2012; Purevdorj, Costerton & Stoodley, 2002). The final stage of the biofilm lifecycle is the dispersion of planktonic cells, which colonize new surfaces, repeating the cycle (McDougald et al., 2012). The linear model (figure 2) is somewhat misleading, as the detachment of motile cells can occur at any phase if conditions deteriorate (Watnick & Kolter, 2000).

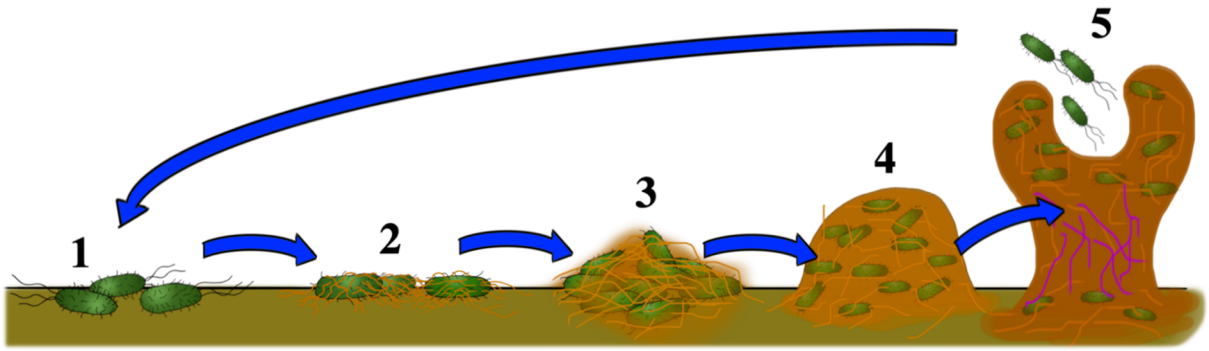

Fig. 2: *Stages in the development of a biofilm. (1) Planktonic cells attach to a suitable surface, (2) EPS is secreted to irreversibly attach cells to surface, (3) microcolonies are formed, (4) more complex biofilm develops and (5) new planktonic cells are dispersed from the biofilm to colonize new surfaces.*

1.4.1 Multispecies biofilm

As biofilms in nature are inhabited by a multitude of species, true replication *in vitro* and constructing models for their behaviour is challenging (Watnick & Kolter, 2000; Yang et al., 2011). This is linked to the emergent properties of such communities, as well as the exceedingly complex web of symbiotic and antagonistic interactions between inhabitants (Burmølle et al., 2006; Flemming et al., 2016; Røder et al., 2015). These interactions are often mediated by quorum sensing, as the signal molecules (autoinducers) in many cases are similar enough that cellular communication between different species can occur (Dickschat, 2010; Parsek & Greenberg, 2005). Gradients of oxygen, nutrients and metabolites in the matrix introduce environmental niches for microorganisms with specific requirements (Stewart & Franklin, 2008). Certain species or strains have been shown to take on the role of primary surface colonizers, quickly adhering to the surface and producing large amounts of matrix that can be inhabited by other species at later times (Salta et al., 2013; Sasahara & Zottola, 1993). These other species may either contribute to the overall success of the community or exploit the matrix structure and nutrients for their own gain: so-called “selfish cheaters” (Sasahara & Zottola, 1993; West et al., 2006).

1.4.2 Pseudomonas and L. monocytogenes in biofilm

Listeria monocytogenes and *Pseudomonas spp.* are commonly isolated from the same biofilms in salmon processing plants and survive CD procedures better than much of the competing microflora (Fagerlund et al., 2017; Langsrud et al., 2016). The disparities in growth rate and EPS-production as well as environmental requirements suggest that these species may take on distinct roles in the colonization process (Sasahara & Zottola, 1993).

Pseudomonas spp. are known as formidable biofilm producers and construct complex, highly differentiated structures. The utilization of matrix-building subpopulations seems to be a part of the biofilm production strategy from the onset (Mann & Wozniak, 2012). Certain subsets of the population will overproduce one or a few matrix components, providing a valuable service for the whole community (Mann & Wozniak, 2012). Early stages of development depend predominantly upon one or two polymers, and for *Pseudomonas* biofilms these are largely polysaccharides (nucleic acids also play an important structural role in new matrices) (Mann & Wozniak, 2012; Whitchurch et al., 2002). The polysaccharides can be further divided into capsular, which coat cells (alginate/levan/Psl), and aggregative, providing structural integrity and interaction with matrix components (cellulose/Pel) (Mann & Wozniak, 2012). Mature structures can exhibit a "mushroom" morphology, where the "stalks", the interior of the "caps" and especially cavities inside the "caps", containing cells that will be dispersed, contain large concentrations of eDNA (Allesen-Holm et al., 2006; Ma et al., 2009). The increase of nucleic acids in the stalks and caps corresponds with decreasing concentration of eDNA in the rest of the structure (Allesen-Holm et al., 2006). The mechanism for DNA-release is based on controlled cell death and lysis and is mediated by quorum-sensing (Ma et al., 2009; Webb et al., 2003; Yang et al., 2009). Cells have also been observed to migrate from the underlying layer into the "caps" of the "mushroom" structure as the biofilm matures, resulting in a cell concentration gradient (Klausen et al., 2003).

Listeria monocytogenes shows a weak ability to produce biofilm compared to most *Pseudomonas spp.,* with lower overall production of matrix components and a thinner, simpler structure, in some cases described as surface adhesion rather than biofilm production (Bridier et al., 2010; Chae & Schraft, 2000; Kalmokoff et al., 2008; Rieu et al., 2008). There are significant differences in biofilm forming ability when comparing strains of *L. monocytogenes*, but whereas food industry persistent strains commonly are good biofilm formers, a relationship cannot be clearly established between good biofilm formers and strains linked to listeriosis outbreaks (Borucki et al., 2003; Barbosa et al., 2013; Fagerlund, Langsrud & Møretrø, 2021). This indicates that *L. monocytogenes* may utilize an alternative strategy for surviving in food processing environments.

L. monocytogenes exists in, and interacts with, a complex mixture of background microflora in the food processing environment, which to a large part decide its survival both long- and

short-term (Fagerlund, Langsrud & Møretrø, 2021; Fox et al., 2014). When it comes to growth, *L. monocytogenes* appears to be throttled in co-culture with background microbiota, including *Pseudomonas spp*. Several studies have observed that *L. monocytogenes* constitute less than 10% of cells in such biofilms and experience significant reduction in total growth compared to monoculture (Heir et al., 2018; Pang & Yuk, 2019; Puga, Orgaz & SanJose, 2016). There are also many advantages to cohabitation. Bremer, Monk & Osborne (2001) found increased attachment to stainless steel and better desiccation survival when *L. monocytogenes* was co-cultured with *Flavobacterium spp*. compared to monoculture. This corresponds with the results of Hassan, Birt & Frank (2004), where *L. monocytogenes* attached significantly better to stainless steel covered with *Pseudomonas putida* biofilm than a clean surface. Several studies also indicate that tolerance to disinfectants, desiccation and nutrient starvation is increased in co-cultures of *L. monocytogenes* and *Pseudomonas spp.* compared to monoculture biofilms (Ammor et al., 2004; Giaouris et al., 2013; Ibusquiza et al., 2012; Pang & Yuk, 2019).

When biofilms containing *L. monocytogenes* and *Pseudomonas* are further studied, some of the mechanisms that may be responsible for this effect are unveiled. *L. monocytogenes* has been shown to induce EPS over-production when introduced to *Pseudomonas* biofilms, with the new matrix being denser but containing a similar total number of cells per area unit (Puga et al., 2018; Puga, Orgaz & SanJose, 2016). Such communities also establish two distinct layers, where the bottom is inhabited by *L. monocytogenes* while *Pseudomonas* dominates the surface, somewhat similar to what is observed in monoculture (Fagerlund et al., 2017; Puga, SanJose & Orgaz, 2014). A comparable result was found in biofilm made up of quickly growing *E. coli* and slowly growing *L. monocytogenes* and *Salmonella enterica*, where *E. coli* established a layer above the others after a short period of incubation (Almeida et al., 2011). Interestingly, *L. monocytogenes* has been shown to migrate to the deeper levels of the matrix when introduced to pre-existing *Pseudomonas* biofilms, suggesting some form of tropism (Puga, Orgaz & SanJose, 2016). *L. monocytogenes* synthesize flagella when grown at lower temperatures, which may play a role in such a relocation (Lemon, Higgins & Kolter, 2007; Peel, Donachie & Shaw, 1988). There are also examples of *Pseudomonas spp.* utilizing eDNA structures in the matrix for cell migration, but whether this is relevant for *L. monocytogenes* is not known (Barken et al., 2008). The tendency for tropism may be further amplified by the atmospheric preferences of these species, as microaerophilic *L. monocytogenes* may grow better in the minimally oxygenated environment deep inside the

matrix. Finally, this may partially explain the significantly lower transfer-rate of *L. monocytogenes* onto salmon fillets when it is co-cultured with *P. fluorescens* compared to monoculture, demonstrated by Pang & Yuk (2019). *L. monocytogenes* is then less likely to come in contact with the salmon but is more protected from chemical disinfectants and desiccation.

1.5 Disinfectants in food processing

Some of the most widely utilized disinfectants in the food industry have peracetic acid (PAA) or quaternary ammonium compounds (QAC), such as benzalkonium chloride (BAC), as main active ingredients. PAA is a strong oxidizing agent and disrupts a wide range of cellular functions, most importantly the cellular membrane and a number of intracellular proteins (Kitis, 2004; Fue, McCue & Boesenberg, 2007, p. 587-588). QACs primarily disrupt the cell membrane through the action of N-alkyl groups with specific chain lengths which bind to membrane phospholipids (Gerba, 2015; Gilbert & Moore, 2005). Other chemicals may be added to increase the efficacy of the disinfectant, such as surfactants, which reduce surface tension and facilitate even spread on surfaces and into crevices. Additives are especially important for PAA-based disinfectants, as hydrogen peroxide and acetic acid both stabilize the otherwise rapidly deteriorating PAA and increase overall efficacy (Briñez et al., 2006; Kunigk et al., 2001).

