School of Public Health

Understanding the role of biofilm formation in the predominance of meat spoilage pseudomonads

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This thesis is presented for the Degree of

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature- Nirmani Wickramasinghe

Date- 12/11/2020

Authorship attribution statement for the thesis

Paper 1- located at chapter 02

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ABSTRACT

The red meat industry is Australia's largest food manufacturing sector and bacterial spoilage of meat causes significant financial losses to the industry. Despite using refrigerated temperatures to minimize bacterial growth, psychrotrophic bacteria can still multiply on meat and cause spoilage. *Pseudomonas fragi* is the most predominant microorganism on aerobically stored chilled meat. *Pseudomonas lundensis* is another bacterial species that grow on chilled meat and currently limited knowledge is available on them. These bacterial species readily form biofilms on meat under refrigerated and temperature abuse conditions. When biofilms mix with meat exudates it leads to slime formation which is a key quality defect. Biofilms provide many benefits to the residing bacteria to withstand stressful, chilled environments. Limited research has been done on biofilm formation on complex food materials and about their structural and genomic characteristics. The main objective of this research was to study how biofilm formation can aid these psychrotrophic pseudomonads to withstand chilled temperatures and overcome the competition from other microorganism to become the predominant organisms on aerobically stored meat. The degradation pattern of meat muscle when these biofilms are formed on meat was also studied.

In the first part of this research, a model system that closely mimics the practical industry conditions was designed to study biofilm formation *in-situ* on raw beef muscle. Five strains of *P. fragi* and 5 strains of *P. lundensis* were selected to study their cellular and structural arrangement when formed as mono-species biofilms on meat under refrigerated and ambient temperature conditions. Biofilms formed on surface sterilized raw beef were stained with fluorescent dyes and imaged using confocal laser scanning microscope. The biofilm biovolume on image stacks were quantified using COMSTAT and three-dimensional renderings of the scanned biofilms were obtained using AVIZO application. It was found that *P. fragi* and *P. lundensis* form structurally different biofilms despite their close taxonomic arrangement. *Pseudomonas fragi* produce highly dense, compact, lawn-like biofilms where the rod-shaped bacteria vertically oriented themselves within the biofilm. In contrast, *P. lundensis* produced loosely arranged, disorganized biofilms with much intercellular spacing, gaps and voids. Nematic ordering was not observed in *P.*

lundensis biofilms. The structural arrangement of *P. fragi* is likely to provide these biofilms with strong mechanical properties that can withstand environmental forces. The tight cellular arrangement can help increase resistance to biofilm invasion by foreign species. Circular and elliptical voids on meat tissues with mature biofilms showed that membranes of bovine nuclei do not get degraded as easily as muscle fibers. Extracellular DNA (eDNA) content in biofilms does not appear to change with temperature conditions and strain-level variations in eDNA production were observed.

Since the matrix of a biofilm plays a key role in its stability, the composition of key matrix components of selected *P. fragi* and *P. lundesnis* strains were determined using chemical analysis and Raman spectroscopy. The biofilms were grown at 10 °C and 25 °C on nitro-cellulose membranes placed on surface sterilized beef cuts. Extra-cellular polymeric substances (EPS) of the matrix were extracted in soluble and bound forms. Chemical analysis was done to quantify the total carbohydrates, proteins and extracellular DNA content of the matrix. Chemical analysis revealed that when biofilms are formed under low temperature conditions, the matrix carbohydrate and protein contents increase at statistically significant levels compared to biofilms formed at ambient temperature.

Raman spectra were obtained from planktonic bacteria and membrane grown biofilms at 10 °C and 25 °C. The results of Raman spectroscopy correlated with the results of chemical analysis where high levels of matrix carbohydrate and protein contents were detected in low temperature stored samples. Carbohydrate concentration was higher in biofilm samples of all four strains compared to planktonic samples. Planktonic bacteria contained high amounts of guanine in the chemical composition compared to biofilm bacteria. Guanine at biofilm stage could be used for the development of cyclic di-GMP which can lower the guanine levels in biofilm samples. Thus, the detection of low levels of guanine in biofilms samples could be because guanine is utilized for cyclic-di-GMP formation.

All biofilms follow a cycle of bacterial irreversible attachment, maturation and dispersal and these stages are genetically regulated. RNA sequencing was performed at initiation, maturation and the dispersal stages of the biofilm cycle of *P. fragi* 1793 strain, grown on aerobically stored beef, kept at 10 °C. Differential expression analysis revealed that genes coding for Flp family type IVb pilin, ribosome modulation factor, creatininase and pyruvate dehydrogenase were among the most upregulated genes while genes encoding for iron uptake systems and taurine transport were significantly down regulated. The results also showed that protein synthesis and cellular multiplication cease after the population maximum of the biofilm has been reached.

Certain chemical compounds and environmental cues have been shown to trigger biofilm dispersal. In the final section of this research, the potential of nitric oxide (NO) donor compounds to act as dispersing agents on *P. fragi* biofilms formed on chilled beef was assessed. It was found that a concentration of 40 µmols/l of sodium nitroprusside can cause two log reductions in viable *P. fragi* biofilms formed on meat compared to the no treatment controls. The correct rate of nitric oxide release is important to cause dispersal in *P. fragi* biofilms.

The findings of this research provided insight into how biofilm formation can aid the long-term survival of *P. fragi* on chilled meat. New knowledge was also presented that may help to design novel biofilm controlling compounds that can be used in the meat industry. These findings can be used as a platform to study future biofilm research under *in situ* conditions.

Abbreviations

AHL,	acyl homoserine-lactone
AMPC,	Australian Meat Processor Corporation
ANOVA,	analysis of variance
BEPS,	bound extra cellular polymeric substances
CIP,	cleaning in place
CFC,	centrimide–fucidin–cephaloridine
CFU,	colony forming units
CLSM,	confocal laser scanning microscopy
COGs,	clusters of orthologous groups
CSIRO	Commonwealth Scientific and Industrial Research Organization
eDNA,	extra cellular DNA
EDTA,	ethylene diamine tetra acetic acid
EPS,	extra cellular polymeric substances
DEGS,	differentially expressed genes
MAP,	modified atmospheric packaging
MEF,	moderate electric field
MRD,	maximum recovery diluent
NO,	nitric oxide
PBS,	phosphate buffered saline
PCA,	principal component analysis
PDH,	pyruvate dehydrogenease
PI,	propidium iodide
SEM,	scanning electron microscope

SEPS,	soluble extra cellular polymeric substances
SNP,	sodium nitroprusside
SSO,	specific spoilage organisms
QS,	quorum sensing
UHT	ultra-high temperature
VOC,	volatile organic compounds
VP,	vacuum packaging

PUBLICATIONS AND CONFERENCE PRESENTATIONS

Nirmani Wickramasinghe, Josh T Ravensdale, Ranil Coorey, Gary A Dykes, P. Scott Chandry (2018). Development of a method for *in-situ* characterization of biofilms grown on meat muscle. *Poster abstract*.ASM annual conference, Brisbane, Australia. July 1st-4th .

N.Wickramasinghe, M.M.Hlaing , J.T.Ravensdale , R. Coorey , G.A.Dykes , P. S. Chandry (2018). Structural transformation and matrix composition of biofilms formed on meat by psychrotrophic pseudomonads *Poster abstract* The 8th ASM Biofilms conference, Washington, USA.

Wickramasinghe, N. N., Ravensdale, J., Coorey, R., Chandry, S. P. & Dykes, G. A (2019). The Predominance of Psychrotrophic Pseudomonads on Aerobically Stored Chilled Red Meat. *Comprehensive Reviews in Food Science and Food Safety* **18**, 1622-1635, doi:10.1111/1541-4337.12483

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Wickramasinghe, N. N., Ravensdale, J. T., Coorey, R., Dykes, G. A., & Scott Chandry, P. (2021). Transcriptional profiling of biofilms formed on chilled beef by psychrotrophic meat spoilage bacterium, *Pseudomonas fragi* 1793. *Biofilms Journal*

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CHAPTER 1

General introduction

1.1 Microbial growth on meat

The red meat industry is Australia's largest manufacturing sector and red meat is an important component of the Australian daily diet (Pointon et al., 2006). Consumer demand for products that are fresh and minimally processed is increasing. It is therefore important to maintain and improve the quality and safety attributes of fresh meat (Edmund, 2016). Meat is a highly perishable food product due to its rich nutrient composition, high water activity, moderate pH and inherent enzymatic activity (Ercolini, Russo, Torrieri, Masi, & Villani, 2006). Despite advancements in food preservation methods, bacterial spoilage is a substantial issue in the meat industry. Microorganisms originating from the animal's intestinal tract or transferred from the environment can contaminate meat during processing and handling. Bacterial colonization on meat can cause degradation of meat muscles.

During commercial production, meat is usually stored at temperatures of ~2 °C and at ~ 4 °C during transportation to limit the growth of spoilage microorganisms (Coombs, Holman, Friend, & Hopkins, 2017). However, a group of microorganisms collectively known as psychrotrophic bacteria can still grow and multiply under chilled chain conditions. Small fluctuations in temperature can dramatically increase the bacterial population and decrease the time to spoilage (Chen, Godwin, & Kilonzo-Nthenge, 2011). Psychrotrophic microorganisms such as psychrotrophic *Pseudomonas*, *Brochothrix thermosphacta*, lactic acid bacteria, and psychotropic *Clostridium* spp. dominate spoilage flora of meat stored at low temperatures (Lebert, Begot, & Lebert, 1998). These proteolytic bacteria can disrupt the meat structure, allowing penetration into the muscle tissue (Gill & Penney, 1977).

***Psychrotrophic pseudomonads*:** Among the key meat spoilage bacteria, psychrotropic *Pseudomonas* species dominate on aerobically stored meat at refrigerated temperatures (2–8 °C), (Ercolini et al., 2007). To date, *Pseudomonas* is the largest genus of Gram-negative bacteria containing 202 species (Garrity, 2001). Pseudomonads are motile, gamma proteobacteria belonging to the family *Pseudomonaceae.* Members of the *Pseudomonas* genus colonize soil, plants and, water as well as raw and processed meat (Robertson, Hapca, Moshynets, & Spiers, 2013). Spoilage pseudomonads are generally recruited from the local environment and are further selected for by meat processing and storage conditions (Ercolini et al., 2007).

Under chilled, aerobic conditions, *Pseudomonas fragi*, *Pseudomonas lundensis*, *Pseudomonas fluorescens* and *Pseudomonas putida* are the primary meat spoilage bacteria (Ercolini et al., 2007). These are psychrotropic bacteria and their growth is favored by the chain of chilled temperatures applied in the meat industry. They can cause meat deterioration due to their ability to produce extracellular proteases and lipases during chilled conditions. A key characteristic of meat spoilage pseudomonads is that they readily form biofilms under low temperature conditions. Robertson et al. (2013), found that biofilm formation is more common in meat affiliated psychrotropic pseudomonads than the rest of the pseudomonad community. Here I discuss the characteristics of *P. fragi* and *P. lundensis* which are the most prevalent species on chilled beef.

***Pseudomonas fragi*:** Of the psychrotrophic pseudomonads, *P. fragi* is the predominant bacterial species on meat of different biological origins worldwide including beef, pork and chicken (Drosinos & Board, 1995; G. Y. Wang, Ma, Wang, Xu, & Zhou, 2017). *Pseudomonas fragi* has a fast growth rate under chilled temperature conditions (Drosinos & Board, 1994). Due to its high metabolic requirement for iron, it is believed that meat could be the ecological niche for *P. fragi.* This organism can cause meat discolorations through enzymatic oxidation of pigments and through oxygen tension caused by increasing growth of bacteria (Bala, Marshall, Stringer, & Naumann, 1978).

Although different food additives have been used in levels safe for human consumption and under different pH ranges in attempts to control *P. fragi* on meat, currently no additive has been shown to be effective (Tapas, Kumar Patel, Dhindwal, & Tomar, 2011). The exact reasons for the dominance of this species on aerobically stored fresh meat are still unknown. Due to the many benefits biofilms provide to its residing bacteria, it is likely that biofilm formation plays a role in the dominance of these species. It is therefore highly beneficial to the meat industry to study the biofilm formation characteristics of this species in the complex meat environment.

***Pseudomonas lundensis*:** According to the limited amount of research on *P. lundensis*, it was first isolated from fresh meat and named as a new species of meat spoilage pseudomonad in 1986 (Molin, Ternstrom, & Ursing, 1986). The temperature range for its growth is between 0 °C and 33 °C. Studies have reported *P. lundensis* to have strong proteolytic activity, causing spoilage of chilled pork (Molin & Ternstrom, 1986). According to phenotypic cluster analysis, its closest relative appears to be *P. fragi* (Stanborough et al., 2018). *Pseudomonas lundensis* has a significant capacity to produce biofilms at low temperatures as compared to ambient room temperatures (Liu et al., 2015). At the same time, higher protease activity was observed in extracted biofilms than in planktonic cultures (Liu et al., 2015). Currently, no information is available on the dynamics of *P. lundensis* biofilm formation, its gene expression within the biofilms, and the composition of the biofilm matrix *in situ.*

1.2 Bacterial biofilms

Biofilms are usually defined as bacterial cells attached to a surface or to each other, embedded in a self-produced or an acquired exopolysaccharide matrix (Hans-Curt et al., 2016). Free living planktonic bacteria go through a series of metabolic and phenotypic changes during transformation into sessile cells in a biofilm (Barraud, Kjelleberg, & Rice, 2015). Biofilms form when planktonic bacteria attach themselves irreversibly to surfaces and start secreting exo-polymeric substances (EPS) (Valentini & Filloux, 2016). The biofilm life cycle consist of several stages including reversible attachment, irreversible attachment, micro colony formation, maturation and dispersal (Hans-Curt et al., 2016).

Studies have determined that the shift between free living, planktonic mode and the formation of structured, sessile communities embedded in an exo-polymeric matrix is a staple for the majority of bacterial species (van Der Veen & Abee, 2011). The transition between planktonic and sessile modes in the bacterial life cycle is regulated by gene expression in response to environmental stimuli (Fagerlind et al., 2012). Since formation of biofilms is the norm for the majority of bacteria in the natural environment, spoilage microbes also exist as biofilms in food processing plants and on food surfaces.

***The biofilm matrix*:** The biofilm matrix is composed of mainly water, polysaccharides, proteins, lipids, extra cellular vesicles and extra cellular DNA (eDNA) (Carey, Knut, Ned, & Bonnie, 2015). Biofilm's extra-cellular polymeric substance (EPS) is a complex mixture of high molecular weight polymers secreted by microorganisms, products of cell lysis and hydrolysis as well as organic matter adsorbed from the substrates that they are grown on (Mann & Wozniak, 2012). The composition as well as the quantity of EPS can vary depending on substrate, microbial flora and environmental conditions (Liang, Li, Yang, & Du, 2010). The content of carbohydrates, proteins and eDNA can have a substantial effect on the biofilm structure and stability (Lal, Sharma, Pruthi, & Pruthi, 2010).

The matrix provides structure and, mechanical stability to the biofilm as well as protecting the microorganisms within it from hazardous environmental effects such as desiccation, predation, radiation, antibiotics and host immune defenses (Flemming, Neu, & Wozniak, 2007). Furthermore, the matrix aids in transportation of small molecules used in communication and is the location of numerous extracellular enzymatic reactions (Mann & Wozniak, 2012). Therefore, the biofilm matrix should not be considered as a passive or an inert material but as an active component of a biofilm. In order to establish an in-depth view of the biofilms, it is necessary to study both the cellular and the EPS components together. Currently no knowledge is available of EPS composition of biofilms formed by meat spoilage pseudomonads. Therefore, this project aims to study the composition of matrix EPS of selected *P. fragi* and *P. lundensis* strains.