1.5.1 Tolerance and adaptation to disinfectants

A range of concerns have been raised regarding microbial adaptation to QACs. Heterogeneities in the processing environment facilitate a vast number of "natural experiments", where enclaves composed of diverse mixtures of microorganisms are regularly exposed to different concentrations of the disinfectant. The result of this is a selection pressure favouring communities that are able to adapt to the stress imposed by QACs; a widely reported phenomenon (Aase, Sundheim, Langsrud & Rørvik, 2000; Fagerlund et al., 2017; Langsrud, Sundheim & Borgmann-Strahsen, 2003; Møretrø et al., 2016). This also happens in nature, and there are many rapports of microbiological adaptation in downstream ecosystems from large-scale QAC use (Buffet-Bataillon et al., 2012; Gerba, 2015; Jennings, Minbiole & Wuest, 2015; Zhang et al., 2015). The mechanisms that facilitate resistance to QACs include efflux pumps, modification of membrane fatty acids, slime production, and adhesion/biofilm formation (Guerin-Mechin et al., 2000; Jaglic & Cervinkova, 2012; Jiang et

al., 2016; Langsrud, Sundheim & Borgmann-Strahsen, 2003; Pirone-Davies et al., 2018; Wassenaar et al., 2015). Many of the genes that code for these mechanisms are located in plasmids and can therefore readily be transferred by horizontal gene transfer, which may explain the number of shared resistance genes in *L. monocytogenes* and other microflora isolated from food and food processing environments (Jiang et al., 2016; Pirone-Davies et al., 2018). The close link between these genes and resistance to certain antibiotics is also a troubling aspect that further incentivises increased control of the microbial populations in question, as well as limiting the use of QACs (Buffet-Bataillon et al., 2012; Han et al., 2019; Hegstad et al., 2010; Mulder et al., 2018; Walsh & Fanning, 2008).

The low specificity of PAA means that adaptation and resistance is a much greater challenge for microbes. There are no reports of specific PAA resistance genes in any microorganism to date and cross-tolerance to other oxidizing agents is documented, but exceedingly rare (Kampf, 2018). *E. coli* has been shown to adapt when exposed to sublethal concentrations and major changes in the *Pseudomonas aeruginosa* transcriptome, mostly related to membrane proteins and metabolism, have been observed after sublethal exposure (Chang et al., 2005; Zook, Busta & Brady, 2001). However, *L. monocytogenes* did not show any ability to adapt after long-term exposure to sublethal concentrations of PAA in a study by Kastbjerg & Gram (2012). The adaptation of microorganisms in downstream environments is not regarded as a problem with PAA, as the active ingredient rapidly deteriorates into acetic acid and oxygen when diluted (Kunigk et al., 2001; Monarca et al., 2002).

1.5.2 Processing environment-enabled disinfection survival

Hurdles faced in a real-life food processing environment represent major challenges for obtaining satisfactory disinfections. A prerequisite for chemical disinfection is the removal of soil and food residue from the production environment by rinsing with water, application of detergent and scrubbing. The presence of organic matter can reduce the effectiveness of the disinfectant by diluting and interfering with it, as well as altogether shielding the microbes from the agent (Aarnisalo et al., 2000; Briñez et al., 2006; Fatemi & Frank, 1999; Rossoni & Gaylarde, 2000). The niches which are most in danger of accumulating soil and food residue (like drains, the undersides of conveyor belts and other hard-to-reach spots on equipment) are also hotspots for biofilm formation (Cappitelli, Polo & Villa, 2014; Møretrø & Langsrud, 2017). The matrix acts as a diffusion barrier (relevant for QACs) and EPS interfere with the disinfecting agent (relevant for PAA). This effect is especially prominent with mature

biofilms, which may be related to internal structural changes of the matrix or phenotypic changes in its inhabitants between approximately 48h and 96h of incubation, depending on environment (Akinbobola et al., 2017; Ibusquiza, Herrera & Cabo, 2011; Lee et al., 2016).

Although most studies look at the short-term effect of disinfection, the long-term and real-life application is important to take into account. One of the often-overlooked aspects is the fate of the matrix after disinfection. When measuring the effect of a disinfectant, it is often just the efficacy for reducing CFU-count that is measured, while the matrix itself is subject to relatively little scrutiny (Loukili et al., 2006). In a scenario where all biofilm inhabitants have been exterminated, the matrix structure can be recolonized by planktonic cells from the environment. A study by Akinbobola et al. (2021) observed a dramatic increase in tolerance to a PAA-based disinfectant for *P. aeruginosa* when it was introduced to mature, disinfected biofilm matrix. The matrix may also be repopulated by its old inhabitants after disinfection. This can be badly injured cells which are provided with the nutrients and protection against desiccation and other stresses needed to regain reproductive abilities (Wesche et al., 2009). "Persister cells", which make up effectively dormant subpopulations with significantly better tolerance to stress than growing cells, may also play an important role in repopulation (Lewis, 2005; Roberts & Stewart, 2005). Some survival in established biofilms in food processing environments is assumed, as the goal in to supress and limit microbial action, not sterilize. The required disinfectant efficacy in the EU is a 5,0 log reduction of viable cells on a surface after 5 minutes of exposure to the disinfectant (Fue, McCue & Boesenberg, 2007, p. 582)

1.6 Project objective

This bachelor's thesis has the objective of investigating the growth dynamics in biofilm and suspension and the biofilm disinfection survival characteristics of a *L. monocytogenes* isolate and a mix of five *Pseudomonas spp.* isolates sampled at a Norwegian salmon processing facility. This objective will be reached through the following steps:

1. Study growth of separate and mixed bacterial cultures in suspension and biofilm on stainless steel coupons.
2. Investigate survival characteristics of separate and mixed biofilms grown on coupons when exposed to a peracetic acid disinfectant.

2 Materials and methods

2.1 Bacterial isolates used in experiments

All bacterial isolates used in this project were sampled from equipment and product contact surfaces at a Norwegian salmon processing facility. *Pseudomonas* isolates were sampled during the first year of production at the facility (between 25/05/18 and 22/05/19), as part of an ongoing PhD project at the institute (G.M.B Thomassen, personal communication, 2021). Samples were collected early in the morning before the start of production, on the clean surfaces of processing or transportation equipment that would come in contact with salmon during production. A set of these samples were screened for growth on Pseudomonas CFC Selective Agar (PA) (CM0559, Oxoid, with Supplement SR0103E, Oxoid) at 25 ºC. Isolates that grew on PA were genetically sequenced (partial *rpoD*-sequencing) to determine species and strain (table 1), and biofilm growth characteristics on 96-well polystyrene peg lids were tested (data not shown) (Boyko, 2020). Minimum Inhibitory Concentration (MIC), Minimum Biocidal Concentration (MBC) and Minimum Biofilm Eradication Concentration (MBEC) for the Peracetic acid-based disinfectant used at the facility "Aqua Des" (PAA) (H661, Aquatic Chemistry AS, Norway) was tested for these isolates (table 1) (T. Reiche, personal communication, 2021). One *Listeria monocytogenes* isolate was used in the experiments. This was isolated from a scanner in a filleting machine (non-contact surface) by staff at the facility in a *Listeria* monitoring program, on the 19/12/2019. The isolate was screened for growth on Brilliance™ Listeria agar (BLA) (CM1080, Oxoid, with Supplement SR0227E, Oxoid) and whole genome sequencing revealed that it is of sequence type (MLST) 8, part of lineage II and clonal complex 8 (G.M.B Thomassen, personal communication, 2021). Isolates were stored in approximately 20 % glycerol at -80 ºC and cultured at least twice on tryptic soy agar (TSA) (84602.0500, VWR Chemicals) before use.

Five *Pseudomonas*-isolates (LJP040, LJP042, LJP321, LJP760 and LJP788) were selected to use in this project. Selection criteria were differences in the *rpoD*-gene, being good or adequate biofilm producers and, in the case of LJP042, a remarkably high MIC value (table 1). The *L. monocytogenes* isolate was used as strains of this sequence type are known to persist in food processing environments and have been implied in foodborne outbreaks of listeriosis from salmon products (EFSA, 2018; Fagerlund et al., 2016).

Table 1: *Original sampling point and date, species determined by BLAST search using partial* rpoD *sequencing, as well as Minimum Inhibitory Concentration (MIC), Minimum Biocidal Concentration (MBC) and Minimum Biofilm Eradication Concentration (MBEC) for the* Pseudomonas *isolates used in the project in relation to the same PAA-based disinfectant as is used in the sampling facility. Columns marked "GT" represent work done by G.M.B. Thomassen, (personal communication, 2021), "VB" is retrieved from Boyko (2020) and "TR" represents work done by T. Reiche (personal communication, 2021).*

Isolate ID	Sampling point (GT)	Sampling date (GT)	BLAST hit (VB)	ID % (VB)	MIC (TR)	MBC (TR)	MBEC (TR)
LJP040	Steel tray on orientation rig	25/05/18	*Pseudomonas fluorescens*, strain: RF 59	99,03	0,25 %	0,5 %	2 %
LJP042	Steel tray on orientation rig	25/05/18	*Pseudomonas sp.* CCUG 62357 or *Pseudomonas marginalis*	99,85 or 99,56, resp.	2 %	0,5 %	2 %
LJP321	Water from wellboat	31/10/18	*Pseudomonas fluorescens,* strain: ATCC 17573	99,71	0,25 %	0, 25%	0,5 %
LJP760	Tail cutter at the end of heading machine	22/05/19	*Pseudomonas putida,* strain: CFBP 5933	99,26	0,25 %	1 %	2 %
LJP788	Conveyor belt leading to skinning machine	22/05/19	*Pseudomonas lundensis*, strain not determined	99,60	0,25 %	1 %	2 %

2.2 Growth and survival experiment

The method for biofilm growth and disinfection survival was based on the biofilm experiment described in Heir et al. (2018) with modifications (figure 3). The main experiment was performed in four replicates (A1, A2, B1, B2), with a modification of the method in the two final replicates (results from the two first (A1, A2) were discarded due to contamination). Materials were prepared according to manufacturer's specifications unless otherwise stated.

The day before each experiment, isolates were grown 24 h on TSA at 25 ºC for *Pseudomonas spp.* and 37 ºC for *L. monocytogenes*. Colonies were transferred to a sterile glass tube containing 10 mL 0,9 % NaCl using a sterile cotton swab and standardized to the McFarland Equivalence Turbidity Standard 3.0 (McFarland, 1907). As the McFarland standard is intended for gram-negative, rod shaped bacteria, it is more suitable for *Pseudomonas spp.* than *L. monocytogenes*, but all isolates were nevertheless standardized using this method. An

equal volume of each standardized *Pseudomonas sp.* isolate was transferred to a separate, sterile container and mixed.

Three sets of coupons were inoculated (table 2): four coupons with *L. monocytogenes* (Lm), five with *Pseudomonas spp.* (Ps) and four with a combination of both (PsLm), as well as a negative control. Tryptic soy broth (84675.0500, Oxoid) was made to half the specified concentration and added 2,5 g/L NaCl (27808.297, VWR Chemicals) to compensate for osmotic pressure (½TSB). The four petri dishes used for inoculation were each added 19,6 mL ½TSB. Petri dishes used to inoculate Ps and PsLm were added 400 µL standardized suspension of *Pseudomonas spp.* to obtain approximately 10^7 CFU/mL in the inoculum. The dishes used to inoculate Lm and PsLm were added 40 µL standardized *L. monocytogenes* suspension. No bacterial suspension was added to negative control. Sterile stainless-steel coupons (25mm x 5mm x 1mm, AISI 316) were aseptically placed in the inoculum solutions (including negative) and bacteria were given 3 h in room temperature (approx. 23 ºC) to attach to the coupons. Inoculation in the first replicate was carried out using 6-well polystyrene plates (Costar® Ref 3516, Corning Incorporated) with equal cell concentration but using ¼ the original volume per well. Each inoculum solution was enumerated immediately after bacteria were added.

Table 2: *Codes for different cultures used to inoculate coupons in experiments. Number of coupons inoculated of each set and the subsequent treatment in the main experiment is also presented.*

Code	**Lm**	**Ps**	**PsLm**
Isolates used in inoculation	*L. monocytogenes*	*Pseudomonas spp. (n=5)*	*L. monocytogenes* and *Pseudomonas spp. (n=5)*
Number of coupons for each replication	4 (2 Disinfected, 2 not disinfected)	5 (3 Disinfected, of these 1 reinoculated w/Lm (Lm+Ps(m)), 2 not disinfected)	4 (2 Disinfected, 2 not disinfected)

All enumeration was done using a modified microspot technique (Harrigan, 1992, p. 58-59). 10-fold dilutions were made in a 96-well plate, pipetting 20 µL bacterial suspension into 180 µL 0,9 % NaCl and mixing vigorously with the pipette. A multichannel pipette was used to carefully deposit 8 spots containing 10 µL diluted suspension in three rows on each lightly

dried agar plate. Plates were incubated at 37 ºC for 24 h when enumerating *L. monocytogenes* and 12 ºC for 48 h when enumerating *Pseudomonas spp.*

Inoculated coupons were transferred with sterile forceps to 6-well plates in the two first replicates and modified 5 mL Eppendorf Tubes® (Cat. no.: 0030119401, Eppendorf AG) in the two final replicates. Eppendorf Tubes® were modified to be more suitable for handling and aerobic incubation by removing an appendage on the lid, intended to "snap" it into closed position, and poking a "breathing hole" in the top of the lid with a hollow needle. Growth media (½TSB) was added aseptically to incubation vessels: 5 mL to each well in the 6-well plates and 3 mL to Eppendorf Tubes®. Samples were incubated with shaking (70 rpm) at 12 ºC for 72 h.

On day 3, after 72 h of incubation, enumeration of planktonic growth was performed for all samples including negative control, and two coupons from each set (three for Ps) were disinfected. Coupons were rinsed carefully in 0,9 % NaCl, before being submerged in a 1 % solution of the disinfectant, all using sterile forceps. The disinfectant used in this project was the same PAA based disinfectant used at the facility where samples were isolated (and used to test MIC/MBC/MBEC). As specified by the manufacturer, concentrate was diluted with sterile tap-water and used immediately after dilution. The diluted suspension contained 0,05 % peracetic acid, 0,2 % hydrogen peroxide and 0,1 % acetic acid. After having been submerged for 10 min, the disinfection procedure was ended by rinsing coupons in 0,9 % NaCl.

To test bacterial growth and survival in biofilm, one untreated and one disinfected coupon from each set were sonicated, and the resulting solutions were enumerated. Untreated coupons were rinsed carefully in 0,9 % NaCl and transferred to a solution of ½TSB with 1 % Tween® 20 (0777-1L, Lot: 19G2956482, VWR Chemicals), while disinfected coupons were transferred directly from the rinsing solution, all done with sterile forceps. Samples were sonicated for 15 min at 40 kHz and 24 ºC in a sonication bath (Branson 5800 Ultrasonic Cleaner). In the first two replicates, 6-plate wells with 5 mL Tween®-solution were used, while unmodified Eppendorf Tubes® with 3 mL solution were used in the two final replicates. Tween® was added as a surfactant to aid removal of the biofilm matrix during sonication (Chen & Stewart, 2000).

Remaining coupons were prepared for another incubation sequence. Disinfected coupons were transferred using sterile forceps to new modified Eppendorf Tubes® (or an unused well in a 6-well plate) and provided with fresh growth medium. Bacterial suspension for untreated coupons was discarded, and new growth medium was added. One of the disinfected Ps-coupons was reinoculated with Lm-inoculum (Lm+Ps(m)), performed as described for the initial inoculation (not shown in figure 3). This coupon was transferred to an incubation vessel with ½TSB after 3 h inoculation. Samples were incubated for another 72 h at 12 ºC with shaking at 70 rpm.

On day 6, 144 h after inoculation, samples were taken from all suspensions, including negative control, and enumerated. All remaining coupons, including negative control, were sonicated, and the resulting solutions enumerated. All disinfected coupons were stored for an additional period (4 days, total of 7 days after disinfection) after enumeration to test for survival under the initial detection limit (<10 CFU/mL) (not shown in figure 3). Survival after day 6 was recorded as a positive or negative result, logging the number of days after treatment when applicable.