***Gene expression during biofilm formation*:** The formation of complex three dimensional biofilms is a dynamic process and it involves a coordinated series of molecular events that are regulated by gene expression (Schembri, Kjærgaard, & Klemm, 2003). Studies have found considerable differences in gene expression profiles between populations of biofilms and planktonic bacteria (Marvin et al., 2001). Bacteria use quorum sensing to measure cell numbers in a population and regulate gene expression in response to environmental stimuli (Gram et al., 2002). Quorum sensing is used to regulate genes involved in biofilm formation, biofilm dispersal, motility, bioluminescence, toxin production, exo-polymeric substance production and production of virulence factors (Skandamis & Nychas, 2012). Due to the minimal overlap between functions involved in biofilm formation by different bacterial species in vastly different environments, finding common gene-expression patterns through global expression analysis is difficult in biofilm studies. Currently, very limited information is available on the key genes that govern biofilm formation, maturation and dispersal of psychrotrophic pseudomonads when formed on meat under chilled temperature conditions.

1.3 Control of biofilms

The matrix of a biofilm is a highly hydrated structure that protects bacteria from adverse environmental conditions such as antibiotics, disinfectants, sanitizers and desiccation (Flemming et al., 2007). Furthermore, bacterial cells embedded in a biofilm matrix live in sessile mode where they are metabolically less active and as a result, their control by the host immune system or antibiotics if formed in living organisms is difficult (Luyan et al., 2009). Since bacterial species live in close proximity to each other within the biofilm, plasmid transfer can occur at a higher rate. This is beneficial to bacteria because the transferred DNA may carry resistance that could enhance the survival of cells which were previously susceptible to an antibiotic or a disinfectant (van Der Veen & Abee, 2011). Due to these factors, biofilm bacteria are up to several hundred times more resistant to cleaning, sanitizing and antimicrobial agents than planktonic cells (Mretr & Langsrud, 2004). Once biofilms are formed on biotic or abiotic surfaces, eradication or removal can be extremely difficult.

1.4 Slime formation on meat

Slime is formed when bacteria grow as a biofilm on meat and combine with meat exudes. Slime formation leads to organoleptic degradation which leads to consumer rejection of meat. To date, extremely limited research is available on biofilm and slime formation on meat despite the economic losses caused by spoilage pseudomonads. One of the first known studies to explore biofilm formation on meat muscles was undertaken in 1982 (Yada & Skura, 1982). The researchers used scanning electron microscopy (SEM) to image biofilms of *P. fragi* on beef *longissimus dorsi* muscles and observed the degradation of stromal proteins. In another study, *Escherichia coli* was seeded onto the surface of beef and chicken muscles (Mattila & Frost, 1988) and biofilm formation was observed via SEM and Transmission Electron Microscope (TEM). Initially, small bacterial colonies were observed on the meat surfaces, but after 24 hours, bacteria were widely dispersed through the matrix. Subsequent studies of bacterial growth on meat were undetaken by other researchers (Delaquis & McCurdy, 1990). They observed *P.*

fragi and *P. fluorescence* biofilms using Confocal Laser Scanning Microscopy (CLSM) and established that spoilage bacteria grow vigorously on meat surfaces. It was concluded that the slime layer attached to the surface of spoilt, low-temperature stored meat is in fact a biofilm. Another group assessed biofilm formation of meat spoilage pseudomonads at an air-liquid interphase and liquid-solid interphases and they raised the questions as to whether unattached 'floating' biofilm formation is a significantly different colonization strategy compared to air-liquid-solid interphase biofilm formation (Liu et al., 2015)

1.5 Significance of the study

Since a large number of bacterial cells are packed on a small surface area of meat, biofilms can degrade meat tissues much faster than individual planktonic bacteria. Disruption of colonies and biofilms can occur during meat transport and handling which can release fragments of these aggregates containing high numbers of bacteria. Biofilms formed on chilled meat can therefore be more effective sources of cross contamination and colonizing of new habitats than individual bacteria. Furthermore, studies have shown that biofilms can harbor harmful pathogenic bacteria and protect them from disinfectants (Mretr & Langsrud, 2004). These biofilms also protect pathogenic bacteria from sanitation procedures applied in the industry and enhance their survival which can cause concerns for food safety (H. Wang, Ding, Wang, Xu, & Zhou, 2013). Biofilms are extremely difficult to be eradicated with current cleaning in place (CIP) procedures used in the meat industry(van Der Veen & Abee, 2011).

Biofilm formation is a very common phenomenon for many pathogenic and spoilage bacteria in the food industry. Despite the significant losses caused by psychrotropic pseudomonads in the meat industry, surprisingly very limited research has addressed their biofilm formation characteristics.

Pseudomonas fragi is the most dominant bacterial species in aerobically stored chilled meat worldwide (Drosinos & Board 1994) and it is important to identify the characteristics which allow them to thrive in chilled conditions. Despite being a predominant psychrotrophic, meat spoilage organism, currently limited information is available on phenotyphic, metabolic and biofilm formation characteristics of *P. lundensis*.

To date, the vast majority of research on meat spoilage pseudomonads have used broth culture models to study planktonic bacteria. Only limited studies have examined their biofilms and abiotic surfaces, rather than meat, to grow biofilms. The metabolic pathways and metabolic products of microorganism vary substantially based on the environmental conditions they are grown under. The extent of proteolytic activities or other metabolic activities which allow their growth at chilled temperatures cannot be interpreted with relevance to meat industry without s suitable experimental model. It has been found that *P. fragi* do not produce proteolytic enzymes under *in vitro* conditions unless the medium is supplemented with organic nitrogen (Ercolini et al., 2010). It is therefore clear that the outcome of experiments can vary greatly depending on the environmental conditions used. For this reason, it is extremely important to study the biofilm formation of psychrotrophic meat spoilage pseudomonads using experimental models that mimic practical industry conditions.

The main objective of this study is to investigate the biofilm formation characteristics of *P. fragi* and *P. lundenis in situ* on meat under industry applicable conditions. In so doing is was anticipated a greater understanding of how biofilm formation can play a role in the ability of these bacteria to withstand the harsh environmental conditions of chilled temperatures would be gained. In addition, I sought to understand how these biofilms aid the bacteria in overcoming competition from other microorganisms, allowing them to become dominant on chilled meat. Ultimately, the work aimed to suggest potential control mechanisms for these biofilms to reduce spoilage in the meat industry

The main hypothesis of this research is that biofilm formation can aid psychrotrophic pseudomonads to survive chilled temperature conditions used in the meat industry and become the predominant spoilage flora.

1.6 Key Objectives

1. To grow biofilms of selected strains of psychrotropic *P. fragi and P. lundensis* on aerobically stored chilled beef to establish the spatial and cellular arrangement as well as meat degradation pattern within the biofilm.
2. To quantify and characterise the key matrix components of *P. fragi* and *P. lundensis* biofilms when grown on aerobically stored chilled beef.
3. To establish the gene expression profile, and its significance, of *P. fragi* when grown as a biofilm on aerobically stored chilled beef.
4. To apply a novel method to enhance biofilm dispersal and reduce spoilage of *P. fragi* biofilms formed on aerobically stored chilled beef.

1.7 References

Bala, K., Marshall, R. T., Stringer, W. C., & Naumann, H. D. (1978). Effect of endocellular enzymes from *Pseudomonas fragi* on the colour of beef and aqueous beef extract. *Journal of Food Science, 43*(3), 684-688. doi:10.1111/j.1365-2621.1978.tb02393.x

Barraud, N., Kjelleberg, S., & Rice, S. A. (2015). Dispersal from microbial biofilms. *Microbiology spectrum, 3*(6). doi:10.1128/microbiolspec.MB-0015-2014

Carey, D. N., Knut, D., Ned, S. W., & Bonnie, L. B. (2015). Extracellular matrix structure governs invasion resistance in bacterial biofilms. *The ISME Journal, 9*(8). doi:10.1038/ismej.2014.246

Chen, F.-C., Godwin, S., & Kilonzo-Nthenge, A. (2011). Relationship between cleaning practices and microbiological contamination in domestic kitchens. *Food Protection Trends, 31*(11), 672-679.

Coombs, C. E. O., Holman, B. W. B., Friend, M. A., & Hopkins, D. L. (2017). Long-term red meat preservation using chilled and frozen storage combinations: A review. *Meat Science, 125*, 84-94. doi:10.1016/j.meatsci.2016.11.025

Delaquis, P. J., & McCurdy, A. R. (1990). Colonization of beef muscle surfaces by *Pseudomonas fluorescens* and *Pseudomonas fragi*. *Journal of Food Science, 55*(4), 898-902. doi:10.1111/j.1365-2621.1990.tb01560.x

Drosinos, E. H., & Board, R. G. (1994). Metabolic activities of pseudomonads in batch cultures in extract of minced lamb. *The Journal of applied bacteriology, 77*(6), 613. doi:10.1111/j.1365-2672.1994.tb02809.x

Drosinos, E. H., & Board, R. G. (1995). Microbial and physicochemical attributes of minced lamb: sources of contamination with pseudomonads. *Food Microbiology, 12*, 189-197. doi:10.1016/S0740-0020(95)80097-2

Edmund. (2016). Growth of spoilage bacteria during storage and transport of meat. *EFSA Journal, 15*(6), 38. doi:10.2903/j.efsa.2016.4523

Ercolini, D., Casaburi, A., Nasi, A., Ferrocino, I., Di Monaco, R., Ferranti, P.,Villani, F. (2010). Different molecular types of *Pseudomonas fragi* have the same overall behaviour as meat spoilers. *International Journal of Food Microbiology, 142*(1), 120-131. doi:10.1016/j.ijfoodmicro.2010.06.012

Ercolini, D., Russo, F., Blaiotta, G., Pepe, O., Mauriello, G., & Villani, F. (2007). Simultaneous detection of *Pseudomonas fragi*, *P. lundensis*, and *P. putida* from meat by use of a multiplex PCR assay targeting the carA Gene. *Applied and Environmental Microbiology, 73*(7), 2354. doi:10.1128/AEM.02603-06

Ercolini, D., Russo, F., Torrieri, E., Masi, P., & Villani, F. (2006). Changes in the spoilage-related microbiota of beef during refrigerated storage under different packaging conditions. *Applied and Environmental Microbiology, 72*(7), 4663. doi:10.1128/AEM.00468-06

Fagerlind, M. G., Webb, J. S., Barraud, N., McDougald, D., Jansson, A., Nilsson, P., Rice, S. A. (2012). Dynamic modelling of cell death during biofilm development. *Journal of Theoretical Biology295*, 23-36. doi:10.1016/j.jtbi.2011.10.007

Flemming, H.-C., Neu, T. R., & Wozniak, D. J. (2007). The EPS matrix: the "House of biofilm cells". *Journal of bacteriology*, *189*(22), 7945-7947. doi:10.1128/JB.00858-07

Flemming H.C., Wingender J., Szewzyk U., Steinberg P., Rice S.A., Kjelleberg S. (2016). Nature Reviews Microbiology, 14(9), 563. doi:10.1038/nrmicro.2016.94

Garrity, G. M. (2001). *Bergey's manual of systematic bacteriology / George M. Garrity, editor-in-chief* (2nd ed.). New York: New York:Springer

Gill, C. O., & Penney, N. (1977). Penetration of bacteria into meat. *Applied and Environmental Microbiology, 33*(6), 1284. doi:10.1128/AEM.33.6.1284-1286.1977

Gram, L., Ravn, L., Rasch, M., Bruhn, J. B., Christensen, A. B., & Givskov, M. (2002). Food spoilage- interactions between food spoilage bacteria. *International Journal of Food Microbiology, 78*(1), 79-97. doi:10.1016/S0168-1605(02)00233-7

Lal, P., Sharma, D., Pruthi, P., & Pruthi, V. (2010). Exopolysaccharide analysis of biofilm-forming *Candida albicans*. *Journal of Applied Microbiology, 109*(1), 128-136. doi:10.1111/j.1365-2672.2009.04634.x

Lebert, I., Begot, C., & Lebert, A. (1998). Growth of *Pseudomonas fluorescens* and *Pseudomonas fragi* in a meat medium as affected by pH (5.8–7.0), water activity (0.97–1.00) and temperature (7–25°C). *International Journal of Food Microbiology, 39*(1), 53-60. doi:10.1016/S0168-1605(97)00116-5

Liang, Z., Li, W., Yang, S., & Du, P. (2010). Extraction and structural characteristics of extracellular polymeric substances (EPS), pellets in autotrophic nitrifying biofilm and activated sludge. *Chemosphere, 81*(5), 626-632. doi:10.1016/j.chemosphere.2010.03.043

Liu, Y. J., Xie, J., Zhao, L. J., Qian, Y. F., Zhao, Y., & Liu, X. (2015). Biofilm formation characteristics of *Pseudomonas lundensis* Isolated from meat. *Journal of Food Science, 80*(12), M2904-M2910. doi:10.1111/1750-3841.13142

Luyan, M., Matthew, C., Haiping, L., Matthew, R. P., Kenneth, B., & Daniel, J. W. (2009). Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. *PLoS Pathogens, 5*(3), e1000354. doi:10.1371/journal.ppat.1000354

Mann, E. E., & Wozniak, D. J. (2012). *Pseudomonas* biofilm matrix composition and niche biology. *FEMS Microbiology Reviews*, 893-916.doi: 10.1111/j.1574-6976.2011.00322.x

Marvin, W., Bangera, M. G., Roger, E. B., Matthew, R. P., Gail, M. T., Stephen, L., & Greenberg, E. P. (2001). Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature, 413*(6858), 860.doi:10.1038/35101627

Mattila, T., & Frost, A. J. (1988). Colonization of beef and chicken muscle surfaces by *Escherichia coli. Food Microbiology, 5*(4), 219-230. doi:10.1016/0740-0020(88)90021-4

Molin, G., & Ternstrom, A. (1986). Phenotypically based taxonomy of psychrotrophic *Pseudomonas* isolated from spoiled meat, water, and soil. *International Journal of Systematic Bacteriology, 36*(2), 257-274.

Molin, G., Ternstrom, A., & Ursing, J. (1986). *Pseudomonas lundensis*, a new bacterial species isolated from meat. *International Journal of Systematic Bacteriology, 36*(2), 339-342. doi:10.1099/00207713-36-2-339

Mretr, T., & Langsrud, S. (2004). *Listeria monocytogenes* : biofilm formation and persistence in food-processing environments. *Biofilms, 1*(2), 107-121. doi:10.1017/S1479050504001322

Pointon, A., Jenson, I., Jordan, D., Vanderlinde, P., Slade, J., & Sumner, J. (2006). A risk profile of the Australian red meat industry: Approach and management. *Food Control, 17*(9), 712-718. doi:10.1016/j.foodcont.2005.04.008

Robertson, M., Hapca, S., Moshynets, O., & Spiers, A. (2013). Air–liquid interface biofilm formation by psychrotrophic pseudomonads recovered from spoilt meat. *Journal of Microbiology, 103*(1), 251-259. doi:10.1007/s10482-012-9796-x

Schembri, M. A., Kjærgaard, K., & Klemm, P. (2003). Global gene expression in *Escherichia coli* biofilms. *Molecular Microbiology, 48*(1), 253-267. doi:10.1046/j.1365-2958.2003.03432.x

Skandamis, P., & Nychas, G. (2012). Quorum sensing in the context of food microbiology *Applied Environmental Microbiology*(78)5473-5482).

Stanborough, T., Fegan, N., Powell, S. M., Singh, T., Tamplin, M., & Chandry, P. S. (2018). Genomic and metabolic characterization of spoilage-associated *Pseudomonas* species. *International Journal of Food Microbiology, 268*, 61-72. doi:10.1016/j.ijfoodmicro.2018.01.005

Tapas, S., Kumar Patel, G., Dhindwal, S., & Tomar, S. (2011). In Silico sequence analysis and molecular modeling of the three-dimensional structure of DAHP synthase from *Pseudomonas fragi. Computational Chemistry - Life Science -*

Advanced Materials - New Methods, 17(4), 621-631. doi:10.1007/s00894-010-0764-y

Valentini, M., & Filloux, A. (2016). Biofilms and Cyclic di-GMP (c-di-GMP) Signaling: lessons from *Pseudomonas aeruginosa* and other bacteria. *The Journal of biological chemistry, 291*(24), 12547-12555. doi:10.1074/jbc.R115.711507

van Der Veen, S., & Abee, T. (2011). Mixed species biofilms of *Listeria monocytogenes* and *Lactobacillus plantarum* show enhanced resistance to benzalkonium chloride and peracetic acid. *International Journal of Food Microbiology, 144*(3), 421-431. doi:10.1016/j.ijfoodmicro.2010.10.029

Wang, G. Y., Ma, F., Wang, H. H., Xu, X. L., & Zhou, G. H. (2017). Characterization of extracellular polymeric substances produced by *Pseudomonas fragi* under air and modified atmosphere packaging. *Journal of Food Science, 82*(9), 2151-2157. doi:10.1111/1750-3841.13832

Wang, H., Ding, S., Wang, G., Xu, X., & Zhou, G. (2013). *In situ* characterization and analysis of *Salmonella* biofilm formation under meat processing environments using a combined microscopic and spectroscopic approach. *International Journal of Food Microbiology, 167*(3), 293-302. doi:10.1016/j.ijfoodmicro.2013.10.005

Yada, R. Y., & Skura, B. J. (1982). Scanning electron microscope study of *Pseudomonas fragi* on intact and sarcoplasm- depleted bovine longissimus dorsi muscle. *Applied and environmental microbiology, 43*(4), 905.