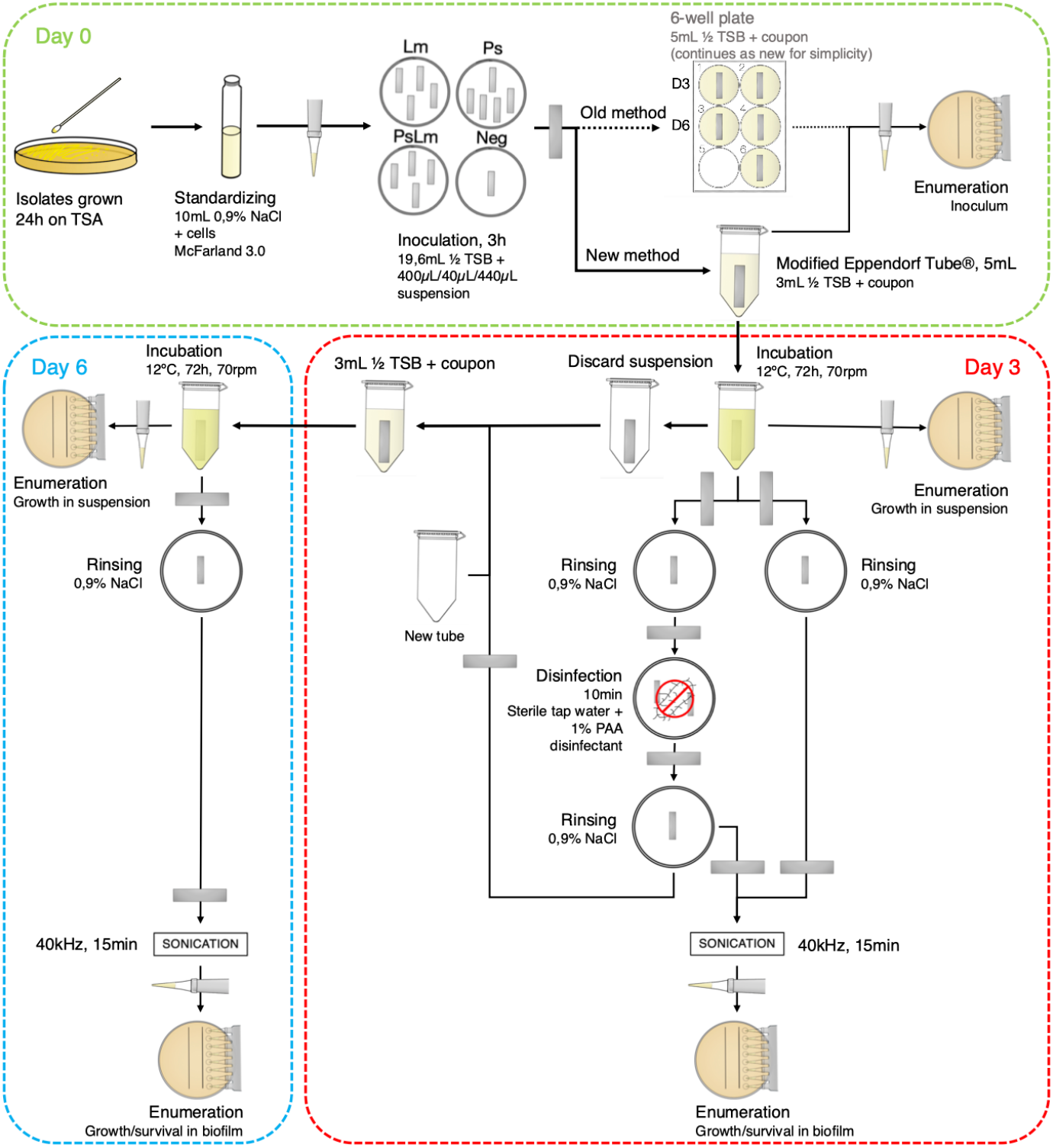

Fig. 3. *Flowchart of biofilm disinfection experiment. Isolates of* L. monocytogenes *and* Pseudomonas spp. *grown on TSA for 24 h and standardized (McFarland 3.0) were used to inoculate stainless steel coupons. Inoculum solutions: Lm*=L. monocytogenes, *Ps*=Pseudomonas spp. *mixture, PsLm*=L. monocytogenes *and* Pseudomonas spp. *coculture. Coupons were transferred to Eppendorf Tubes® (or 6-well plates) containing 3mL (5mL for 6-well plates) ½TSB and incubated 72 h at 12 ºC with shaking (70 rpm). After incubation, half of each set were disinfected (1 % PAA disinfectant, 10 min). One disinfected, one not disinfected coupon of each set was incubated again (same conditions). Enumeration with modified microspot technique was performed for inoculum, suspension and biofilm on coupons after the first and second incubation step, for disinfected and non-disinfected coupons.*

2.3 Growth curve

Individual- and a mix of all *Pseudomonas* isolates were measured spectrophotometrically to map growth patterns. Isolates were prepared and standardized as in the biofilm disinfection experiment. Two millilitres of standardized suspensions (McFarland 3.0) of each isolate and a mix with all *Pseudomonas spp.* were combined with 98 mL ½TSB in 250 mL Erlenmeyer flasks, to a concentration of approximately 10^7 CFU/mL. Flasks were incubated at 12 ºC with shaking at 70 rpm. Every two hours, a 1 mL sample from each flask was transferred to a semi-micro cuvette and absorbance at 600 nm was measured using a spectrophotometer. When absorbency exceeded 0.6, a 10-fold dilution was made using 0,9 mL ½TSB and 100 µL suspension to obtain a value that would better represent the concentration of cells in the solution. All cuvettes were covered with a piece of Parafilm® M and mixed to obtain a more homogenous solution just before measuring. A negative control with only ½TSB was incubated with the other flasks and tested at every point. Flasks were inoculated at 12 h intervals, with one series being measured at 0-12 h, the other at 12-24 h, etc.

2.4 Survival test

Bacterial solutions were standardized, coupons were inoculated and incubated as in the first part of the 6-well plate method of the biofilm disinfection experiment, but using 6 coupons of each variety (Lm, Ps, PsLm). All coupons were disinfected according to the method described above and given new growth media (½TSB) in new 6-well plates. Samples were incubated at 12 ºC with shaking at 70 rpm. Each well was closely examined for any increase in turbidity at least once a day for 7 days after treatment. 72 h after disinfection, all suspensions were tested using traditional plate spreading technique with 100 µL of the suspension on agar plates (TSA, BLA and PA) to assess survival or contamination. Selective agar media (PA and BLA) were used to differentiate populations in co-culture. Samples which were negative on first assessment were tested again 144 h after disinfection. Some samples of Ps and PsLm were enumerated on PA using traditional plate spreading technique, 96 h after treatment.

2.5 Statistical analysis

Statistical differences between populations were calculated using unpaired Tukey's HSD function in Minitab® (Version 19.2020.2.0, 2020, www.minitab.com). Population sizes and standard errors calculated from two replicate experiments, creation of log-transformed growth curve from mean of two (0-12 h) or one (14-36 h) replicate and all other manipulation of raw data was performed using Microsoft Excel® (Version 16.48, 2021).

3. Results

3.1 Growth curve

Relative growth for individual *Pseudomonas* isolates and a *Pseudomonas* mixture was measured spectrophotometrically, illustrated as a log-transformed growth curve (figure 4). All *Pseudomonas spp.* grew quickly from the onset, entering exponential growth after a very short lag phase. After approximately 8-10 hours, the individual isolates formed two distinct groups. A quickly growing group, consisting of LJP760 and LJP788, measured above all other samples for the duration of the test. A slowly growing group, with LJP040, LJP042 and LJP321, appeared to exhibit a shorter exponential phase, but would approach the quickly growing group towards the end of the test. It must be noted that LJP321 would show visible cellular aggregation after approximately 12 h of growth, making accurate measurement challenging. The mixed culture and the calculated average value of individual strains were between the two groups from the end of the exponential phase to the end of the experiment, with the mixture approaching the quickly growing group and straying away from the average after approximately 24 h.

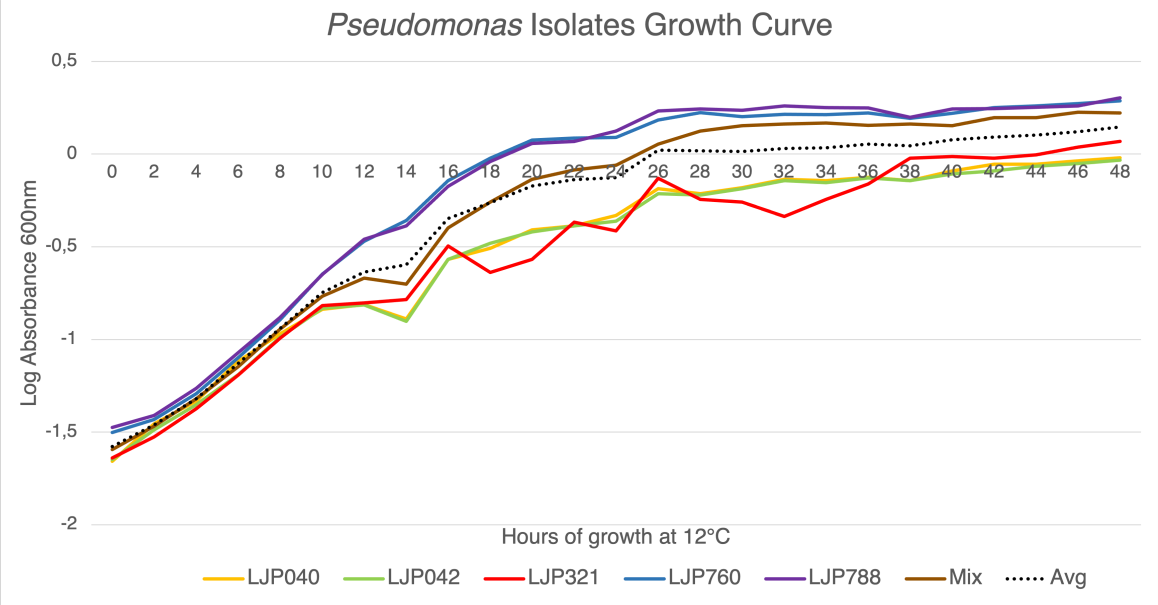

Fig. 4. *Growth curve of individual and mix of* Pseudomonas *isolates, also showing the calculated average of all individual cultures at each point. Values measured spectrophotometrically at 600 nm every two hours. Cultures were grown in 12 ºC with shaking (70 rpm) in 250 mL Erlenmeyer flasks using ½TSB as growth media. Inoculation with standardized cultures to approx. 10^7 CFU/mL and a total volume of 100 mL at start. All isolates exhibit a very short lag-phase, growing exponentially until approximately 18-24 h.*

3.2 Growth and survival of bacterial cultures

The main focus of the project was determining the effect of a disinfection routine on biofilm of *L. monocytogenes* and *Pseudomonas spp.* grown alone and in combination. Both planktonic and biofilm growth were measured at two timepoints, 72 and 144 h after inoculation, as well as the inoculum suspension itself. Enumeration of the inoculum is not included in the following figures or statistical analysis, as adherent cells, and not the suspension, make up the basis for further growth. Concentration of *Pseudomonas spp.* in the inoculum solutions was between approximately 7,1 and 7,2 log CFU/mL for both Ps and PsLm, while concentration of *L. monocytogenes* was between approximately 6,5 and 6,6 log CFU/mL for both Lm and PsLm, with no substantial deviations from the respective means (>0,2 log) in any repetition. *Pseudomonas spp.* were not detected in Lm+Ps(m) cultures, so these are regarded as *L. monocytogenes* monocultures with 72 h-*Pseudomonas* matrices.

3.2.1 Growth in non-disinfected suspension and biofilm

Results from the two final replicates (using Eppendorf Tubes®) of the experiment are used due to a persistent problem of contamination in the original method. As two replicates are included in this result, with one coupon or suspension being measured for each parameter, the mean value for these results are used with no standard error. The exception to this is suspensions measured on day 3, where all four parallels were enumerated on each replicate, providing data to calculate standard error and significant differences. Other values are therefore compared to these where applicable (figure 5).

After 72 h of growth, the total population in suspensions were similar for all cultures, with some deviations in biofilm. Suspensions of Lm (8,9 log), Ps (8,9 log) and PsLm (9,0 log) were not significantly different (P>0,05), with Lm+Ps(m) in between these at 8,9 log. After 144 h, mean value for Lm receded somewhat compared to 72 h, from 8,9 to 8,8 log, but this was within one unit of standard error. Mean values for Ps and PsLm increased in the same period, from 8,9 and 9,0 log to 9,3 and 9,3 log, respectively. The increase was greater than one unit of standard error for Ps and greater than two units for PsLm. In 72 h biofilms, Lm and Lm+Ps(m) both contained 5,8 log CFU per coupon, while Ps and PsLm contained respectively 6,7 and 6,8 log CFU per coupon; approximately equivalent to a 1:10 ratio. The difference was however much smaller after 144 h, with the mean value for Lm biofilm at 6,5 log, while Ps and PsLm remained at 6,7 and 6,8 log CFU/coupon.