CHAPTER 02

The predominance of psychrotrophic pseudomonads on aerobically stored chilled red meat

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2.1 Abstract

Microbial spoilage of meat during chilled aerobic storage causes significant financial losses to the industry. Even with modern day preservation techniques, spoilage remains an unsolved problem. Spoilage of meat is a complex process that involves the activity of endogenous enzymes and microorganisms. Psychrotrophic *Pseudomonas* species are the key microorganisms that cause spoilage in aerobically stored chilled meat. Spoilage pseudomonads are highly robust and able to withstand stressful environmental conditions that would otherwise inhibit the growth of other spoilage organisms. In order to implement efficient control measures, and to minimize spoilage, a thorough understanding of the characteristics of spoilage pseudomonads is essential. This review focuses on the spoilage process and the key metabolic attributes of the main psychrotrophic spoilage *Pseudomonas* species to explain their predominance on meat over other psychrotrophic bacteria. This review also highlights less studied, but important, characteristics of psychrotrophic pseudomonads such as biofilm formation and quorum sensing in the context of meat spoilage. The importance of the use of model systems that are closely applicable to the food industry is also discussed in detail.

2.2 Introduction

Meat is a rich source of nutrients, including proteins, vitamins, iron, and other minerals, and has high water activity, which makes it a highly perishable food commodity (Doulgeraki, Ercolini, Villani, & Nychas, 2012). The industry uses a number of methods to extend the shelf life of meat including freezing to subzero temperatures, chilling, vacuum, and modified atmosphere packaging, canning, curing, and drying (EFSA Panel on Biological Hazards (BIOHAZ), 2016; Nychas, Skandamis, Tassou, & Koutsoumanis, 2008). Modified atmosphere packaged (MAP) and cured meat products have longer shelf-life compared to aerobically stored meat (Ercolini, Russo, Torrieri, Masi, & Villani, 2006). Lactic acid bacteria can still cause spoilage even under MAP conditions and some *Pseudomonas* species have been isolated from MAP meat (Wang et al., 2017). The high CO_2 content associated with MAP lowers the meat pH causing muscle deterioration and water loss (Doulgeraki et al., 2012; Ercolini et al., 2006; Skandamis & Nychas, 2012). MAP is therefore not an entirely effective solution for the preservation of meat. Consumer demand for safe, minimally processed meat products with high nutritive and functional properties is increasing. For commercial reasons, it is important to focus on ways to extend the shelf-life of meat with minimal additives, curing agents and chemical preservatives. Much scientific attention is directed at improving the long-term storage of minimally processed refrigerated meat. The spoilage potential of refrigerated meat can vary depending on the muscle pH, water activity, storage temperature, oxygen availability, and initial number of the microbial population present on meat (Doulgeraki et al., 2012).

2.3 Spoilage characteristics of aerobically stored meat

Spoilage is a complex process that can occur due to the activities of indigenous enzymes and the existing microflora of meat. The pH of the muscle decreases after slaughter, and when it reaches below pH 5, the activity of indigenous enzymes ceases. For this reason, the contribution of indigenous enzymes post-slaughter is negligible compared to spoilage caused by microorganisms (Tsigarida & Nychas, 2001). Pre- and post-slaughter stress can increase spoilage activities, and during spoilage, the microorganisms produce various enzymes, which preferentially act upon different proteins, fats and carbohydrates of the muscle.

Spoilage characteristics become evident after microorganisms have exhausted the glucose and lactate present in meat and begin to utilize the proteins (Doulgeraki et al., 2012). During enzymatic breakdown of amino acids, volatiles with sensory significance such as ethyl and methyl esters, fatty acids, amines as well as sulfur-containing compounds, such as methyl sulfide and dimethyl sulfides, are released (Ercolini et al., 2010). Often multiple signs of spoilage, such as off-odors, discolorations, and slime formation, become detectable when the microbial populations reach 10^7 to 10^8 colony forming unit (CFU) cm^{-2} (Dainty, Edwards, Hibbard, & Marnewick, 1989; Gram et al., 2002; Nychas et al., 2008). The consequent release of undesired metabolites leads to organoleptic degradation and product rejection by consumers.

Production of volatile organic compounds (VOCs) is the first sign of organoleptic degradation in meat spoilage. The most common VOCs released during spoilage of raw meat are alcohols, aldehydes, ketones, fatty acids, esters, and sulfur compounds (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015). Production of extensive amounts of methyl acetate and ethyl acetate was apparent when *Pseudomonas* species are grown on a meat paste under aerobic, chilled conditions (Stanborough et al., 2018). In naturally spoilt meat, more than one bacterial strain of the same species as well as multiple bacterial species coexist. The volatile profile is therefore likely to vary based on the abundance of microbial species and strains in that particular sample.

The types of physical and chemical changes during spoilage and the rate of spoilage can vary depending on the microbial flora present on meat and meat product composition. A study by Ercolini, Russo, Nasi, Ferranti, and Villani (2009) found that specific molecules were identified in the volatile profile only when the meat was contaminated by a specific microbial community. The spoilage potential of a microorganism is the ability of a pure culture to produce metabolites that are associated with the spoilage of a particular product (Gram et al., 2002). The presence of *Pseudomonas* species leads to discolorations and slime formation on meat (Motoyama, Kobayashi, Sasaki, Nomura, & Mitsumoto, 2010), but microbial interactions including antagonism, commensalism, and quorum sensing determine the ultimate spoilage characteristics of a product.

2.4 Post slaughter microflora of meat and the spoilage process

Post-slaughter microbial contamination of meat is unavoidable in practical situations, particularly during mass production in the meat industry. Microorganisms from the animal skin, hide, hooves, ruptured intestines, and fecal matter, as well as the abattoir environment, can easily contaminate meat despite the regulations applied in the meat industry (Doulgeraki et al., 2012). Even though manufacturers practice stringent quality control measures, elevated temperature conditions and their associated impact on microbial growth are common during storage and transportation of meat (Nychas et al., 2008). For example, survey studies have shown that the required maximum temperature limits for refrigerated storage are often not met in practice and temperatures higher than 10 °C are not unusual during transportation (Koutsoumanis, Stamatiou, Skandamis, & Nychas, 2006). During transportation and storage in retail cabinets, temperature fluctuations can significantly affect the growth of microorganisms (Kennedy et al., 2005). Furthermore, minimal temperature control is practiced by the consumer between purchase and final home processing. Unless the storage temperatures are kept below 0 °C, microorganisms on the carcass will multiply rapidly. Small fluctuations in temperature can significantly reduce the time to spoilage as psychrotrophic bacteria rapidly increase their populations (Nychas et al., 2008).

Raw meat is exposed to a diverse community of spoilage and pathogenic microorganisms including Gram-negative and Gram-positive bacteria, and yeasts and molds (Gram et al, 2002). Gram-negative bacteria represent the group with the highest meat spoilage potential (Mohareb et al, 2015) and they can be difficult to control due to their complex cell wall structure (Hyldgaard et al, 2015). Furthermore, the storage conditions affect the composition of the microbial community on meat. High oxygen affinity leads to a high growth rate of aerobic bacteria, which results in rapid catabolism of glucose and lactate (Nychas et al, 2008). An increase in pH and titrimetric acidity as well as a decrease in glucose and lactic acid concentrations are also associated with aerobic spoilage (Koutsoumanis et al, 2006).

Among the spoilage bacteria growing on meat, several bacterial species are only associated with meat, slaughterhouses, and other facilities used for meat processing. They are also known as specific spoilage organisms (SSOs) and their presence is a function of meat composition, processing, preservation, and storage conditions (Gram et al, 2002). *Pseudomonas, Brochothrix , Acinetobacter,* and *Shewanella* are genera commonly isolated from aerobically stored chilled meat, whereas *Enterobacteriaceae* and LAB dominate on vacuum-packed (VP) meat (Nychas et al., 2008; Pennacchia, Ercolini, & Villani, 2011). The survival, growth, and succession of specific spoilage bacteria can be affected by a diversity of eco-physiological factors of the substrate (Gram et al, 2002).

Chilled storage is considered to range between –1.5 and 5 °C (Coombs, Holman, Friend, & Hopkins, 2017) and it reduces the growth rate of all bacteria. The maintenance of the chilled chain condition significantly narrows the diversity of the microflora that can grow and multiply on meat (Doulgeraki et al., 2012; Shaw & Dainty, 1985). During chilled storage, psychrotrophic bacteria, which are best adapted to cold temperatures, will outgrow the mesophilic bacteria and reach high cell densities.
Psychrotrophic *Pseudomonas* species are the most predominant microorganisms found in aerobically stored chilled meat (Ercolini et al., 2007). Despite the presence of other psychrotrophic spoilage organisms, this genus dominates in the long run.

2.5 Dominance of meat spoilage pseudomonads on aerobically stored chilled meat

Pseudomonas is a diverse genus of Gram-negative, rod-shaped, obligatory aerobic, and motile (by one or several polar flagella) bacteria (Garrity, 2001). To date, it is the largest genus of Gram-negative bacteria containing 202 species according to the classification based on analysis of the cellular fatty acids, 16S rRNA, and classical physiological and biochemical tests (Özen & Ussery, 2012). This genus is ubiquitous in nature and commonly found in soil, water, plants, and raw meat.

Psychrotrophic *Pseudomonas* species contribute significantly to spoilage of aerobically stored chilled meat, fish, and dairy products (Dainty et al., 1989; Koutsoumanis et al., 2006; Lebert, Begot, & Lebert, 1998). *Pseudomonas fragi* , *Pseudomonas lundensis* , *Pseudomonas fluorescens* , and *Pseudomonas putida* are the four main *Pseudomonas* spp. responsible for causing meat spoilage during chilled storage (Delaquis, Gariépy, & Montpetit, 1992; Edwards, Dainty, & Hibbard, 1987; Ercolini et al., 2007; Raposo, Pérez, de Faria, Ferrús, & Carrascosa, 2017; Sundheim, Sletten, & Dainty, 1998).

Pseudomonas fragi is a nonpigmented species, whereas *P. lundensis* , *P. fluorescence* , and *P. putida* are pigmented species (Mead & Adams, 1977). The taxonomic classification of some *Pseudomonas* spp. as psychrotrophic is a relatively recent approach (Molin & Ternström, 1982). The study of spoilage pseudomonads has improved significantly after the development of the centrimide–fucidin–cephaloridine (CFC) medium, which can successfully isolate *Pseudomonas* spp. from other organisms in food samples (Mead & Adams, 1977). The CFC media contain the selective agents cephaloridine and fucidin, which inhibit the growth of a range of bacteria as well as most strains of *Acinetobacter* and *Moraxella* spp. (Mead & Adams, 1977).

Studies on the growth of psychrotrophic bacteria on minced beef and pork at low temperature under different pH values have shown that changes in *Pseudomonas* populations are closely followed by sensory changes during storage (Koutsoumanis et al., 2006). The growth of psychrotrophic *Pseudomonas* spp. on meat leads to the development of off-odors and slime production (Raposo et al., 2017).

Volatile compounds produced by *Pseudomonas fragi* and mixed natural floras on beef of normal and high-pH meat consist of a range of alkyl esters and a number of sulfur-containing compounds including dimethyl sulfide (Dainty et al., 1989). Dimethyl sulfide is responsible for the "cabbage-like" odor of spoiled meat (Stanborough et al., 2018).

Spoilage *Pseudomonas* spp. that grow on chilled meat, fish, and in milk have a range of characteristics that aid their survival under low-temperature conditions. These organisms are highly proteolytic, a feature that provides them with better access to nutrients compared to nonproteolytic psychrotrophic bacteria that grow on meat such as *Brochothrix* spp. (Drosinos & Board, 1995; Koutsoumanis et al., 2006).When the substrate is depleted of glucose and lactate, proteolytic bacteria secrete extracellular proteases that break down the connective tissue between muscle fibers and allow the bacteria to penetrate into the muscle tissue (Koutsoumanis et al., 2006). This provides the proteolytic bacteria with a competitive advantage over non-proteolytic bacteria since they have continued access to nutrients.

Early studies have reported that penetration of the meat by proteolytic *Pseudomonas* spp. appears to be caused by lysis of the endomysium rather than the densely packed proteins of the muscle fibers (Bloom, 1968). Some earlier studies done on *P. fragi* growth on porcine muscle using scanning electron microscopy (SEM) have shown that myofibrils were extremely disrupted compared to uninoculated controls (Yada & Skura, 1982). Studies conducted at 37 °C on beef muscle to assess the proteolytic and penetration ability of *P. putida*, *P. fragi*, *Lactobacillus plantarum*, and *Lactobacillus casei* have shown that *P. putida* and *P. fragi* penetrate along the muscular septa of the poultry muscle, whereas *Lactobacillus* spp. did not invade the muscle strips at all (Gupta & Nagamohini, 1992). It is clear that *Pseudomonas* spp. have better penetration of the muscle via proteolysis compared to other spoilage species.

Psychrotrophic spoilage pseudomonads have the ability to secrete cold-active proteases and lipases even at low cell numbers (Haryani, Datta, Elliott, & Deeth, 2003; Merieau, Gugi, Guespin-Michel, & Orange, 1993). Therefore, even with the control measures applied in the industry to limit their growth, these organisms can still cause product deterioration at low cell densities. In a study evaluating proteases extracted from

Pseudomonas spp. isolated from a chicken carcass, it was noted that the proteolytic clearing of the zones on protein agar plates occurred prior to noticeable change in the density of bacterial growth suggesting that the protease synthesis occurs at low cell numbers (Sikes, 1979). In another study evaluating the metalloprotease activity of *P. fluorescence* strain 07A in sterile reconstituted milk, it was demonstrated that the expression of this protease was higher during the lag phase at 4 °C than when during the logarithmic phase at 25 °C (Alves et al., 2018).

Production of the metalloprotease AprX begins at the early stages of bacterial growth cycle at refrigerated temperatures. A study assessing the lipolytic activity of spoilage pseudomonads at low temperatures has shown that the amount of enzyme produced per cell at 5 °C was equal to that produced at 20 °C (Alford & Elliott, 1960). Similar results were reported in a more recent study that assessed the lipase activity of *P. fluorescence* strain SMD 31. This study found that the activity in full strength peptone was greater at 5 °C than at 20 °C (Rajmohan, Dodd, & Waites, 2002). The secreted enzymes can accumulate during refrigerated storage and cause product deterioration even at low bacterial numbers. Furthermore, most of these enzymes can withstand high-heat treatments used in the food industry (Von Neubeck et al., 2015). The residual activity of these spoilage enzymes can cause quality defects prior to the expiry date of the product. This scenario is more of a concern in the dairy industry, for products such as ultra-high temperature (UHT) processed milk or milk powder, than in the meat industry. Studies on proteolytic and lipolytic activity of spoilage pseudomonads have reported an increase in lipase activity during a decline in protease levels (Rajmohan et al., 2002). At the same time, it has been observed that lipase production by psychrotrophic pseudomonads is generally high at temperatures below the optimum growth temperature, whereas protease production is high at slightly above the optimum growth temperature.

The genes encoding protease (*aprX*) and lipase (*lipA*) are encoded in an operon conserved in pseudomonads (Zhang et al, 2019) and located at opposite ends of the same operon (Woods, Burger, Beven, & Beacham, 2001). Several factors, such as temperature, iron content, and quorum sensing, affect the regulation of lipase and peptidase production in psychrotrophic *Pseudomonas* (Machado et al., 2017; Woods et al., 2001). AprX has an

optimum production temperature slightly above the optimum temperature for bacterial growth, whereas LipA has a production temperature below the optimum bacterial growth temperature (Merieau et al., 1993). Along with the expression levels, the levels of secretion can also vary. The differences in the regulation of lipase and protease secretion may likely be related to the proximal and distal locations of *aprX* and *lipA* in the operon (Woods et al., 2001).

Even though *Pseudomonas* are known to cause deterioration of meat products worldwide, not all psychrotrophic meat spoilage *Pseudomonas* have the ability to grow equally on different types of meat. The predominant bacteria in chilled chicken are *P. fragi* and *P. fluorescens* , whereas *P. lundensis* is found more commonly on red meat (Arnaut-Rollier et al., 1999; Arnaut-Rollier, De Zutter, & Van Hoof, 1999). A study characterizing spoilage, associated to *Pseudomonas* species on gilt-head Mediterranean sea bream fish, has shown that *P. lundensis* was the predominant species on fish, while *P. fragi* and *P. putida* were detected less frequently (Tryfinopoulou, Tsakalidou, & Nychas, 2002). Such results show that the growth rate of different *Pseudomonas* spp. can vary based on the nutrient composition of specific muscle types. A study comparing growth of *Pseudomonas* spp. on chilled chicken and pork showed that the growth rate was faster on fresh poultry than on fresh pork but the population maximum that was around 9 to 10 $\log^{10}$ $CFUg^{-1}$ was similar in both meat types (Bruckner, Albrecht, Petersen, & Kreyenschmidt, 2012).

In addition to traits that are common to the *Pseudomonas* species in general, it is important to focus on the attributes of the four key spoilage pseudomonads in detail (Table 1) to better understand their growth on chilled meat

Table 2.1. Beneficial traits of psychrotrophic *Pseudomonas* spp. that likely aid them in becoming the predominant flora in the meat spoilage community

Metabolic trait	Species	Benefit	Reference
Proteolytic ability	*P. fragi*, *P. lundensis*, *P. fluorescens*, *P. putida*	Better access to nutrients compared to non-proteolytic spoilage organisms	(E. H. Drosinos & R. G. Board, 1995a), (Koutsoumanis, Stamatiou, Skandamis, & Nychas, 2006)
The ability to utilize a range of substrates	*P. fragi*, *P. lundensis*, *P. fluorescens*, *P. putida*	Access to a diverse range of nutritional resources	(Singha, Kotoky, & Pandey, 2017) (Adeline, Carol, & Aw, 2009)
The ability to utilize cold active lipases and proteases	*P. fluorescens*, *P. fragi*,	Access to nutrients under temperatures which inhibit competing bacterial species	(Rajmohan, Dodd, & Waites, 2002), (Alquati et al., 2002)
The ability to utilize creatine and creatinine	*P. fragi*	Access to nutrients during substrate depletion	(E. H. Drosinos & R. G. Board, 1995a)
Fast growth under chilled temperatures	*P. fragi*, *P. lundensis*, *P. fluorescens*, *P. putida*	The ability to increase the cell population under stressful environments	(W. Chen et al., 2017)
Shorter lag periods	*P. fragi*	The ability to quickly reach the rapidly multiplying exponential phase	(Lebert et al., 1998)
Extracellular enzyme secretion via 'blebbing' of the outer cell wall	*P. fragi*	The efficient invasion of the substrates	(Dutson, Pearson, Price, Spink, & Tarrant, 1971), (Thompson, Naidu, & Pestka, 1985), (Labadie, 1999)
Xenobiotic efflux and antibiotic resistance	*P. fragi*, *P. fluorescens*	The ability to overcome inhibitory compounds	(De Filippis, La Storia, Villani, & Ercolini, 2019)
Bacteriocin production	*P. fragi*, *P. fluorescens* *P. putida*	Inhibit growth of competing organisms	(Bosch et al., 2006) (Champomier-Verges & Richard, 1994)

Diversity of strains	*P. fragi*	The ability to multiply in diverse environmental conditions	(Doulgeraki, Ercolini, Villani, & Nychas, 2012) (Georgnaras, Kunene, von Holy, & Hastings, 1999)
Trehalose production	*P. fragi* strain 121	Minimizes cell lysis under freezing conditions	(Yanzhen, Yang, Xiangting, & Wei, 2016)
Biofilm formation	*P. fragi*, *P. lundensis*, *P. fluorescens*, *P. putida*	Currently unknown	(G. Y. Wang et al., 2017) (Y. J. Liu et al., 2015) (Mastropaolo, Silby, Nicoll, & Levy, 2012) ("Growth of spoilage bacteria during storage and transport of meat," 2016)
Pathogenic ability	*P. lundensis*, *P. putida*	Currently unknown	(Scales, Erb-Downward, Falkowski, Lipuma, & Huffnagle, 2018) (Bogaerts et al., 2008)

2.5.1 *Pseudomonas lundensis*

Pseudomonas lundensis was initially isolated from refrigerated beef and regarded as the second most prevalent meat-spoilage bacterium (Molin, Ternstrom, & Ursing, 1986). Prior to its identification as a separate species, *P. lundensis* strains may have been misidentified as *P. fluorescens* due to its production of fluorescent pigments, which were at that time used to identify them from non-pigment species such as *P. fragi*. It was further established that *P. lundensis* is closely related to the *P. fragi* and *P. fluorescens* DNA–DNA homology group. These organisms contribute to meat spoilage by producing off-odors and slime during chilled storage. Characterization of the VOC profile of *P. lundensis* on chilled minced beef during spoilage is marked by production of large amounts of 1-undecene, 5-methyl-2-hexanone, and methyl-2-butenoic acid (Stanborough et al., 2018). *Pseudomonas lundensis* cells grown on meat are smaller than cells grown in batch cultures, and a greenish tint on meat can be observed when they are

present (Molin et al., 1986). The greening color is due to its production of pyocyanin pigment. At the same time, higher protease activity was observed in extracted biofilms than in planktonic cultures (Liu et al., 2015). Currently no information is available on the iron acquisition process, means of quorum sensing and matrix composition of *P. lundensis* biofilms formed *in situ*.

This organism can tolerate a wide temperature range between 0 and 33 °C, with 30% of the strains capable of growing at 37 °C (Molin et al., 1986). In addition to its ability to spoil raw meat, this proteolytic bacterium was isolated from human lungs of several patients with cystic fibrosis (Scales, Erb-Downward, Falkowski, Lipuma, & Huffnagle, 2018). These results indicate that this species has the potential to act as a pathogenic organism and more attention should be given to this infrequently studied species.

Biodegradation activity was detected when this bacterium formed large clearing zones on Bushnell Hass agar plates, with the largest diameter observed on plates supplemented with paraffin, followed by mineral oil and gasoline (Adeline, Carol, & Aw, 2009). Such results indicate that *P. lundensis* can also utilize a number of substrates as its carbon source. These results show that, apart from the meat spoilage ability, some strains of this species have the potential to act as bioremediation agents in hydrocarbon-polluted environments.

2.5.2 *Pseudomonas fluorescens*

Pseudomonas fluorescens is another psychrotrophic bacterium responsible for causing meat spoilage (Gonçalves, Piccoli, Peres, & Saúde, 2017). A study done by Zhu et al, 2019, has shown that *P. fluorescens* dominated in mixed species biofilms with *Shewanella baltica*. Biofilm formation and adhesion were higher for mixed and mono-species biofilms at 4 °C than at 30 °C.

Some strains of *P. fluorescens* isolated from petroleum hydrocarbon-contaminated soil have shown the ability to utilize a diverse range of hydrocarbon sources (Barathi & Vasudevan, 2001). *Pseudomonas fluorescens* strains have been recommended for bioremediation of environments contaminated with diesel fuel (Kumar et al., 2014).

Mellor, Bentley, and Dykes (2011) demonstrated that *P. fluorescens* has the ability to degrade fat associated with chicken skin. This attribute is linked to their production of a lipopeptide biosurfactant known as viscosin (Maier, 2003). The ability to produce biosurfactants enhances the bioavailability of water-immiscible substrates, adhesion to surfaces, motility, and antibacterial activity (Mellor et al., 2011).

A study assessing proteolytic and lipolytic activity of *Pseudomonas* spp. isolated from Moroccan sardines demonstrated that *P. fluorescens* had a considerable level of lipolytic activity in fresh, ice-stored, and spoiled product. Rancidity development is a key cause of degradation in Moroccan sardines, and *P. fluorescens* is considered as a SSO of fish (Gennari, Tomaselli, & Cotrona, 1999). Similarly, another study that characterized the phenotypic and genotypic traits of spoilage pseudomonads isolated from poultry fillets at refrigerated temperatures found that *P. fluorescens* produced enzymes with proteolytic, lipolytic, and lecithinase activities, whereas *P. fragi* produced mainly proteolytic enzymes (Morales, Aguirre, Troncoso, & Figueroa, 2016). Strain diversity is another key feature of this species. Amplified fragment length polymorphism studies have shown significant strain diversity in *P. fluorescens* isolated from carcasses at different processing stages and from different environmental sources of a poultry abattoir (Geornaras, Kunene, von Holy, & Hastings, 1999).

Pseudomonas species are known to cause discolorations on meat during spoilage. Studies have found that the rate of discolorations on meat is highest during the log phase of bacterial growth (Robach & Costilow, 1961). It was suggested that aerobic bacteria rapidly consume oxygen in the storage environment and cause metmyoglobin formation. This hypothesis was confirmed in a study where *P. fluorescens* accelerated oxymyoglobin oxidation on beef (Chan, Joo, Faustman, Sun, & Vieth, 1998; Piette & Idziak, 1992).

Pseudomonas spp., and in particular, the fluorescent group, produce a range of antibacterial and antifungal compounds such as antibiotics and cyanide (Ganeshan & Manoj Kumar, 2005). The ability to produce antibacterial compounds that suppress the growth of competing microorganisms is clearly an advantage in becoming the dominant component in an ecological niche. *Pseudomonas fluorescens* strain F18 isolated from fish produced a bacteriocin-like substance, which inhibited the growth of *P. fragi* ATCC 4973 (Champomier-Verges & Richard, 1994). This species can also enhance the growth of non-pathogenic and pathogenic bacteria by harboring them in preformed biofilms (Eriksson, Di Paola, Pasetti, & Manghi, 1995). The pathogenic bacteria taking refuge in biofilms of other bacterial species can evade many sanitization procedures applied in the food industry. Recent work done by Liu et al. (2018) has found that the RpoS sigma factor plays a crucial role in survival of *P. fluorescens* under stressful environmental conditions including carbon starvation, heat, and low-dose ethanol exposure. The RpoS sigma factor was related to food spoilage by regulating extracellular protease secretion and total volatile basic nitrogen in *P. fluorescens* when grown in sterilized salmon juice.

2.5.3 *Pseudomonas putida*

Pseudomonas putida is predominantly found in environmental niches such as soil, freshwater, and on the surfaces of living organisms. *Pseudomonas putida* is another bacterium known to proliferate on meat stored at low temperatures (Bruckner et al., 2012; Hyldgaard et al., 2015). It is also known as the predominant bacterium on poultry meat (Bruckner et al., 2012). In common with the rest of the spoilage pseudomonads, the majority of *P. putida* strains isolated from different environmental niches are able to metabolize a wide range of biogenic and xenobiotic compounds. Seeding of naturally present *P. putida* in contaminated waters can be used as a potential tool in bioremediation of oil spills (Raghavan & Vivekanandan, 1999). *Pseudomonas putida* was considered as a mildly virulent pathogen causing bacteremia in immunocompromised patients (Bouallègue et al., 2004). However, more recent studies have reported the emergence of multi-drug and carbapenem-resistant *P. putida* strains, which highlights the importance of this spoilage organism as a potential pathogen (Bogaerts et al., 2008; Hardjo, Nawangsih, Moksidy, Kurniawan, & Tjiang, 2015). Studies have also reported the ability of *P.*

putida strain ATCC 12633 and F1.3 isolated from meat to produce bacteriocin-like substances, which can inhibit the growth of *P. fragi* type strain ATCC 4973 and other 10 meat isolates identified as *P. fragi* in iron-depleted media (Champomier-Verges & Richard, 1994).

It was suggested that these inhibitory substances are strong siderophores, which make iron unavailable for sensitive strains. Protease treatment of the supernatant of the inhibitory *P. putida* strains resulted loss of inhibitory potential, which indicates the proteinaceous nature of the substance.

2.5.4 *Pseudomonas fragi*

Among the psychrotrophic pseudomonads, *P. fragi* is well known as the most dominant species on aerobically stored refrigerated meat (Drosinos & Board, 1995; Ercolini et al., 2007; Koutsoumanis et al., 2006; Lebert et al., 1998). It is also the predominant bacterial component on meat of different biological origins including beef, pork, and chicken (Drosinos & Board, 1995; Tarrant, Pearson, Price, & Lechowich, 1971).

Pseudomonas fragi has a fast growth rate under chilled temperature conditions. Its production of spoilage-related off-odors and slime quickly leads to rejection of meat products by consumers (Ercolini et al., 2006). Short-chain fatty acids esters are responsible for the "fruity odor," which is characteristic in meat spoiled by most *P. fragi* strains (Edwards et al., 1987).

Taxonomically *P. fragi* is classified within the *P. fluorescens* rRNA branch and it is closely related to *P. fluorescens* and *P. lundensis* (Arnaut-Rollier et al., 1999). This organism grows at temperatures ranging from 2 to 35 °C (Chen et al., 2017) and survives under the strict chill chain conditions applied during fresh meat production and transportation. Even though *P. fragi* is associated with spoilage of several foods, including refrigerated dairy products and fish, meat may well be its ecological niche for several reasons that are discussed in detail below.

***Enzymatic activity*:** *Pseudomonas fragi* is a highly proteolytic bacterium and its ability to secrete extra-cellular proteases and lipases could be one of the key factors that determine its rapid growth on meat. In an earlier study assessing *P. fragi* growth on rabbit and porcine muscle, it was shown that it causes extensive protein degradation, which results in muscle breakdown (Hasegawa, Pearson, Price, Rampton, & Lechowich, 1970). *Pseudomonas fragi* secreted aldolase, glyceraldehyde phosphate dehydrogenase, lactic dehydrogenase, and creatine kinase in the sarcoplasm of rabbit and porcine muscles, which led to muscle breakdown (Hasegawa et al., 1970). Early electron microscopic images of *P. fragi* growth on porcine muscle have depicted extensive degradation of myofibrils compared to the uninoculated controls (Dutson, Pearson, Price, Spink, & Tarrant, 1971). Authors also reported marked proteolysis where there was a severe loss of material in the H zone and marked disruption of the A band, as well as loss of dense material from the Z line of the porcine skeletal muscle tissue (Dutson et al., 1971; Hasegawa et al., 1970). Enzymatic activity of *P. fragi* has a deleterious effect on the color of meat stored at chilled temperatures. The enzymatic oxidation of myoglobin to metmyoglobin causes browning of the meat (Bala, Marshall, Stringer, & Naumann, 1977, Ercolini, 1978; Ercolini et al., 2010). As the surface growth of *P. fragi* increased on chilled meat, the percentage of myoglobin decreased from 100 % to 0 %, whereas the pH and free fatty acid values increased (Motoyama et al., 2010).