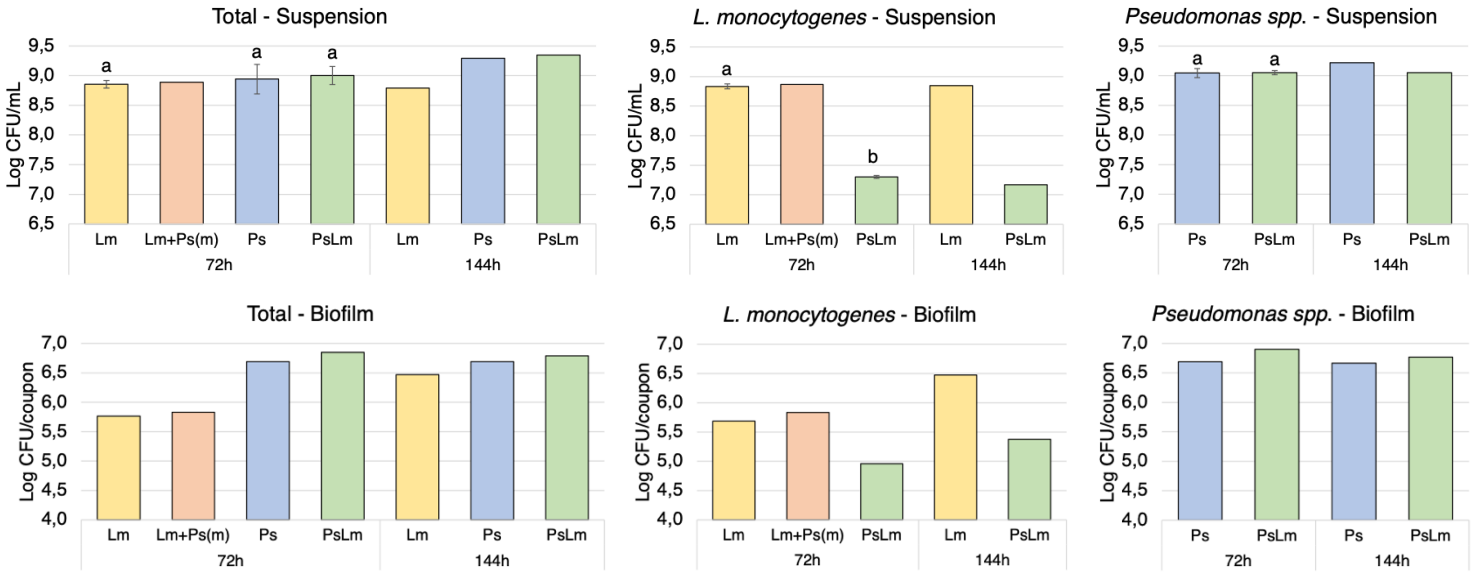

Fig. 5. *Log-transformed growth in suspension and biofilm on stainless steel coupons of different bacterial cultures, measured 72 h and 144 h after inoculation. Standard error is shown when possible, columns with different letters are significantly different (P<0,05). Lm* = L. monocytogenes*, Lm+Ps(m) = disinfected 72 h* Pseudomonas spp. *biofilm reinoculated with* L. monocytogenes*, Ps =* Pseudomonas spp. *and PsLm* = L. monocytogenes *and* Pseudomonas spp. *Upper graphs show population in suspension surrounding coupons, measured in CFU/mL, while the lower graphs show growth in biofilm on coupons, measured in CFU/coupon. Total population (graphs to the left) is determined by growth on non-selective agar media (TSA), while number of* L. monocytogenes *(graphs in the middle) and* Pseudomonas spp. *(graphs to the right) are determined by growth on selective agar media: BLA and PA respectively. No survival was detected for disinfected* Pseudomonas spp. *in Lm+Ps(m) cultures, so these are regarded as being* L. monocytogenes *monocultures with* Pseudomonas *matrices.*

L. monocytogenes grew substantially less when co-cultured with *Pseudomonas spp.* (PsLm) compared to monoculture (Lm), in suspension. *L. monocytogenes*, with 7,3 log CFU/mL, made up approximately 2% of the total population in PsLm suspension after 72 h, and approximately 3 % of equivalent Lm and Lm+Ps(m) cultures. After 144 h, the mean value for the *L. monocytogenes* subpopulation in PsLm suspension was reduced compared to 72 h, from 7,3 to 7,2 log, making up less than 1 % of the total population.

L. monocytogenes, with 5,0 log CFU/coupon made up between 1 and 2 % of PsLm biofilm after 72 h, but this ratio increased to approximately 4 % after 144 h, when a mean value of 5,4 log CFU per coupon was measured. Compared to equivalent monoculture, *L. monocytogenes* in coculture biofilm grew to a ratio of 1:5 after 72 h and 1:10 after 144 h, roughly approximated.

Little difference could be measured for *Pseudomonas spp.* in both suspension and biofilm between either sampling time or mono-/co-culture. Minor differences in total population of suspension were registered, as mentioned above, but biofilm was very stable.

3.2.2 Survival after disinfection

When the sonicated solution was enumerated immediately after disinfection, no colonies were observed from any coupon in any repetition, indicating survival of less than 30 culturable cells per coupon (the detection limit for this experiment). After coupons were incubated 72 hours after disinfection (144 h in total), only one PsLm parallel in total exhibited regrowth. A total population in suspension of 8,7 log CFU/mL with the vast majority (8,7 log) *Pseudomonas spp.* and 6,6 log *L. monocytogenes,* as well as 6,3 log CFU/coupon (vast majority *Pseudomonas spp.* at 6,3 log) and 5,4 log *L. monocytogenes* in biofilm was measured. When disinfected coupons were incubated for another 24 h, there was observed regrowth in one Ps parallel and an additional PsLm parallel (only *Pseudomonas spp.*) in the third replication, while one PsLm parallel was positive for both *Pseudomonas spp.* and *L. monocytogenes* on the final replication. In total, one of four Ps parallels survived disinfection, while three of four PsLm parallels survived, of which all contained *Pseudomonas spp.* but only two contained *L. monocytogenes*. Parallels testing negatively for survival 72 h after disinfection did not test positively at the end of the testing period, 168 h after disinfection. No *L. monocytogenes* monoculture parallel survived in any replication.

3.3 Survival test

As the disinfection survival results from the main experiment were not conclusive and tested very few parallels (n=2) of each set per replication, another experiment was carried out to further investigate the relationship between bacterial culture and survival (table 3). An increase in turbidity was not observed for any parallel on the first day after disinfection. On the second day, 4 of 18 Ps- and 7 of 18 PsLm parallels exhibited visible growth, while 13 of 18 Ps- and 9 of 18 PsLm parallels were positive on the third day. From day four until the end of the period samples were observed, no further change in turbidity was seen, with the final result being 15 of 18 Ps parallels and 13 of 18 PsLm parallels. No change in turbidity was observed in Lm cultures.

On day 3, 72 hours after disinfection, all parallels were tested for growth by plating on agar media; non-selective TSA for Lm and Ps, and selective PA and BLA for PsLm. A majority (10 of 18) of the PsLm parallels tested positive for growth on BLA, indicating survival of *L. monocytogenes*. Although only one concentration of the suspension was tested for each parallel, differences between positive samples were observed. This ranged from 5 colonies to densely overgrown petri dishes. Parallels which did not test positively in the first round were tested again on day 6 (144 h after disinfection). No additional positive survival results were observed from the second round of plating. Visible growth corresponded with results from plating for *Pseudomonas spp.* No parallel from the Lm-culture tested positively in any test.

Table 3. *Test for survival in disinfected coupons. Coupons inoculated with* L. monocytogenes *(Lm),* Pseudomonas spp. *(Ps) or a combination (PsLm) were incubated with 5mL ½TSB in 6-well plates at 12 ºC and 70 rpm shaking for 72h before disinfection. Green/text = survival, red/dash = no survival. PsLm is subdivided into* Pseudomonas spp. *and* L. monocytogenes *subpopulations. Lowest number of days after disinfection where an increase in turbidity is observed is noted where appropriate (T = high turbidity, t = low turbidity). PsLm parallels were tested for* L. monocytogenes *by plating on selective agar, 72 h (Day 3) and 144 h (Day 6) after disinfection. First positive test noted.*

	Rep 1				Rep 2				Rep 3			
	Lm	Ps	PsLm		Lm	Ps	PsLm		Lm	Ps	PsLm	
			Lm	Ps			Lm	Ps			Lm	Ps
1	-	-	Day 3	T4	-	T4	-	T4	-	t3	-	-
2	-	-	-	t4	-	t3	-	T4	-	T2	-	-
3	-	T4	Day 3	T2	-	t2	-	-	-	T3	Day 3	t4
4	-	t3	Day 3	T2	-	T3	Day 3	T2	-	T3	-	-
5	-		Day 3	t2	-	t2	Day 3	t2	-	T3	Day 3	t4
6	-	t3	Day 3	T2	-	T2	Day 3	t2	-	T3	-	-

3.4 *Pseudomonas* isolates colony characteristics and relative growth

As all *Pseudomonas* isolates were grown on TSA in the same conditions (25 ºC, 24 h) before being standardized and used in experiments, colony characteristics could be observed and systematized. Streaks of LJP040 and LJP042 were fluorescent under UV-light (group F), LJP321 formed slightly fluorescent, mucoid colonies, with streaks coalescing into slimy aggregations (group M), and LJP760 and LJP788 grew very quickly, forming prominent streaks and large colonies with no fluorescence (group Q) (table 4, figure 6: picture 1 and 4).

***Table 4**: Colony characteristics of* Pseudomonas *isolates used in the project. Three groups were established based on characteristics when grown at 25 ºC for 24 h on TSA: F (strong fluorescence), M (connecting, mucoid colonies and weak fluorescence), and Q (quickly growing, no fluorescence).*

Group	Isolate ID	Fluorescence	Colony morphology	Growth rate
F	LJP040	Strong	Separate, dry	Moderate
	LJP042	Strong	Separate, dry	Moderate
M	LJP321	Weak	Connected, mucoid	Moderate
Q	LJP760	None	Separate, dry	Quick
	LJP788	None	Separate, dry	Quick

These characteristics could be used to roughly quantify *Pseudomonas* isolates at different points in the previously mentioned experiments. During enumeration in the main experiment, some observations were made of the composition of *Pseudomonas* colonies from growth in different lifestyles (biofilm or planktonic growth). As a modified microspot technique was used, each spot contained 10 µL of suspension, growing in a small area (approximately 8 by 15 mm) and colonies were challenging to differentiate compared to traditional plate spreading technique. A certain difference was observed, with a domination of large colonies (Q) in planktonic growth while a more even spread between small (F/M) and large colonies was seen in solution from biofilm (figure 6: picture 2). These were not further distinguishable as colonies grew too densely to be picked and fluorescence was not expressed when colony size was less than 1 mm in diameter.

In the "Survival test" experiment, cultures were enumerated 72 h after disinfection using traditional plate spreading technique. Colonies could then be better separated into morphological groups, as the size where fluorescence was expressed could be reached before the plate was overcrowded. A large degree of variation was observed between different parallels. This ranged from only Q colonies (figure 6: picture 5a) to mostly F with some Q (5b) and some in between (5c). No pattern was observed differentiating coupons inoculated with *Pseudomonas spp.* in monoculture compared to co-culture with *L. monocytogenes*. Colonies with mucoid morphology (M) were not observed in enumeration of disinfected biofilm.

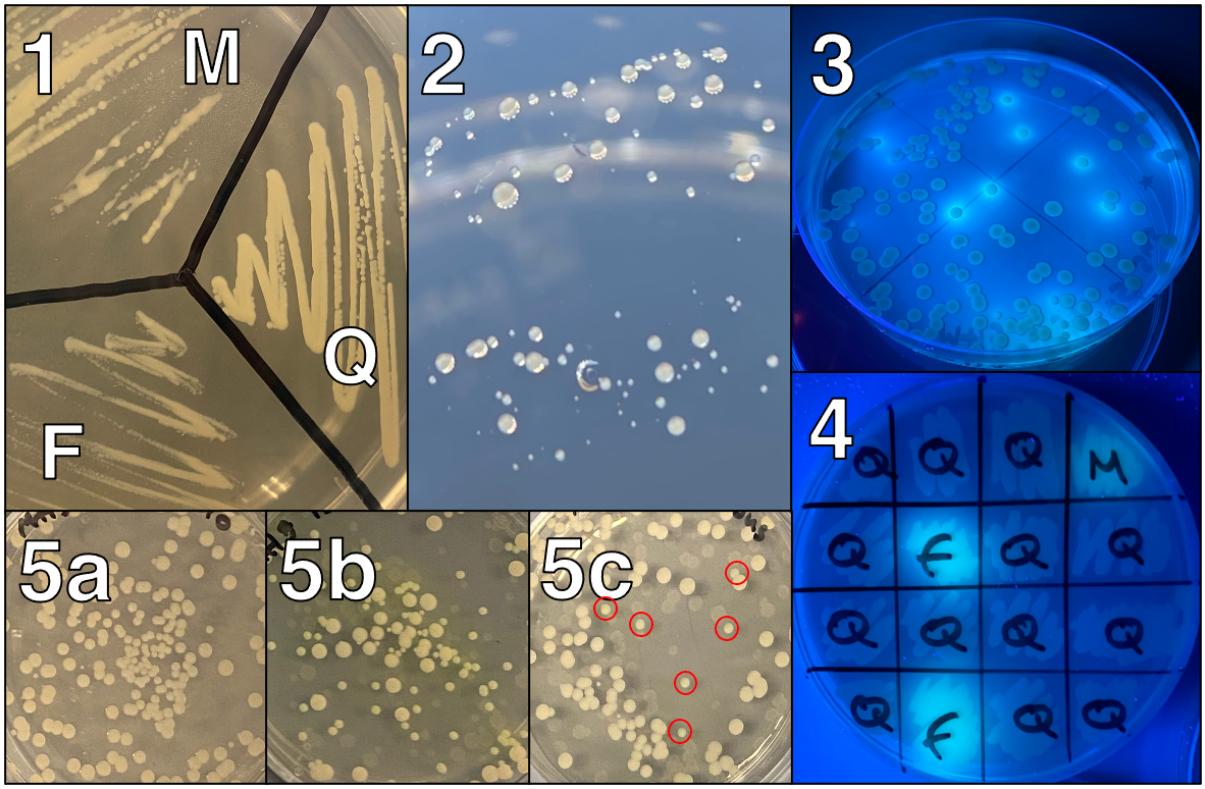

Fig. 6: *Pictures of colonies from* Pseudomonas *isolates used in this project. 1: Representative of each group based on colony characteristics; F (strong fluorescence), M (connecting, mucoid colonies and weak fluorescence), and Q (quickly growing, no fluorescence). 2: Two spots of the same dilution from enumeration of biofilm (using modified microspot plating technique), major variation in the size of colonies. 3: Colonies under UV light, majority non-fluorescent with some strongly fluorescent. 4: Colonies picked and transferred to new agar plate to better characterize, picture under UV light (note majority Q with some M and F). 5: Variations in surviving* Pseudomonas *isolates after disinfection, a = only Q, b = majority F or M, some Q, c = majority Q, some F or M (marked with red circle).*

4 Discussion

4.1 Growth in planktonic and biofilm cultures

The main experiment (chapter 2.2) in this project elucidated the growth dynamics in suspension and biofilm of *Listeria monocytogenes* and *Pseudomonas spp.* isolated from processing equipment and contact surfaces in a salmon processing facility (figure 5). Three sets of bacterial cultures; monoculture of *L. monocytogenes* (Lm), *Pseudomonas spp.* (Ps) and co-culture of *L. monocytogenes* and *Pseudomonas spp.* (PsLm) were used to inoculate stainless steel coupons. Bacterial cultures were grown at 12 °C, the same temperature as the processing facility, and measured 72 and 144 h after inoculation.

Planktonic growth was similar for all cultures at 72 h but deviated for cultures containing *Pseudomonas spp.* after the second incubation step. The initial (72 h) difference in population was small (<0,2 log) and not significant (P>0,05) but Ps and PsLm cultures reached a higher population density at 144 h compared to Lm (0,5 log) and the corresponding cultures at 72 h (0,3 log). It was expected that cultures would reach a similar maximum capacity after this amount of time, with exponential growth lasting approximately 24 h, as seen in the growth curve (chapter 3.1). The Ps and PsLm cultures did however have approximately double the population density at 144 h compared to 72 h. This pattern was not observed in *L. monocytogenes* monoculture, as the same concentration was measured at both timepoints, for both standard Lm and Lm with disinfected *Pseudomonas spp.* matrix (Lm+Ps(m)). Growth kinetics for the *L. monocytogenes* isolate were not examined for practical concerns. The literature supports the view that *L. monocytogenes* grows slower but reaches similar cell density in stationary phase compared to *Pseudomonas spp.* in these conditions (Duffy et al., 1994; Lebert, Robles-Olvera & Lebert, 2000; Mellefont, McMeekin & Ross, 2008).

The doubling of cell density in Ps and PsLm cultures might be due to more cells at the onset of incubation, or a change in the quantitative composition of isolates. At the initial inoculation, the solution used to inoculate coupons contained approximately the same concentration of all isolates. Coupons were transferred to incubation vessels, given fresh growth medium, and incubated. After 72 h of growth, the cultures would be more conditioned to the growth medium (½TSB) and the temperature (12 °C compared to 25 °C before standardization) and might have a shorter lag phase when the medium was rejuvenated. There

would also be more cells in biofilm on the coupon and walls of the incubation vessel, was well as some remaining suspension which could not be removed by pipette. With isolates having different growth characteristics, as was the case in this experiment, a gradual population shift could lead to the quickly growing isolates outcompeting the others over successive cycles of incubation. A more homogenous culture might have fewer detrimental inter-species interactions and the available nutrients could be channelled to cell growth rather than production of EPS or other nutrient-demanding activities. It was noted that non-disinfected planktonic cultures after incubation contained more cells of the Q (quickly growing) group (figure 6, picture 4). This pattern is contrasted with the homogenous Lm culture, which reached the same concentration after both incubation steps. The Ps and PsLm cultures could also simply grow quicker as a result of higher density of cells at the start of the second incubation step.

Biofilm population dynamics showed other trends than planktonic cultures. The difference was substantial at 72 h between cultures with and without *Pseudomonas spp.*, with Ps and PsLm approximately 1,0 log higher than Lm and Lm+Ps(m). There was not measured any difference between *L. monocytogenes* with or without the disinfected *Pseudomonas spp.* matrix. The difference was reduced after the second incubation step, with *L. monocytogenes* monoculture biofilm slightly lower than corresponding Ps and PsLm biofilms (0,2 and 0,3 log resp.). These differences might be a result of the biofilm development cycle. A biofilm is not measured solely on the number of cells contained in the matrix, with differentiation and complexity of the structure taking place as it develops (Flemming & Wingender 2010; Tolker-Nielsen, 2015). This corresponds with the results from this experiment, as biofilm populations of cultures with strong biofilm-producers (*Pseudomonas spp.*) appeared to stagnate compared to weak biofilm-producers (*L. monocytogenes*). It is also important to note that the biofilm is not removed when new media is added, as is the case for the suspension, so this is representative of a community at 144 h of development. The disinfection survival of *L. monocytogenes* with or without *Pseudomonas spp.* matrix, disinfection of biofilms grown for 72 h compared to 144 h or the matrix structure at these intervals were not investigated but would be worth looking further into.