Another interesting observation is that when *P. fragi* cells colonize the muscle tissue, globular protrusions or "bleb"-like structures appear on the outer cell wall surfaces (Dutson et al., 1971; Thompson, Naidu, & Pestka, 1985) (Figure 1). Electron microscopic observations have shown that these structures are covered with multiple layered membranes identical to the cell wall and they form into detached globules that migrate into the muscle mass (Thompson et al., 1985). Studies have also shown that *P. fragi* secretes proteases into these "bleb" structures and it is present there in high concentrations. Secretion of proteases via these structures can help to efficiently hydrolyze the meat proteins and thereby provide a source of amino acids for cellular metabolism (Labadie, 1999; Thompson et al., 1985). Similar "bleb" structures were observed in the human pathogen *Pseudomonas aeruginosa* in which those structures seem to aid in the penetration of the bacterium into the infected tissues (Kadurugamuwa

& Beveridge, 1995) and this could be a mechanism facilitating the efficient invasion of the tissues to be degraded. Some studies have reported that, despite being a highly proteolytic organism, *P. fragi* is unable to excrete proteases when grown *in vitro* (Dutson et al., 1971; Labadie, 1999; Molin & Ternström, 1982). It was assumed that meat proteins or peptides are necessary for the secretion of proteases into the extracellular medium (Labadie, 1999). This shows that even if the bacterial cells have access to sufficient nutrients when grown in broth culture models, the metabolic pathways can vary when grown *in vitro*.

In addition to proteases, some *P. fragi* strains produce cold-active lipases that are active at temperatures around 10 °C. The lipases secreted by the *P. fragi* strain IFO 3458 had large numbers of charged residues and fewer disulfide bridges, which provide better flexibility at low temperatures compared to lipases secreted by mesophilic bacterial species (Alquati et al., 2002).

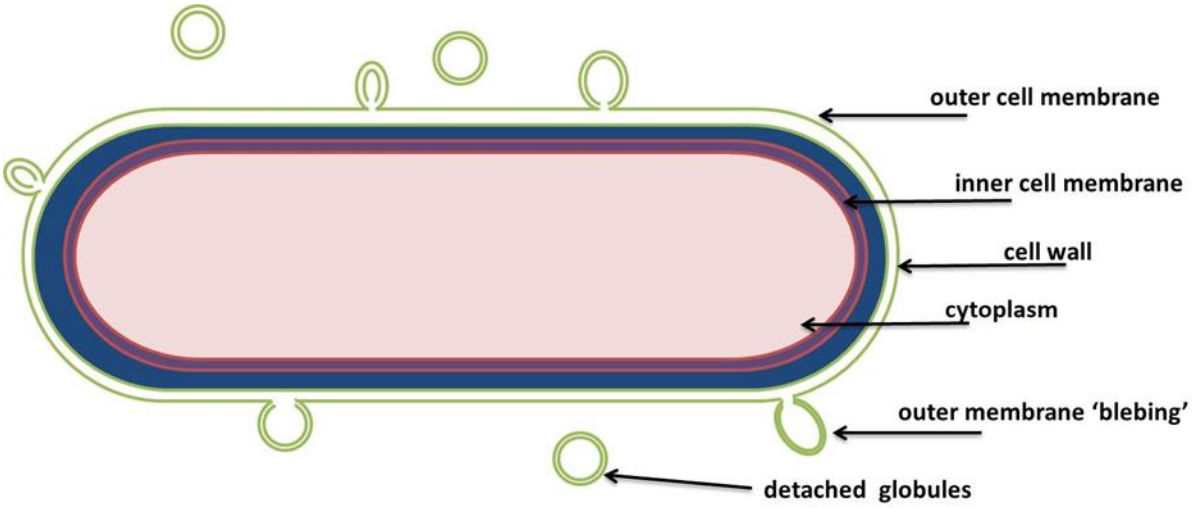

Figure 2.1 Extracellular enzyme secretion by *Pseudomonas fragi* via "bleb"-like protrusions in the outer cell wall surfac

***Substrate utilization*:** Studies on *P. fragi* , *P. fluorescens*, and *P. lundensis* in extracted meat juice models have determined the order of substrate catabolism by these species when stored at 4 °C for several days (Drosinos & Board, 1995). Glucose was catabolized in the early stages of exponential phase and lactate in the latter stages. Among the three species tested, *P. fragi* was the only species that had the ability to utilize creatine and creatinine when the substrates were depleted of glucose and lactate. This metabolic versatility could be one of the reasons *P. fragi* dominate even over other psychrotrophic *Pseudomonas* spp. during the latter stages of chilled storage.

Pseudomonas fragi strain DBC isolated from crude oil-contaminated soil samples in northeast India demonstrated an ability to utilize high-molecular-weight polyaromatic hydrocarbons as the sole carbon source (Singha, Kotoky, & Pandey, 2017). Draft genome sequence analysis has shown that this strain contains genes involved in aromatic hydrocarbon metabolism, biofilm formation, stress adaptation, and quorum sensing. This demonstrated that *P. fragi* strains have the ability to utilize different carbon sources and survive under nutrient-limited environments.

***Iron acquisition*:** The mechanisms used by *P. fragi* for the acquisition of iron were unclear with earlier studies claiming that siderophores, which are used for iron uptake, are not produced by this species (Champomier-Vergès, Stintzi, & Meyer, 1996). Because *P. fragi* is a non-pigmented species that does not produce the green fluorescent pigment pyoverdine and does not grow in the presence of iron chelators, it was assumed that *P. fragi* was unable to produce siderophores. Their siderophore production was tested by growing bacteria on Chrome-Azurol-S medium and then testing the spent-culture supernatant. Siderophores could not be detected using this assay (Champomier-Vergès et al., 1996). According to Labadie (1999), *P. fragi* has the ability to utilize diverse sources of iron present on fresh meat. The majority of *P. fragi* strains are able to utilize complex iron sources such as transferrin, lactoferrin, and hemoglobin, which are naturally present in meat and milk. Studies have found that these organisms are able to remove tightly bound iron present in a succinate medium as insoluble phosphate salts of hydroxides (Ferrocino, Ercolini, Villani, Moorhead, & Griffiths, 2009). It was suggested that *P. fragi* uses siderophores of foreign origin and competes for the siderophores

produced by other bacterial species (Molin & Ternström, 1982). For successful growth, *P. fragi* requires twice as much iron than the pathogenic strain *P. aeruginosa* ATCC 15692 (Champomier-Vergès et al., 1996). It was assumed that the inability to form siderophores by this high-iron requiring species could be the reason that meat became its ecological niche as meat is a rich source of iron (Labadie, 1999).

Recent studies have found that siderophore production does indeed occur in *P. fragi*. Positive results for siderophore production were obtained in experiments conducted for siderophores in plant-growth-promoting bacteria (Farh, Kim, Sukweenadhi, Singh, & Yang, 2017). *Pseudomonas fragi* strain ATCC 4973, which is an environmental isolate from grass lands, produces plant-growth-promoter IAA (Farh et al., 2017). Plant growth promoting bacteria use siderophores to chelate metals, which help plant uptake for metals such as iron, zinc, and copper from the soil while reducing the bioavailability of heavy metals in the rhizosphere. This observation was further confirmed by genomic analysis that identified a conserved gene cluster responsible for siderophore production. *Pseudomonas fragi* strains were found to produce α-hydroxycarboxylate under iron starvation at optimum growth and refrigeration temperatures (Stanborough, Fegan, Powell, Tamplin, & Chandry, 2017).

***Strain diversity*:** The *Pseudomonas fragi* species has a large number of biotypes and the biotype diversity could also affect their ability to grow successfully on meat (Doulgeraki et al., 2012). Because not all strains of the same species have the equivalent spoilage potential, it is quite important to characterize meat spoilage at the strain level. Analyzing the volatile profile of 12 *P. fragi* strains grown on chilled beef paste showed strong differences in the total amounts of VOCs between the isolates. Pangenome analysis showed a high degree of genomic diversity among the tested strains (Stanborough et al., 2018).Even though the genus *Pseudomonas* is considered aerobic, cells are often isolated from VP and MAP food (De Filippis, La Storia, Villani, & Ercolini, 2019). This is due to strain level diversity where different strains suited to specific environmental conditions are able to survive and grow. Metagenomic characterization of spoiled beef has found that different strains of *P. fragi* can be selected based on packaging conditions, and these strains may also produce different metabolites during spoilage. *Pseudomonas*

fragi strains that were prevalent in vacuum packed meat had a lower prevalence of genes involved in oxidative stress and a higher prevalence in proteolysis and amino acid degradation (De Filippis et al., 2019). In contrast, strains present in aerobically stored meat had higher lipolytic potential.

Dainty et al. (1989) stated that despite the significance of *P. fragi* in the predominant spoilage flora on chilled meat, the contribution of VOCs produced by this bacterium was not significant in the profile of spoiled meat samples with high and normal pH. It is important, however, to highlight the fact that this conclusion was made by analyzing only a single strain of *P. fragi* isolated from spoiled fish. More meat isolates should be tested under different storage conditions to confirm this statement. More recent studies have also claimed that despite the proteolytic and lipolytic potential of *in vitro* studies, different molecular types of *P. fragi* play a similar overall role as spoilage agents when grown on meat (Ercolini et al., 2010). In contrast, strong differences were detected in total amounts of VOCs among *P. fragi* strains when grown on meat paste under chilled conditions (Stanborough et al., 2018). Due to these contradictory claims, it is important to further assess the spoilage potential of psychrotrophic pseudomonads at strain level (Casaburi et al., 2015).

The bacterial strain P121, identified as *P. fragi*, is an isolate from Arctic ocean coast soil where the average temperature is below 10 °C. It is a marine bacterium that can grow between 4 and 25 °C in culture medium with added sea salt (Mei, Huang, Liu, He, & Fang, 2016). Studies have found that the survival rate of the P121 strain was far higher than the survival rate of the *P. fragi* type strain ATCC 4973 after the same cold shock exposure. In addition, P121 has the ability to transform maltose to trehalose, an ability that was not detected in the *P. fragi* type strain.

Trehalose is a sugar containing two glucose molecules. Certain bacterial and fungal species produce this compound under freezing conditions (Ohtake & Wang, 2011). Trehalose provides a protective effect and minimizes cell lysis (Mei et al., 2016). P121 also has the ability to provide protection to its cells by detoxifying harmful substances and using aromatic compounds as carbon and nitrogen sources, which is beneficial in surviving very low temperatures (Yanzhen, Yang, Xiangting, & Wei, 2016). These results led to the conclusion that some *P. fragi* strains can tolerate extreme cold and harsh environments.

***Response to competition*:** Extracellular polymeric substances (EPS) extracted from *P. fragi* strains grown on chilled chicken breast were able to reduce biofilm formation of *Serratia* spp. and *P. fluorescence* strains grown in microtiter plates (Bosch et al., 2006). *Pseudomonas fragi* strains may also have the capacity to withstand antibacterial components secreted by competing organisms. Pangenome analysis of *P. fragi* strains isolated from VP beef found a higher prevalence of genes coding for proteins involved in xenobiotic efflux, which is linked with drug and antibiotic resistance (De Filippis et al., 2019). The complete genome sequence of *P. fragi* strain P121 has revealed the existence of gene clusters encoding for bacteriocins and genes that can degrade toxic compounds (Yanzhen et al., 2016). The ability to inhibit the growth of competing organisms as well as to withstand antibacterial components of other species is likely to aid *P. fragi* to become dominant in the long run.

***Adaptation to stress*:** Psychrotrophic bacteria have the ability to carry out metabolic processes at chilled temperatures. Understanding the physiological and molecular basis of growth at low temperature in *Pseudomonas* is therefore important. Although an early initiation of protein synthesis is blocked below 8 °C in mesophilic bacteria, protein synthesis is maintained at 0 °C in *P. fragi* K1 strain isolated from minced beef (Hebraud, Dubois, Potier, & Labadie, 1994). *Pseudomonas fragi* displays a relatively high synthesis of cellular proteins under low temperatures, which is significantly different from mesophilic bacterial species. The level of proteins from 20 *P. fragi* Kl strains were higher at 4 °C than at 30 °C and their relative rate of synthesis increased systematically with a decrease in temperature (Hebraud et al., 1994).

Moderate electric field (MEF) technology is used as a preservation strategy in the food industry. Subjecting *P. fragi* cells to a continuous MEF has shown that the impact of MEF application gradually weakens with time as the cells adapt to the stress by modifying their metabolism and gene expression. Transmission electron microscopy observations have shown that the disrupted cellular membranes of *P. fragi* cells were reestablished rapidly over time. No difference could be observed in MEF-treated cells and control samples over time (Chen et al., 2017). It appears that this organism has the ability to rapidly adapt to stress conditions in the environment, which helps cells to survive for long durations.

It has been observed in several independent investigations that *P. fluorescens* is more abundant than *P. fragi* on fresh meat at the beginning of the slaughter line. The latter species becomes dominant in long-term stored meat (Lebert et al., 1998). *Pseudomonas fluorescence* was prevalent in several environmental samples taken from chillers in which beef carcasses were stored (Gustavsson & Borch, 1993). One explanation could be that meat is considered as the ecological niche of *P. fragi* (Delaquis & McCurdy, 1990), whereas *P. fluorescens* cells are found more often in environmental samples.

Growth rate experiments carried out on minced meat have shown that *P. fragi* strains have shorter lag times than *P. fluorescens* (Lebert et al., 1998). Such results could explain the succession of flora that is observed in long-term stored meat where species with the shorter lag phase reach the rapid multiplying exponential phase and become the predominant flora. It is also important to highlight that these experiments were done using planktonic bacteria in broth culture models and the environmental conditions in a broth cultures are quite different to complex meat muscles.

2.6 *Pseudomonas* biofilms

Psychrotrophic *Pseudomonas* spp. readily form biofilms under the chilled conditions used in the meat industry. Studies have indicated that the slime layer that grows on the surface of low-temperature stored meat during spoilage is in fact a biofilm (Gonçalves et al., 2017; Jay, Vilai, & Hughes, 2003). The layer of slime formed on spoiled meat surfaces is composed of bacterial biofilm and meat exudate. Slime formation is a key spoilage characteristic on aerobically stored meat and generally precedes the production of undesirable odors. Even though much research has been done on the volatile components during meat spoilage, only limited work has addressed formation of biofilms and slime.

A biofilm can be regarded as a large community of bacterial cells attached to a surface or to each other embedded in self-produced and/or acquired EPS (Flemming et al., 2016). Biofilms are formed when free-living planktonic bacteria attach irreversibly to a surface and embed themselves in an extracellular polymeric matrix (Ghannoum, Parsek, Whiteley, & Mukherjee, 2015). When the environmental conditions become unsuitable for the bacteria to survive, the matured biofilms disrupt and release the cells to recolonize new habitats (Baudin, Cinquin, Sclavi, Pareau, & Lopes, 2017). Formation of biofilms is a staple mode of growth for the majority of bacterial and fungal species (Barraud, Kjelleberg, & Rice, 2015).

The biofilm matrix plays a key role in the survival of bacteria under harsh environmental conditions. It shields the residing bacteria from antimicrobial agents, radiation, predation, and desiccation. At the same time, the biofilm matrix immobilizes bacterial cells and keeps them in close proximity, which enhances horizontal gene transfer that aids in the spread of antibiotic resistance (Flemming, Neu, & Wozniak, 2007). The biofilm matrix also contains amino sugar complexes that have been shown to increase along with the spoilage process (Jay et al., 2003). However, the matrix composition of biofilms formed by meat spoilage pseudomonads remains largely unknown.

Studies of attachment of bacteria to different meat surfaces have shown that some species are better able to attach to these surfaces than others. *Pseudomonas* spp. have considerably higher attachment rates compared to other meat spoilage organisms (Firstenberg-Eden, Notermans, & Schothorst, 1978). Because irreversible attachment is the initial step of biofilm formation, this trait could be one of the reasons that make these organisms strong biofilm formers and why high numbers of pseudomonads remain on meat after the rinsing process. Bacterial adhesion to meat and the factors that affect adhesion have been studied in detail by Piette and Idziak (1989) who found that *P. fluorescens* and *Acinetobacter* remained on surfaces after rinsing, whereas the majority of *Lactobacillus* cells were washed away.