4.1.1 Interactions between *L. monocytogenes* and *Pseudomonas spp.*

Much of the value in understanding these communities is tied to the interactions between *L. monocytogenes* and the microflora, here represented by five isolates of *Pseudomonas*. A lot of

studies have focused on this subject, all showing different dynamics and interactions, from antagonistic to synergistic and neutral (Carpentier & Chassaing, 2004; Fox et al., 2014; Heir et al., 2018; Ibusquiza et al., 2012; Langsrud et al., 2015; Lebert, Robes-Olvera & Lebert 2000; Mellefont, McMeekin & Ross, 2008; Papaioannou et al., 2018; Puga, SanJose & Orgaz, 2014). In this project, *L. monocytogenes* in co-culture with *Pseudomonas spp.* constituted approximately 2 % at 72 h and less than 1 % at 144 h in suspension. The opposite pattern was observed in biofilm, as the *L. monocytogenes* subpopulation increased compared to the total population, with between 1 and 2 % at 72 h to approximately 4 % at 144 h. This pattern corresponds with the results of, amongst others, Fagerlund et al. (2017).

The results from this project showed that *Pseudomonas spp.* supressed the *L. monocytogenes* population in both suspension and biofilm compared to monoculture. The nature of these interactions was not further investigated and can therefore not be determined. Although *Pseudomonas spp.* supressed *L. monocytogenes*, there did not appear to be an active antagonistic relationship, such as is often observed with non-*Pseudomonas* species present in the microflora. For some of these species, there is documented anti-listerial activity, with bacteriocins or other mechanisms impeding growth or inactivating *L. monocytogenes* altogether (Carpentier & Chassaing, 2004; Fox et al., 2014; Papaioannou et al., 2018).

The relative decrease of *L. monocytogenes* in suspension might be explained by the selection pressure in this environment favouring quickly growing isolates. At inoculation, *L. monocytogenes* made up approximately 20 % of the population, was reduced to one tenth of this at 72 h and less than one twentieth at 144 h. It is probable that such a disparity would continue to increase with successive incubations and lends credence to the idea that a population shift is happening for *Pseudomonas spp.* as well. The gradual shift towards a more homogenous mixture can be explained with the “Jameson Effect”, where the first species in a mixed culture to reach stationary phase and exhaust the available nutrients halts the growth of all other inhabitants (Mellefont, McMeekin & Ross, 2008).

There are a number of documented mechanisms used by *Pseudomonas spp.* to adversely affect competing species. These include iron sequestration, production of bacteriocins and other toxic metabolites (Gram et al., 2002; Palleroni, 2015). There are however examples of the same kind of suppression of *L. monocytogenes* as is seen in this project when *Pseudomonas* strains which do not produce siderophores or bacteriocins are used (Buchanan

& Bagi, 1999; Mellefont, McMeekin & Ross, 2008). The bacteriocins or other suppression mechanisms produced by *Pseudomonas spp.* might simply not target the *L. monocytogenes* strain used in this project. Furthermore, if such mechanisms are at play, it does not fit with the increase of *L. monocytogenes* in co-culture biofilm over time.

The relative amount of *L. monocytogenes* was lower in biofilm compared to suspension at 72h, but the relationship switched at 144, with biofilm approximately five times higher. This is while the total population and subpopulation of *Pseudomonas spp.* in the biofilm remained stable. The increase might be the result of either *L. monocytogenes* having better growth conditions in biofilm or a "migration" of nearby planktonic cells into the matrix. Environmental niches in the biofilm, especially in terms of oxygen saturation, might facilitate the growth of *L. monocytogenes* (Fagerlund, Langsrud & Møretrø, 2021; Giaouris et al., 2015; Stewart & Franklin, 2008). The bottom layer of the *Pseudomonas*-dominated biofilm might become a more attractive environment for *L. monocytogenes* as nutrients get depleted and the build-up of toxic metabolites in suspension halt planktonic growth. As *Pseudomonas spp.* tend to migrate to the upper layers and *L. monocytogenes* are shown to favourably colonize the bottom layers, it is probable that a species-based differentiation of the biofilm plays a role in the dynamic observed in this experiment (Almeida et al., 2011; Fagerlund et al., 2017; Klausen et al., 2003; Puga et al., 2018; Puga, SanJose & Orgaz, 2014).

These results paint a picture of *L. monocytogenes* primarily living in biofilm reservoirs and dispersing small numbers of planktonic cells into the environment. Langsrud et al. (2015) found *L. monocytogenes* primarily in biofilm in the food processing environment, while the surrounding suspension was dominated by other species. *L. monocytogenes* can then circumvent the harsh, competitive suspension, while dispersing small numbers of cells which can grow if conditions are favourable and there isn't excessive competition, as seen by Pang & Yuk (2019) (Fagerlund, Langsrud & Møretrø, 2021; Lianou et al., 2020).

4.2 Survival of biofilms exposed to disinfectant

This project demonstrated that the disinfection treatment was sufficient at reducing biofilm populations, but cohabitation with *Pseudomonas spp.* can be the key to survival and persistence for *L. monocytogenes* in conditions relevant to a salmon processing facility. The experiments simulated the conditions of a stainless-steel surface directly exposed to the

disinfectant for an extended period of time (10 min). The effect of the treatment was measured in two ways, one with high resolution but few parallels (in the "Growth and survival experiment") and one with low resolution but many parallels (in the "Survival test").

The immediate result of disinfection was a dramatic reduction in viable cells from biofilms of all cultures. In fact, no colonies were observed on any agar plate from a disinfected coupon immediately after treatment, e.g., a surviving population under the detectable limit of 30 CFU/coupon. This corresponded to a reduction of 4,3, 5,2 and 5,4 log for Lm, Ps and PsLm cultures respectively. The number of cells in Lm biofilm at 72 h was too low to measure a 5,0 log reduction with the current experimental parameters, but the two other cultures are within the required efficacy for industrial surface disinfectants at 5,0 log (although the exposure time was longer than the specified 5 minutes in this experiment) (Fu, McCue & Boesenberg, 2007, p. 852). As Lm cultures did not grow back within a one-week window after disinfection, it can be ascertained that the real number of viable cells was zero (at least in practice), which translates to more than a 5,0 log reduction. The disinfection treatment, under the parameters used in the current project, was effective in reducing both *L. monocytogenes* and *Pseudomonas spp.* in 72 h-biofilm to a sufficient extent.

The cultures which survived disinfection did so as the result of a small number of surviving cells. These had a long lag-phase, some parallels not showing visible signs of regrowth before four days after treatment (table 3) indicating sublethal damage. A long lag-phase might also be the result of survivors consisting of largely dormant persister cells which require more time to become active (Lewis, 2005; Roberts & Stewart, 2005). There appeared to be some correlation between the amount of time before visible growth and the survival of *L. monocytogenes* subpopulation in surviving PsLm-cultures, as 3 of 6 PsLm parallels without visible growth before day 4 and 7 of 7 with visible growth before day 4 had surviving *L. monocytogenes*. There was also no discernible pattern within the surviving *Pseudomonas* populations after disinfection, with a large degree of variation seen between different parallels (figure 6, picture 5a,b,c). This indicates that the survival of these cultures was the result of a small number of cells, which resulted in a skewed ratio between isolates compared to non-disinfected cultures. Disinfected and non-disinfected *Pseudomonas* isolates from the Ps and PsLm culture were originally supposed to be sequenced, but the seemingly random nature of the survival process made this seem superfluous compared to the work involved.

Although *L. monocytogenes* obtained a major survival advantage in biofilm with *Pseudomonas spp.,* the inverse was not observed. Puga et al. (2018) and Puga, SanJose & Orgaz (2014) noted that the matrix of a *P. fluorescens* biofilm became denser and more compact, while containing the same number of cells, after *L. monocytogenes* was introduced to the community. A denser matrix might limit the diffusion of the disinfectant, thus increasing tolerance. Metabolic cross-feeding and co-aggregation are also mechanisms that could give multi-species biofilms advantages over their mono-species counterparts (Sanchez-Vizuete et al., 2015). These mechanisms can explain why co-cultures of *L. monocytogenes* and members of the ubiquitous microflora can exhibit increased tolerance to disinfectants for both parties (Giaouris et al., 2013; Ibusquiza et al., 2012; Puga, Orgaz & SanJose, 2016; Van der veen & Abee, 2011). There are also numerous examples of mixed biofilms exhibiting reduced or no change in tolerance compared to monospecies biofilms (Fagerlund et al., 2017; Papaioannou et al., 2018). The results from this project indicated no advantage to *Pseudomonas spp.* from growing in coculture.