One of the earliest observations of biofilm formation was reported on chicken meat with the authors noting that an attached bacterial flora on meat was present in a film of water surrounding the chicken skin (Notermans & Kampelmacher, 1975). SEM images of *P. fragi* growing on meat showed preliminary stages of biofilm formation including irreversible attachment and microcolony formation at chilled temperatures (Yada & Skura, 1982). SEM images obtained of *E. coli* seeded on chicken and beef surfaces at 20 °C showed that the organisms grew as separate colonies and the cells were bound together by a dense structure, now termed as glycocalyx (Mattila & Frost, 1988). It was also noted that the glycocalyx that covers the bacteria acts as a nutrient trap and provides protection to the organism. Storage of the samples for prolonged periods of time under chilled temperatures allowed SEM imaging of different colony morphologies growing on the muscle and that psychrotrophic organisms became the predominant flora (Mattila & Frost, 1988). Different microscopic techniques have been used to study the growth of spoilage bacteria on meat. Acridine-orange-stained biofilm cells of *P. fragi* and *P. fluorescence* grown on beef were imaged with confocal laser scanning microscope (Delaquis et al., 1992). The images showed that microcolonies were formed at the surfaces of myofibers, capillaries, and fat cells. Merging of the microcolonies formed a uniform slime layer on meat. The film thickness increased with time and no evidence was found of preferential colonization of specific micro-niches on meat muscle tissue.

Off-odors can be detected when *Pseudomonas* cell counts reach 10^7 to 10^8 $CFUcm^{-2}$ of tissue surface, and slime formation occurs at levels higher than 10^8 $CFUcm^2$ (Nychas et al., 2008). There are large numbers of cells densely packed within biofilms formed on meat, and disruption of these biofilms can release aggregates of cells, which can be effective in cross contaminating the premises and food products with other planktonic bacteria. In natural environments, bacterial biofilms develop as multicellular communities, and several spoilage organisms contribute to the overall spoilage characteristics. These mixed-species biofilms are highly structured and more robust than single-species biofilms (Lee et al., 2014). Most of the time bacterial strains and species are arranged within mixed species biofilms to obtain maximum utilization of nutrients and studies has found that unculturable, low ubadance microorganism survive in these organizations. Also, studies have shown that Pseudomonas biofilms enhance the survival ability of pathogenic Escherichia coli under low temperature conditions by providing ensuring a higher concentration of nutrients (Sterniša, Klančnik, & Smole Možina, 2019).

Although some studies have investigated slime formation by LAB in vacuum-packaged meats (Dykes, Britz, & Holy, 1994; Korkeala, Suortti, & Mäkelä, 1988), very few studies have addressed biofilm formation by *Pseudomonas* species, which is the most dominant meat spoilage organism on aerobically stored chilled meat. Some studies have hypothesized that the bacteria that bring about the highest level of spoilage are those that play specific roles in biofilm formation (Jay et al., 2003). Psychrotrophic pseudomonads are strong biofilm formers and it is clear that biofilm formation does indeed play a key role in the spoilage process (Figures 2.2 & 2.3). Assessments of biofilm growth at the air–liquid interphase by *P. lundensis* in static microcosms using crystal violet staining have found that the biofilm formation ability of psychrotrophic pseudomonads is higher than the rest of the *Pseudomonas* community (Robertson, Hapca, Moshynets, & Spiers, 2013).

Most biofilm studies use microtiter plates, flow cells, or metal coupons to study biofilm growth. It is important to stress the fact that biofilms are extremely complex structures and their composition can vary greatly based on the growth surface and environmental conditions. Even though the results from biofilms grown under controlled laboratory

conditions and on abiotic surfaces are highly insightful, they do not represent conditions of biofilms growing under complex environmental conditions in nature.

In order to study bacterial slime formation on meat, it is important to study them in their natural habitat that is on the muscle tissue itself. At the same time, it is also important to conduct experiments that simulate conditions applied in the food industry as bacterial metabolic pathways can vary based on their environmental conditions.

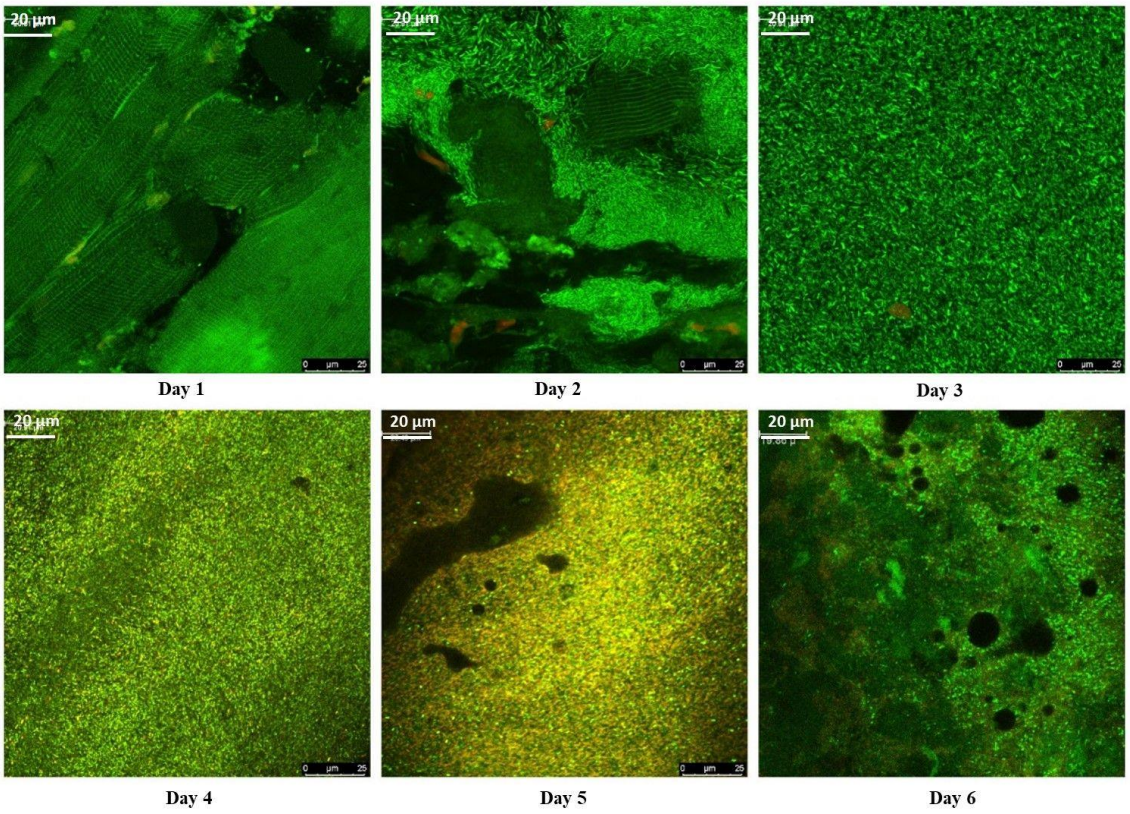

Figure 2.2. Confocal laser scanning micrographs of biofilm formation by *Pseudomonas lundensis* strain, ATCC 49968, on beef skeletal muscle incubated at 10 °C over 6 days. The live cells are stained with SYTO 9 (green), whereas the dead cells are stained with propidium iodide (red). The yellow-colored cells have lost viability and have taken up both red and green stains. The key stages of a biofilms cycle, biofilm initiation, maturation, and dispersal, can clearly be seen

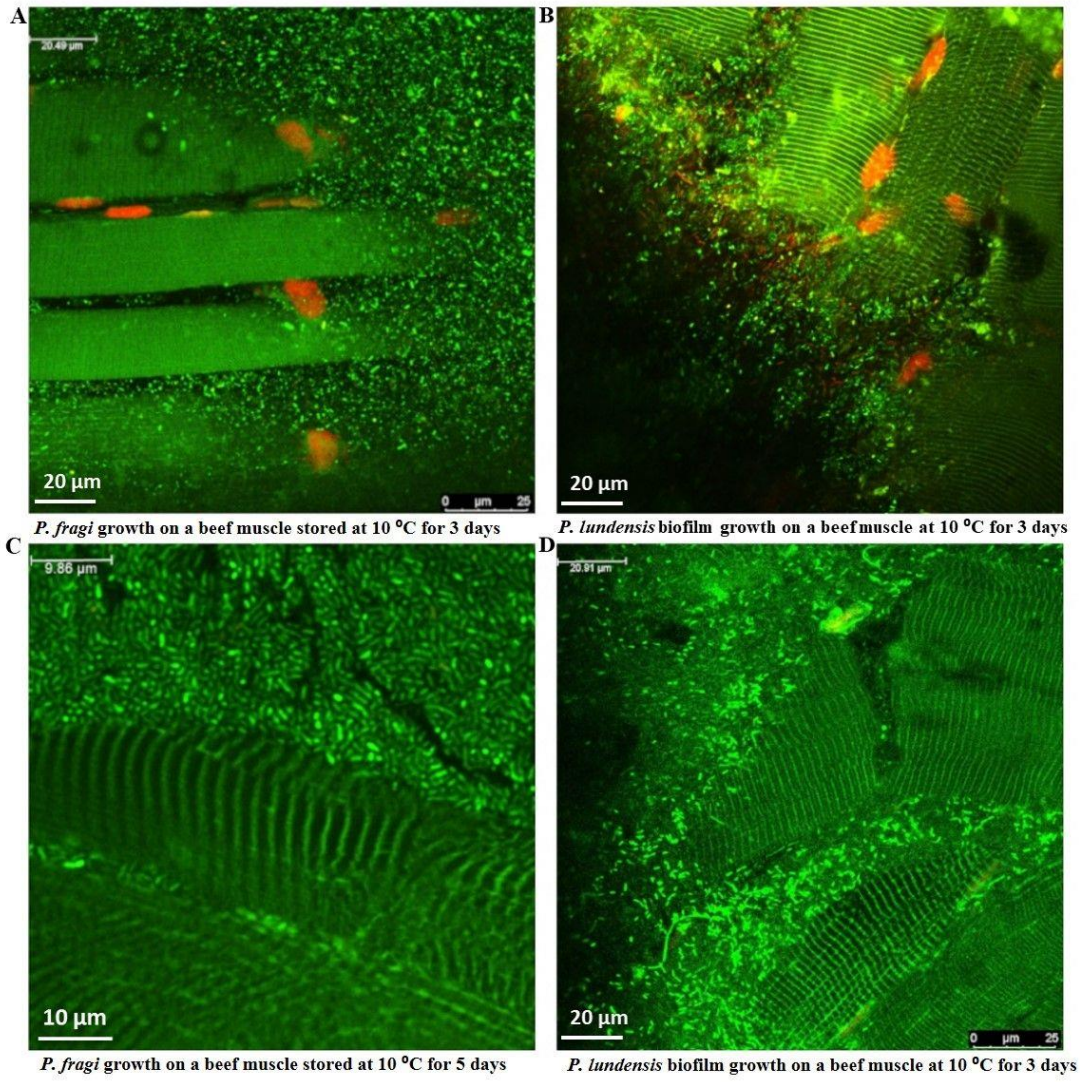

Figure 2.3 Confocal laser scanning micrographs of *Pseudomonas fragi* strains 1832 (A) and 1793 (C), and *Pseudomonas lundensis* strains ATCC 49968 (B) and 1822 (D), grown on beef skeletal muscle at 10 °C. The bacteria are stained with SYTO 9 (green) and propidium iodide (red). Note the nuclei of the meat muscles are also stained in red and beef skeletal muscle is also stained in green

2.7 Quorum Sensing

Quorum sensing (QS) is a mechanism of communication among bacteria. QS can occur within and between bacterial species (Chuan Hao et al., 2015). Small diffusible signaling molecules called autoinducers are used for QS, and these molecules are released by cells to the external environment. QS regulates bacterial gene expression when bacteria live in a large community (Asfahl, Schuster, & Gibbs, 2017; Gobbetti, 2013). When the accumulated signal molecules in the external environment reach a threshold level, all the single-celled bacteria that identify the signaling molecule regulate their gene expression in unison and respond to environmental stimuli as a multicellular organism.

It is important to investigate the role of bacterial communication in food spoilage as it is highly likely that QS plays a role in spoilage . Most Gram-negative bacteria use acyl homoserine lactone (AHL) molecules as their signaling molecule in QS (Asfahl et al., 2017). The role of AHL in food spoilage is currently unknown, but several phenotypic traits including lipolytic, proteolytic, and chitinolytic activities that are potentially involved in spoilage of different foods have been linked to AHL regulation in several bacteria (Nychas et al., 2008). High levels of AHL molecules have been found in a wide range of food products and their concentrations increased as the Gram-negative cell population increased (Gobbetti, 2013).

Pseudomonas psychrophila is taxonomically the closest relative to *P. fragi* and is responsible for spoilage of raw meat and fresh milk stored at refrigeration temperatures (Furmanczyk et al., 2017). In a study assessing the QS regulation of psychrotrophic *P. psychrophila* PSPF19 isolated from refrigerated freshwater fish, it was shown that AHL-signaling molecules increased its proteolytic and lipolytic enzyme production, attachment, and biofilm formation. At the same time, addition of QS inhibitors reduced this strain's biofilm formation and attachment (Bai A & Rai Vittal, 2014). In another study assessing correlation of proteolytic activity by psychrotrophic pseudomonads in chilled meat and AHL signals, it was found that AHL concentration began to increase significantly after onset of proteolysis (Liu, Gray, & Griffiths, 2006).

It has been shown that not all bacterial species and strains use AHL as their signaling molecule. Interestingly, unlike most Gram-negative (Kai & Bonnie, 2016) bacteria *P. fragi* does not appear to produce AHL as its signaling molecule (Ferrocino et al., 2009). When investigating the QS ability of 72 different *P. fragi* strains isolated from fresh and spoiled meat, it was reported that AHL could not be detected by any of the biosensor strains used even when concentrated cells free supernatants were used (Ferrocino et al., 2009). The communication mechanism of key meat spoilage organisms such as *P. fragi* and *P. lundensis* therefore remains unknown.

Some studies were unable to detect AHL molecules in *P. fluorescens* strains isolated from refrigerated raw milk when tested *in vitro* (Martins et al., 2014). It was suggested that the AHL-dependent quorum-sensing system is absent from these strains. In contrast, another study has found that the *P. fluorescence* strain KM120 does indeed produce AHL, and its ability to produce AHL was inhibited when high concentrations of phenolic acid were used (Myszka et al., 2016). Some studies have suggested that QS plays a role in spoilage and slime production (Gram et al., 2002; Skandamis & Nychas, 2012). At the threshold level, QS induces changes in measureable reactions such as pigment production and slime formation. Because the signal molecules have to reach a threshold level, it was hypothesized that QS occurs at least at the biofilm-forming stage (Jay et al., 2003). The detectable AHL concentrations appear on meat when *Pseudomonas* concentrations reach about 10^8 to 10^9 CFU g^{-1}(Liu et al., 2006). It is interesting to note that the measurable spoilage reactions such as pigment production and slime formation are also detectable when the bacterial population reaches around 10^8 CFU g^{-1}.

The potential role of cell-to-cell communication in food spoilage and food safety should be more extensively elucidated. Such information would be helpful in designing approaches for manipulating these communication systems, thereby reducing or preventing spoilage reactions. Understanding QS in food environments may assist in developing quorum-quenching mechanisms or QS inhibitors as food additives or enhancers to prevent bacterial coordination. Such approaches could help prolong the shelf life of perishable food products. Because QS molecules are accumulated in the external environment of the bacteria, it is quite clear that the composition of the external

environment could alter the results of the experiments (Horswill, Stoodley, Stewart, & Parsek, 2007). The signaling molecules are chemical compounds that are released into the food matrix. The rates of its accumulation and degradation are dictated by the structure and composition of the food. The composition of the food matrices is vastly different to the experimental settings that are used in the controlled laboratory settings. Therefore, when studying the role of QS in food spoilage, it is important to design the experimental settings that impersonate the natural food environment.

2.8 Conclusion and perspectives for future research

The evolution of specific *Pseudomonas* spp. to become the dominant flora on chilled meat during storage is a result of metabolic characteristics of specific strains/species, environmental conditions, as well as interactions between other microorganisms in the spoilage community. Based on this review, it is clear that psychrotrophic spoilage *Pseudomonas* spp. are equipped with beneficial characteristics that allow them to withstand harsh environmental conditions and competition.

The research studies done on psychrotrophic pseudomonads so far have shown that their dominance could be attributable to their ability to adapt to stressful environmental conditions that inhibit the growth of most bacterial species. Their efficient mechanisms of iron acquisition, enzyme secretion, strain diversity, and the ability to utilize a range of substrates that are unable to be utilized by other competing species are key factors that assist them in becoming dominant on meat. However, the contribution of biofilm formation and QS to the overall spoilage process, as well as to the predominance of pseudomonads, on chilled meat is yet to be studied in detail. To date, most research has focused on assessing the volatile profile created by pseudomonads on meat to determine their spoilage potential. The assessment of VOC alone is not entirely adequate to determine the spoilage potential of a bacterial species as slime formation and discolorations are also key spoilage characteristics. Biofilm formation is a genetically regulated process, and QS may govern gene expression related to metabolic pathways of spoilage. At this stage, very limited information is available on genetic regulation and

gene expression during biofilm formation on meat by pseudomonads. Little is known about genes regulating biofilm formation, EPS production, and QS of *P. fragi* and even less about *P. lundensis*. Future research should aim to help understand the genetic regulation of the spoilage process. Use of genomic and proteomic approaches should help to elucidate the mechanisms that are activated or repressed during spoilage. Different strains produce distinct spoilage characteristics due to differences in gene the repertoire. Limited research has been done addressing strain specific genomic characteristics of meat spoilage pseudomonads.

It is also important to highlight the conditions of experimental models that have been used. To date, most of the experiments studying spoilage pseudomonads have used broth cultures and microtiter plates. However, the conditions in a well-controlled laboratory environment with abiotic surfaces are vastly different from the complex environment of the meat muscle. Even though the bacterial cells have access to sufficient nutrients when grown in broth culture models, the metabolic pathways can vary when grown *in vitro*. Predictions based on such models are not necessarily valid in complex food environments such as meat. Factors that determine microbial metabolism, such as the structure of the food matrix and interactions between microorganisms, are difficult to be taken into consideration. Use of ground meat has merits but increased spoilage levels can be detected in ground meat compared to solid meat cuts due to the higher surface/weight ratio of the former. In a naturally spoiled meat, different bacterial species grow as a community where interspecies and intraspecies interactions play a key role in their survival. When studying meat spoilage, it is important to use model systems that can accommodate the study of multispecies bacteria and their interactions. Therefore, it is important to design and optimize model systems to study bacterial interactions in natural eco systems that closely resemble conditions used in the meat industry.

2.9 References

Adeline, S. Y. T., Carol, H. C. T., & Aw, C. S. (2009). Hydrocarbon degradation by isolated *Pseudomonas lundensis* UTAR FPE2. *Malaysian Journal of Microbiology, 5*(2), 104-108.

Alquati, C., De Gioia, L., Santarossa, G., Alberghina, L., Fantucci, P., & Lotti, M. (2002). The cold-active lipase of *Pseudomonas fragi. European Journal of Biochemistry, 269*(13), 3321-3328.

Arnaut-Rollier, I., De Zutter, L., & Van Hoof, J. (1999). Identities of the *Pseudomonas* spp. in flora from chilled chicken. *International Journal of Food Microbiology, 48*(2), 87-96.

Arnaut-Rollier, I., Vauterin, L., De Vos, P., Massart, D. L., Devriese, L. A., De Zutter, L., & Van Hoof, J. (1999). A numerical taxonomic study of the *Pseudomonas flora* isolated from poultry meat. *Journal of Applied Microbiology, 87*(1), 15-28.

Asfahl, K. L., Schuster, M., & Gibbs, K. (2017). Social interactions in bacterial cell-cell signaling. *FEMS Microbiology Reviews*, *41*(1), 92-107.

Bai A, J., & Rai Vittal, R. (2014). Quorum sensing regulation and inhibition of exoenzyme production and biofilm formation in the food spoilage bacteria *Pseudomonas psychrophila* PSPF19. *Food Biotechnology, 28*(4), 293-308.

Bala, K., Marshall, R. T., Stringer, W. C., & Naumann, H. D. (1977). Effet of *Pseudomonas fragi* on the color of beef. *Journal of Food Science, 42*(5), 1176-1179.

Bala, K., Marshall, R. T., Stringer, W. C., & Naumann, H. D. (1978). Effect of endocellular enzymes from *Pseudomonas fragi* on the color of beef and aqueous beef extract. *Journal of Food Science, 43*(3), 684-688.

Barathi, S., & Vasudevan, N. (2001). Utilization of petroleum hydrocarbons by *Pseudomonas fluorescens* isolated from a petroleum-contaminated soil. *Environment International, 26*(5), 413-416.

Barraud, N., Kjelleberg, S., & Rice, S. A. (2015). Dispersal from microbial biofilms. *Microbiology spectrum, 3*(6), 1-15. doi:10.1128/microbiolspec.MB-0015-2014.

Baudin, M., Cinquin, B., Sclavi, B., Pareau, D., & Lopes, F. (2017). Understanding the fundamental mechanisms of biofilms development and dispersal: BIAM (Biofilm Intensity and Architecture Measurement), a new tool for studying biofilms as a function of their architecture and fluorescence intensity. *Journal of Microbiological Methods, 140*, 47-57.

Bloom, W. (1968). *A textbook of histology / William Bloom, Don W. Fawcett* (9th ed.). Philadelphia : Saunders.

Bogaerts, P., Huang, T.-D., Rodriguez-Villalobos, H., Bauraing, C., Deplano, A., Struelens, M. J., & Glupczynski, Y. (2008). Nosocomial infections caused by multidrug-resistant *Pseudomonas putida* isolates producing VIM-2 and VIM-4 metallo-β-lactamases. *Journal of Antimicrobial Chemotherapy, 61*(3), 749-751.

Bosch, A., Serra, D., Prieto, C., Schmitt, J., Naumann, D., & Yantorno, O. (2006). Characterization of *Bordetella pertussis* growing as biofilm by chemical analysis and FT- IR spectroscopy. *Applied Microbiology and Biotechnology, 71*(5), 736-747.

Bouallègue, O., Mzoughi, R., Weill, F. X., Mahdhaoui, N., Ben Salem, Y., Sboui, H., . . . Grimont, P. A. D. (2004). Outbreak of *Pseudomonas putida* bacteraemia in a neonatal intensive care unit. *Journal of Hospital Infection, 57*(1), 88-91.

Bruckner, S., Albrecht, A., Petersen, B., & Kreyenschmidt, J. (2012). Characterization and comparison of spoilage processes in fresh pork and poultry. *Journal of Food Quality, 35*(5), 372-382.

Casaburi, A., Piombino, P., Nychas, G.-J., Villani, F., & Ercolini, D. (2015). Bacterial populations and the volatilome associated to meat spoilage. *Food Microbiology,* 45(Pt A), 83-102.

Champomier-Verges, M. C., & Richard, J. (1994). Antibacterial activity among *Pseudomonas* strains of meat origin. *Letters in Applied Microbiology,* 18(1), 18-20.

Champomier-Vergès, M. C., Stintzi, A., & Meyer, J. M. (1996). Acquisition of iron by the non-siderophore-producing *Pseudomonas fragi*. *Microbiology,* 142(5), 1191-1199.

Chan, W., Joo, S., Faustman, C., Sun, Q., Vieth, R. (1998). Effect of *Pseudomonas fluorescens* on beef discoloration and oxymyoglobin oxidation *in vitro*. *Journal of food protection,* 61(10), 1341-6.

Chen, W., Hu, H., Zhang, C., Huang, F., Zhang, D., & Zhang, H. (2017). Adaptation response of *Pseudomonas fragi* on refrigerated solid matrix to a moderate electric field. *BMC Microbiology,* 17(1).

Chuan Hao, T., Kai Shyang, K., Chao, X., Joela, Z., Xiao Hui, T., Guo Ping, L., . . . Staffan, K. (2015). Community quorum sensing signalling and quenching: microbial granular biofilm assembly. *Biofilms and Microbiomes,* 1, 1-9

Coombs, C. E. O., Holman, B. W. B., Friend, M. A., & Hopkins, D. L. (2017). Long-term red meat preservation using chilled and frozen storage combinations: A review. *Meat Science,* 125, 84-94.

Dainty, R. H., Edwards, R. A., Hibbard, C. M., & Marnewick, J. J. (1989). Volatile compounds associated with microbial growth on normal and high pH beef stored at chill temperatures. *Journal of Applied Bacteriology,* 66(4), 281-289.

De Filippis, F., La Storia, A., Villani, F., & Ercolini, D. (2019). Strain-level diversity analysis of *Pseudomonas fragi* after pangenome reconstruction shows distinctive spoilage-associated metabolic traits clearly selected by different storage conditions. *Applied and Environmental Microbiology,* 85(1).

Delaquis, P. J., Gariépy, C., & Montpetit, D. (1992). Confocal scanning laser microscopy of porcine muscle colonized by meat spoilage bacteria. *Food Microbiology, 9*(2), 147-153. doi:10.1016/0740-0020(92)80021-U.

Delaquis, P. J., & McCurdy, A. R. (1990). Colonization of beef muscle surfaces by *Pseudomonas fluorescens* and *Pseudomonas fragi*. *Journal of Food Science* (4), 898-902.

Doulgeraki, A. I., Ercolini, D., Villani, F., & Nychas, G.-J. E. (2012). Spoilage microbiota associated with the storage of raw meat in different conditions. *International Journal of Food Microbiology,* 157(2), 130-141. doi:10.1016/j.ijfoodmicro.2012.05.020.

Drosinos, E. H., & Board, R. G. (1995). Microbial and physicochemical attributes of minced lamb: sources of contamination with pseudomonads. *Food Microbiology*, 12(C), 189-197.

Dutson, T. R., Pearson, A. M., Price, J. F., Spink, G. C., & Tarrant, P. J. (1971). Observations by electron microscopy on pig muscle inoculated and incubated with *Pseudomonas fragi*. *Applied Microbiology*, 22(6), 1152.

Dykes, G. A., Britz, T. J., & Holy, A. (1994). Numerical taxonomy and identification of lactic acid bacteria from spoiled, vacuum-packaged Vienna sausages. *Journal of Applied Bacteriology*, 76(3), 246-252.

Edmund. (2016). Growth of spoilage bacteria during storage and transport of meat. *European Food Safety Authority Journal*, 15(6), 38.

Edwards, R. A., Dainty, R. H., & Hibbard, C. M. (1987). Volatile compounds produced by meat pseudomonads and related reference strains during growth on beef stored in air at chill temperatures. *Journal of Applied Bacteriology*, 62(5), 403-412.

Ercolini, D., Casaburi, A., Nasi, A., Ferrocino, I., Di Monaco, R., Ferranti, P., . . . Villani, F. (2010). Different molecular types of *Pseudomonas fragi* have the same overall behaviour as meat spoilers. *International Journal of Food Microbiology*, 142(1), 120-131.

Ercolini, D., Russo, F., Blaiotta, G., Pepe, O., Mauriello, G., & Villani, F. (2007). Simultaneous detection of *Pseudomonas fragi*, *P. lundensis*, and *P. putida* from meat by use of a multiplex PCR assay targeting the *carA* gene. *Applied and Environmental Microbiology*, 73(7), 2354.

Ercolini, D., Russo, F., Nasi, A., Ferranti, P., & Villani, F. (2009). Mesophilic and psychrotrophic bacteria from meat and their spoilage potential *in vitro* and in beef. *Applied and Environmental Microbiology*, *75*(7), 1990.

Ercolini, D., Russo, F., Torrieri, E., Masi, P., & Villani, F. (2006). Changes in the spoilage-related microbiota of beef during refrigerated storage under different packaging conditions. *Applied and Environmental Microbiology*, 72(7), 4663.

Eriksson, P., Di Paola, G., Pasetti, M., & Manghi, M. (1995). Inhibition enzyme-linked immunosorbent assay for detection of *Pseudomonas fluorescens* on meat surfaces. *Applied and Environmental Microbiology*, 61(1), 397.

Farh, M. E.-A., Kim, Y.-J., Sukweenadhi, J., Singh, P., & Yang, D.-C. (2017). Aluminium resistant, plant growth promoting bacteria induce overexpression of Aluminium stress related genes in *Arabidopsis thaliana* and increase the ginseng tolerance against Aluminium stress. *Microbiological Research,* 200, 45-52.

Ferrocino, I., Ercolini, D., Villani, F., Moorhead, S., & Griffiths, M. (2009). *Pseudomonas fragi* strains isolated from meat do not produce N-acyl homoserine lactones as signal molecules. *Journal of Food Protection,* 72(12), 2597-2601.

Firstenberg-Eden, R., Notermans, S., & Schothorst, M. (1978). Attachment of certain bacterial strains to chicken and beef. *Journal of Food Safety,* 1(3), 217-228.

Flemming, H.-C., Neu, T. R., & Wozniak, D. J. (2007). The EPS matrix: The "House of Biofilm Cells". *Journal of Bacteriology,* 189(22), 7945.

Furmanczyk, E. M., Kaminski, M. A., Spolnik, G., Sojka, M., Danikiewicz, W., Dziembowski, A., . . . Sobczak, A. (2017). Isolation and characterization of *Pseudomonas* spp. Strains that efficiently decompose Sodium dodecyl sulfate. *Frontiers in Microbiology,* 8, 1872.

Ganeshan, G., & Manoj Kumar, A. (2005). *Pseudomonas fluorescens*, a potential bacterial antagonist to control plant diseases. *Journal of Plant Interactions,* 1(3), 123-134.

Garrity, G. M. (2001). *Bergey's manual of systematic bacteriology / George M. Garrity, editor-in-chief* (2nd ed.). New York: Springer.

Gennari, M., Tomaselli, S., Cotrona, V. 1999. The microflora of fresh and spoiled sardines (Sardina pilchardus) caught in Adriatic (Mediterranean) Sea and stored in ice. Food *Microbiology*, 16(1), 15-28.

Georgnaras, I., Kunene, N. F., von Holy, A., & Hastings, J. W. (1999). Amplified fragment length polymorphism fingerprinting of *Pseudomonas* strains from a poultry processing plant. *Applied and Environmental Microbiology,* 65(9), 3828.

Ghannoum, M. A., Parsek, M. R., Whiteley, M., Mukherjee, P. (2015). *Microbial biofilms* (2nd edition.. ed.). Washington, D.C : ASM Press.

Gobbetti, M. (2013). *Bacterial communication in Foods / by Marco Gobbetti, Raffaella Di Cagno*. Boston, MA: Boston, MA : Springer US : Imprint: Springer.

Gonçalves, L. D. D. A., Piccoli, R. H., Peres, A. d. P., & Saúde, A. V. (2017). Predictive modeling of *Pseudomonas fluorescens* growth under different temperature and pH values. *Brazilian Journal of Microbiology*, 48(2), 352-358.

Gram, L., Ravn, L., Rasch, M., Bruhn, J. B., Christensen, A. B., & Givskov, M. (2002). Food spoilage—interactions between food spoilage bacteria. *International Journal of Food Microbiology*, 78(1), 79-97.

Gupta, L., & Nagamohini, Y. (1992). Penetration of poultry meat by *Pseudomonas* and *Lactobacillus* spp. *World Journal of Microbiology and Biotechnology,* 8(2), 212-213.

Gustavsson, P., & Borch, E. (1993). Contamination of beef carcasses by psychrotrophic *Pseudomonas* and *Enterobacteriaceae* at different stages along the processing line. *International Journal of Food Microbiology,* 20(2), 67-83.

Hans-Curt, F., Jost, W., Ulrich, S., Peter, S., Scott, A. R., & Staffan, K. (2016). Biofilms: an emergent form of bacterial life. *Nature Reviews Microbiology, 14*(9), 563. doi:10.1038/nrmicro.2016.94.

Hardjo, L. N., Nawangsih, C., Moksidy, J., Kurniawan, A., Tjiang, M. (2015). Diabetic foot gangrene patient with multi-drug resistant *Pseudomonas putida* infection in Karawaci district, Indonesia. *Journal of Global Infectious Diseases,* 7(1), 37-9.

Haryani, S., Datta, N., Elliott, A., Deeth, H. 2003. Production of proteinases by psychrotrophic bacteria in raw milk stored at low temperature. *Australian Journal of Dairy Technology* 58(1), 15.