The disparity in survival for *L. monocytogenes* in monoculture compared to co-culture with *Pseudomonas spp.* highlights the importance of including the background microflora in the study of *L. monocytogenes* persistence in the food processing environment. Studying *L. monocytogenes* monoculture biofilm can, as seen from this project, give the wrong impression when testing the efficacy of a disinfectant and when evaluating the pathogen's ability to persist in the environment. There is growing agreement that many of the phenotypic characteristics that were thought to be critical for colonization of the processing environment, like biofilm production and synthesis of flagella, only play a part in the exceedingly complex web of interactions with background microflora and the various stresses imposed by the environment which play a role in the persistence of *L. monocytogenes* (Fagerlund, Langsrud & Møretrø, 2021; Giouaris et al., 2015). The results from this project exemplify how a *L. monocytogenes* strain which could not survive disinfection in monoculture and was supressed by the environmental isolates (*Pseudomonas spp.*) could survive on an open stainless-steel surface directly exposed to the disinfectant when grown in co-culture. It is also well established that *L. monocytogenes* strains exhibit a large degree of variation in terms of both interactions with background microflora and their inherent phenotypic characteristics (biofilm production, synthesis of flagella, tolerance to disinfectants, etc), which was not accounted for in this project as only one isolate was used (Borucki et al., 2003; Colagiorgi et al., 2017; Fagerlund, Langsrud & Møretrø, 2021; Giouaris et al., 2015; Heir et al., 2018; Lianou et al.,

2020). Testing different strains of *L. monocytogenes* could therefore be an interesting approach for further studies.

4.3 Evaluation of methods for biofilm growth

The two different methods used in the main experiment to grow biofilm and suspension both had their positive and negative sides. The most important reason for switching to Eppendorf Tubes® was that the first two replicates had problems with contamination, believed to come from cross-contamination within 6-well plates. Three of four Lm- suspensions in the first replicate became contaminated with *Pseudomonas spp.* and the negative control (in the same 6-well plate as Lm-cultures) in the second replicate was contaminated. There were no specific manipulations of samples that the contaminations could be traced back to (using the same pipette tip in different samples or not using sterile forceps, etc.). It was therefore hypothesized that an unnoticeable factor (aerosolized suspension or small drops) could lead to contamination during manipulation of samples in the same 6-well plate. When inoculation was moved from one 6-well plate to individual petri dishes, there was not detected any contamination between cultures. The negative control, located on the same 6-well plate as Lm-cultures, was contaminated with *L. monocytogenes* on day 6 of the second replication. This was also considered to be contamination between wells in the 6-well plate, and another method (using Eppendorf Tubes®) was devised. This seemingly solved the problem of contamination.

The abovementioned contamination events put all the results from the two first replicates into question, especially those involving disinfection, as there was no way to know whether regrowth originated from survival or contamination. The practice of incubating untreated suspension, with approximately 9 log CFU/mL, adjacent to disinfected coupons, which might have 0 CFU/mL, was deemed problematic. Results were therefore discarded, and the method was modified. In Heir et al. (2018), whose method this experiment borrows from, each 6-well plate was used for one culture or treatment, which effectively eliminates the consequences of small amounts of contamination between parallels in 6-well plates. A similar approach could have been taken in the main experiment (chapter 2.2) of this project, but a lower number of parallels was decided upon because of practical concerns. The “survival test” (chapter 2.4) was in this sense more similar to the method as described in Heir et al. (2018), and no instance of contamination was recorded across three replicates in this experiment.

The two methods led to a slight difference in growth patterns. As there were not enough parallels or replicates to determine statistical significance, comparing means are the best tool available. Mean cell density for *Pseudomonas spp.* was in most cases higher when using 6-well plates compared to Eppendorf Tubes®, with some notable exceptions. *L. monocytogenes* in PsLm-culture was lower in 6-well plates on day 3 in both biofilm and suspension, while Ps and PsLm biofilm was lower on day 6. The most probable explanation for this is different levels of aeration for the types of incubation vessels. As 6-well plates were considerably shallower and wider than Eppendorf Tubes®, more surface area in relation to volume would be exposed to air. Lower aeration can explain why the microaerophilic *L. monocytogenes* might grow better with *Pseudomonas spp.*, and in turn why the aerophilic *Pseudomonas spp.* grow slower. These are of course both model systems and will bring their own advantages and disadvantages in terms of replicating the relevant environment and providing measurable results. Both did induce biofilm growth, the same effects (reduction of CFU after disinfection, suppression of *L. monocytogenes* in co-culture) were observed, and the differences were overall small compared to the variation between replicates. The 6-well plate method used in this project was however not fit for purpose as the results could not be trusted due to contamination.

4.4 Experimental parameters and real-life application

The experiments which were carried out in this project were, as always in experimental microbiology, model systems used to approximate the relevant real-life environment in a laboratory setting. It is important to keep this in mind when extrapolating results and findings in the lab to guidelines for use in industry. The *Pseudomonas* isolates used in this project were all isolated on relevant food contact surfaces in the same facility and were picked to be representative of the microflora surviving CD routines. The *L. monocytogenes* isolate was also isolated at the facility and was of the same sequence type which has been implied in a number of foodborne listeriosis outbreaks. Temperature (12 °C) and the surface used to grow biofilm (stainless-steel) were also relevant for the processing environment, but other surface types (transportation belts and transitions between materials) may be more relevant areas for biofilm growth (Fagerlund, Langsrud & Møretrø, 2021). The growth medium (½TSB) could have been changed to fish juice, which would be a better representation of the available nutrients in a salmon processing facility and has been used by other similar studies

(Papaioannou et al., 2018). Inoculation suspensions consisted of an approximately equal concentration of all the isolates which, as seen during the course of experiments, did not remain stable. Bacterial cultures were given three days to grow and form biofilm, relevant for the stop in production during the weekend. No chemical cleaning step was done before disinfection, and the treatment itself consisted of coupons being submerged in a 1 % liquid of the chemical. The producer specifies that the disinfectant should be applied as a foam, which may give a different result. There was no follow-up treatment done to the disinfected coupons.

Comparing the results from this project with Fagerlund et al. (2017), there are some notable differences. *L. monocytogenes* survived disinfection much better in mono-species biofilm, and the overall efficacy of the CD routine was considerably lower. This study used a more diverse mixture of species from the microflora along with *L. monocytogenes*, but *Pseudomonas spp.* dominated after CD. Coupons made of transportation belt material were treated with chemical sanitizer and disinfectant (both QAC and PAA) applied as foam with several consecutive treatments over three days. The exposure time (5 min) was also shorter than in this project. The divergence in results probably stem from the surface typology, a different treatment procedure and possibly antagonistic interactions between species.

This project has demonstrated that the ubiquitous microflora of a food processing environment can assist *L. monocytogenes* survival to an otherwise lethal disinfection routine. Extrapolating these results to any real-life application must be done with care, taking into account the approximations and changes which have been made to be able to effectively and consistently study these communities in the laboratory. Although the disinfection routine reduced the number of viable cells in biofilms to a satisfactory degree, it is not certain that the same effect translates to older biofilm consisting of different species, grown on different materials and treated with disinfectant foam for a shorter period of time. The results nevertheless point to the importance of thorough and frequent CD treatment of the processing environment to supress growth of *L. monocytogenes* in biofilm and limit its regrowth from surviving enclaves.

4.5 Subjects for further study

This project introduced far more questions than in answered. As mentioned above, there are many approximations which have to be made to effectively study a microbiological system in the laboratory. Changing some of these parameters could give other insights into the dynamics of such communities and can be more relevant for the industrial application.

The most relevant parameter is in this regard probably method of application for the disinfectant. Applying the disinfectant as a foam, as is intended by the producer, and adding a chemical cleaning step would be important before the efficacy of the disinfectant can be fully ascertained. Comparing the peracetic acid based "Aqua Des" used in this project to other disinfectants, especially a QAC based alternative, is interesting in terms of both the total efficacy and anti-listerial activity of these products. As the industry is moving away from using QAC based disinfectants, it is important to document that peracetic acid is as or more effective.

Certain parameters were not included for practical concerns but would be worth investigating further. Several cycles of disinfection and longer experimental periods would have a number of advantages. To measure the impact of a disinfection treatment on biofilms grown for 24 h as well as more mature structures, such as 3-, 6- and 9-day biofilms could have practical application and is commonly tested in similar studies (Fagerlund et al., 2017; Papaioannou et al., 2018). One approach which has not been explored in other studies is *L. monocytogenes* survival when introduced to disinfected *Pseudomonas spp.* biofilm matrix. Akinobobola et al. (2021) found a dramatic increase in tolerance for *P. aeruginosa* when new cells were introduced to a dead matrix treated with PAA. This was however in a clinical setting and did not use *L. monocytogenes*. The Lm+P(m) culture was originally supposed to be disinfected, but a single treatment was decided upon for practical concerns. Other *L. monocytogenes* strains and a more diverse microflora was also decided against for practical concerns but could be included to give a deeper understanding of the dynamics in such systems.

5 Conclusion

A *Listeria monocytogenes* ST8 isolate gained a decisive survival advantage grown in co-culture biofilm with *Pseudomonas spp.*, compared to monoculture, when exposed to peracetic acid-based disinfectant. No monoculture *L. monocytogenes* biofilm survived disinfection treatment, while 10 of 18 *L. monocytogenes* subpopulations survived in the mixed culture. There was no observed advantage for *Pseudomonas spp.* being co-cultured with *L. monocytogenes*.

Pseudomonas spp. dominated both suspension and biofilm in co-culture. The relative *L. monocytogenes* subpopulation in suspension decreased in consecutive incubation cycles and increased as a function of time in biofilm. The same pattern was observed for *Pseudomonas spp.*, where slowly growing isolates seemed to be outcompeted in suspension. This points to biofilm as fulfilling a crucial role in enabling the persistence of *L. monocytogenes* in the food processing environment.

The disinfection treatment, as executed in this laboratory setting, successfully reduced the number of viable cells in biofilm by 5 log in all cultures tested. A large fraction of the disinfected *Pseudomonas spp.* parallels (15 of 18) and *Pseudomonas spp.* with *L. monocytogenes* (13 and 10 of 18, resp.) survived the treatment, showing visible regrowth after 2 to 4 days. This exemplifies the importance of frequent and thorough disinfection of the processing environment to supress the growth of microorganisms, including *L. monocytogenes*, in biofilm and limit regrowth from surviving enclaves. A study investigating the survival dynamics of these isolates when exposed to the disinfectant as foam, instead of submerged in solution, is warranted to better translate these results to the processing environment.

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