Hasegawa, T., Pearson, A. M., Price, J. F., Rampton, J. H., & Lechowich, R. V. (1970). Effect of microbial growth upon sarcoplasmic and urea-soluble proteins from muscle. *Journal of Food Science* (6), 720-724.

Hebraud, M., Dubois, E., Potier, P., & Labadie, J. (1994). Effect of growth temperatures on the protein levels in a psychrotrophic bacterium, *Pseudomonas fragi. The Journal of Bacteriology,* 176(13), 4017.

Horswill, A., Stoodley, P., Stewart, P., & Parsek, M. (2007). The effect of the chemical, biological, and physical environment on quorum sensing in structured microbial communities. *Analytical and Bioanalytical Chemistry,* 387(2), 371-380.

Hyldgaard, M., Meyer, R. L., Peng, M., Hibberd, A. A., Fischer, J., Sigmundsson, A., & Mygind, T. (2015). Binary combination of epsilon-poly-l-lysine and isoeugenol affect progression of spoilage microbiota in fresh turkey meat, and delay onset of spoilage in *Pseudomonas putida* challenged meat. *International Journal of Food Microbiology*, 215(C), 131-142.

Jay, J. M., Vilai, J. P., & Hughes, M. E. (2003). Profile and activity of the bacterial biota of ground beef held from freshness to spoilage at 5–7 °C. *International Journal of Food Microbiology*, 81(2), 105-111.

Kadurugamuwa, J. L., & Beveridge, T. J. (1995). Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. *Journal of Bacteriology*, 177(14), 3998.

Kai, P., & Bonnie, L. B. (2016). Quorum sensing signal–response systems in Gram-negative bacteria. *Nature Reviews Microbiology*, 14(9), 576.

Kennedy, J., Jackson, V., Blair, I. S., McDowell, D. A., Cowan, C., & Bolton, D. J. (2005). Food safety knowledge of consumers and the microbiological and temperature status of their refrigerators. *Journal of Food Protection*, 68(7), 1421-1430.

Korkeala, H., Suortti, T., & Mäkelä, P. (1988). Ropy slime formation in vacuum-packed cooked meat products caused by homofermentative *lactobacilli* and a *Leuconostoc* species. *International Journal of Food Microbiology*, 7(4), 339-347.

Koutsoumanis, K., Stamatiou, A., Skandamis, P., & Nychas, G. J. (2006). Development of a microbial model for the combined effect of temperature and pH on spoilage of ground meat, and validation of the model under dynamic temperature conditions. *Applied and Environmental Microbiology*, 72(1), 124-134.

Kumar, V., Singh, S., Manhas, A., Negi, P., Singla, S., Kaur, P., Upadhyay, N. (2014). Bioremediation of petroleum hydrocarbon by using *Pseudomonas* species isolated from petroleum contaminated soil. *Oriental Journal of Chemistry*, 30(4), 1771-1776.

Labadie, J. (1999). Consequences of packaging on bacterial growth. Meat is an ecological niche. *Meat Science*, 52(3), 299-305.

Lebert, I., Begot, C., & Lebert, A. (1998). Growth of *Pseudomonas fluorescens* and *Pseudomonas fragi* in a meat medium as affected by pH (5.8–7.0), water activity (0.97–1.00) and temperature (7–25 °C). *International Journal of Food Microbiology*, 39(1), 53-60. doi:10.1016/S0168-1605(97)00116-5.

Lee, K. W. K., Periasamy, S., Mukherjee, M., Xie, C., Kjelleberg, S., & Rice, S. A. (2014). Biofilm development and enhanced stress resistance of a model, mixed-species community biofilm. *ISME Journal*, 8(4), 894-907.

Liu, M., Gray, J. M., & Griffiths, M. W. (2006). Occurrence of proteolytic activity and N-acyl-homoserine lactone signals in the spoilage of aerobically chill-stored proteinaceous raw foods. *Journal of Food Protection*, 69(11), 2729-2737.

Liu, X., Ji, L., Wang, X., Li, J., Zhu, J., & Sun, A. (2018). Role of RpoS in stress resistance, quorum sensing and spoilage potential of *Pseudomonas fluorescens*. *International Journal of Food Microbiology*, 270, 31-38.

Liu, Y. J., Xie, J., Zhao, L. J., Qian, Y. F., Zhao, Y., & Liu, X. (2015). Biofilm formation characteristics of *Pseudomonas lundensis* isolated from meat. *Journal of Food Science*, 80(12), 2904-2910.

Machado, S. G., Baglinière, F., Marchand, S., Van, Coillie E., Vanetti, M. C. D., De Block J., Heyndrickx, M. (2017). The biodiversity of the microbiota producing heat-resistant enzymes responsible for spoilage in processed bovine milk and dairy products. *Frontiers in microbiology*, 8, 302.

Maier, R. M. (2003). Biosurfactants: Evolution and diversity in bacteria. *Advances in Applied Microbiology*, 52, 101-121.

Martins, M. L., Pinto, U. M., Riedel, K., Vanetti, M. C., Mantovani, H. C., & de Araújo, E. F. (2014). Lack of AHL-based quorum sensing in *Pseudomonas fluorescens* isolated from milk. *Brazilian Jounal of Microbiology*, 45(3), 1039-1046.

Mattila, T., & Frost, A. J. (1988). Colonization of beef and chicken muscle surfaces by *Escherichia coli. Food Microbiology*, 5(4), 219-230.

Mead, G. C., & Adams, B. W. (1977). A selective medium for the rapid isolation of pseudomonads associated with poultry meat spoilage. *British Poultry Science*, 18(6), 661-670.

Mei, Y.-Z., Huang, P.-W., Liu, Y., He, W., & Fang, W.-W. (2016). Cold stress promoting a psychrotolerant bacterium *Pseudomonas fragi* P121 producing trehaloase. *World Journal of Microbiology and Biotechnology*, 32(8), 1-9.

Mellor, G., Bentley, J., & Dykes, G. (2011). Evidence for a role of biosurfactants produced by *Pseudomonas fluorescens* in the spoilage of fresh aerobically stored chicken meat. *Journal of Food Microbiology,* 28(5),1101-1104.

Merieau, A., Gugi, B., Guespin-Michel, J., Orange, N. 1993. Temperature regulation of lipase secretion by *Pseudomonas fluorescens strain* MFO. *Applied Microbiology and Biotechnology,* 39(1), 104-9.

Mohareb, F., Iriondo, M., Doulgeraki, A. I., Van Hoek, A., Aarts, H., Cauchi, M., & Nychas, G.-J. E. (2015). Identification of meat spoilage gene biomarkers in *Pseudomonas putida* using gene profiling. *Food Control,* 57(C), 152-160.

Molin, G., & Ternström, A. (1982). Numerical taxonomy of psychrotrophic pseudomonads. *Journal of General Microbiology,* 128(6), 1249.

Molin, G., Ternstrom, A., & Ursing, J. (1986). *Pseudomonas lundensis*, a new bacterial species isolated from meat. *International Journal of Systematic Bacteriology*, 36(2), 339-342.

Morales, P. A., Aguirre, J. S., Troncoso, M. R., Figueroa, G. O. 2016. Phenotypic and genotypic characterization of *Pseudomonas* spp. present in spoiled poultry fillets sold in retail settings. LWT 73(C), 609-14.

Motoyama, M., Kobayashi, M., Sasaki, K., Nomura, M., & Mitsumoto, M. (2010). *Pseudomonas* spp. convert metmyoglobin into deoxymyoglobin. *Meat Science*, 84(1), 202-207.

Myszka, K., Schmidt, M. T., Majcher, M., Juzwa, W., Olkowicz, M., & Czaczyk, K. (2016). Inhibition of quorum sensing-related biofilm of *Pseudomonas fluorescens* KM121 by *Thymus vulgare* essential oil and its major bioactive compounds. *International Biodeterioration & Biodegradation,* 114, 252-259.

Notermans, S., & Kampelmacher, E. H. (1975). Further studies on the attachment of bacteria to skin. *British Poultry Science,* 16(5), 487-496.

Nychas, G.-J. E., Skandamis, P. N., Tassou, C. C., & Koutsoumanis, K. P. (2008). Meat spoilage during distribution. *Meat Science,* 78(1), 77-89.

Ohtake, S., & Wang, Y. J. (2011). Trehalose: Current use and future applications. *Journal of Pharmaceutical Sciences,* 100(6), 2020-2053.

Özen, A., & Ussery, D. (2012). Defining the *Pseudomonas* genus: Where do we draw the line with *Azotobacter* ? *Microbial Ecology,* 63(2), 239-248.

Pennacchia, C., Ercolini, D., & Villani, F. (2011). Spoilage-related microbiota associated with chilled beef stored in air or vacuum pack. *Food Microbiology,* 28(1), 84-93.

Piette, J. P., & Idziak, E. S. (1989). New method to study bacterial adhesion to meat. *Applied and Environmental Microbiology, 55*(6), 1531.

Piette, J. P., & Idziak, E. S. (1992). A model study of factors involved in adhesion of *Pseudomonas fluorescens* to meat. *Applied and Environmental Microbiology,* 58(9), 2783.

Raghavan, P. U. M., & Vivekanandan, M. (1999). Bioremediation of oil-spilled sites through seeding of naturally adapted *Pseudomonas putida*. *International Biodeterioration & Biodegradation,* 44(1), 29-32.

Rajmohan, S., Dodd, C. E. R., Waites, W. M. (2002). Enzymes from isolates of *Pseudomonas fluorescens* involved in food spoilage. *Journal of Applied Microbiology*, 93(2), 205-13.

Raposo, A., Pérez, E., de Faria, C. T., Ferrús, M. A., & Carrascosa, C. (2017). *Food spoilage by Pseudomonas spp.-An overview*. John Wiley & Sons, Incorporated, 41-71

Robach, D. L., & Costilow, R. N. (1961). Role of bacteria in the oxidation of myoglobin. *Applied Microbiology, 9*, 529.

Robertson, M., Hapca, S., Moshynets, O., & Spiers, A. (2013). Air–liquid interface biofilm formation by psychrotrophic pseudomonads recovered from spoilt meat. *Journal of Microbiology,* 103(1), 251-259.

Scales, B. S., Erb-Downward, J. R., Falkowski, N. R., Lipuma, J. J., & Huffnagle, G. B. (2018). Genome sequences of 12 strains isolated from the lungs of humans. *Genome Announcements,* 6(7).

Shaw, B., & Dainty, R. (1985). Microbiological and biochemical changes during spoilage of meat. *Journal of the Science of Food and Agriculture,* 36, 123-124.

Singha, L. P., Kotoky, R., & Pandey, P. (2017). Draft genome sequence of *Pseudomonas fragi* strain DBC, which has the ability to degrade high-molecular-weight polyaromatic hydrocarbons. *Genome Announcements, 5*(49).

Skandamis, P., & Nychas, G. (2012). Quorum sensing in the context of food microbiology. *Applied Environmental. Microbiology,* 78, 5473-5482.

Stanborough, T., Fegan, N., Powell, S. M., Singh, T., Tamplin, M., & Chandry, P. S. (2018). Genomic and metabolic characterization of spoilage-associated *Pseudomonas* species. *International Journal of Food Microbiology,* 268, 61-72.

Stanborough, T., Fegan, N., Powell, S. M., Tamplin, M., & Chandry, P. S. (2017). Vibrioferrin production by the food spoilage bacterium *Pseudomonas fragi*. *FEMS Microbiology Letters, 365*(6).

Sterniša, M., Klančnik, A., & Smole Možina, S. (2019). Spoilage *Pseudomonas* biofilm with *Escherichia coli* protection in fish meat at 5 °C. J Sci Food Agric, 99(10), 4635-4641. doi:10.1002/jsfa.9703

Sundheim, G., Sletten, A., & Dainty, R. H. (1998). Identification of pseudomonads from fresh and chill-stored chicken carcasses. *International Journal of Food Microbiology,* 39(3), 185-194.

Tarrant, P. J., Pearson, A. M., Price, J. F., & Lechowich, R. V. (1971). Action of *Pseudomonas fragi* on the proteins of pig muscle. *Applied Microbiology,* 22(2), 224.

Thompson, S. S., Naidu, Y. M., & Pestka, J. J. (1985). Ultrastructural localization of an extracellular protease in *Pseudomonas fragi* by using the peroxidase-antiperoxidase reaction. *Applied and Environmental Microbiology,* 50(4), 1038.

Tryfinopoulou, P., Tsakalidou, E., & Nychas, G.-J. E. (2002). Characterization of *Pseudomonas* spp. associated with spoilage of gilt-head sea bream stored under various conditions. *Applied and Environmental Microbiology, 68*(1), 65-72.

Tsigarida, E., & Nychas, G. J. E. (2001). Ecophysiological attributes of a *Lactobacillus* sp. and a *Pseudomonas* sp. on sterile beef fillets in relation to storage temperature and film permeability. *Journal of Applied Microbiology,* 90(5), 696-705.

Von Neubeck, M., Baur, C., Krewinkel, M., Stoeckel, M., Kranz, B., Stressler, T., Fischer L., Hinrichs J., Scherer S., Wenning M. (2015). Biodiversity of

refrigerated raw milk microbiota and their enzymatic spoilage potential. *International Journal of Food Microbiology*, 211, 57-65.

Wang, G.-y., Li, M., Ma, F., Wang, H.-h., Xu, X.-l., & Zhou, G.-h. (2017). Physicochemical properties of *Pseudomonas fragi* isolates response to modified atmosphere packaging. *FEMS Microbiology Letters*, *364*(11).

Woods, R. G., Burger, M., Beven, C. A., Beacham, I. R. (2001). The *aprX-lipA* operon of *Pseudomonas fluorescens* B52: A molecular analysis of metalloprotease and lipase production. *Microbiology*, 147(2):345-54.

Yada, R. Y., & Skura, B. J. (1982). Scanning electron microscope study of *Pseudomonas fragi* on intact and sarcoplasm-depleted bovine *longissimus dorsi* muscle. *Applied and Environmental Microbiology,* 43(4), 905.

Yanzhen, M., Yang, L., Xiangting, X., & Wei, H. (2016). Complete genome sequence of a bacterium *Pseudomonas fragi* P121, a strain with degradation of toxic compounds. *Journal of Biotechnology,* 224, 68-69.

*Z*hang, C., Bijl, E., Svensson B., Hettinga, K. 2019. The Extracellular Protease AprX from *Pseudomonas* and its Spoilage Potential for UHT Milk: A Review. *Comprehensive Reviews in Food Science and Food Safety*, 1541-4337.

Zhu, J., Yan, Y., Wang, Y., & Qu, D. (2019). Competitive interaction on dual-species biofilm formation by spoilage bacteria, *Shewanella baltica* and *Pseudomonas fluorescens*. Journal of Applied Microbiol, 126(4), 1175-1186. doi:10.1111/jam.14187

CHAPTER 3

In situ characterization of biofilms formed by psychrotrophic meat spoilage pseudomonads

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3.1 Abstract

Psychrotrophic *Pseudomonas* species form biofilms on meat during refrigerated and temperature abuse conditions. Biofilm growth leads to slime formation on meat which is a key organoleptic degradation characteristic. Limited research has been undertaken characterizing biofilms grown on meat during chilled aerobic storage. In this work, biofilms formed by two key meat spoilage organisms, *Pseudomonas fragi* and *Pseudomonas lundensis* were studied *in situ* using five strains from each species. Biofilm structures were studied using confocal microscopic images, cellular arrangement, cell counts and biomass quantifications. This work demonstrated that highly dense, compact biofilms are a characteristic of *P. fragi* strains. *Pseudomonas lundensis* formed biofilms with loosely arranged cells. The cells in *P. fragi* biofilm appear to be vertically oriented whereas this characteristic was absent in *P. lundensis* biofilms formed under identical conditions. Despite the continued access to nutrients, biofilms formed on meat by proteolytic *Pseudomonas* species dispersed after population maximum was reached.

3.2 Introduction

The meat industry uses chilled temperature conditions for storage and transportation of raw meat. Psychrotrophic microorganisms can still survive and multiply under chilled-chain conditions which ultimately lead to spoilage. The key organoleptic degradation characteristics which render meat unacceptable for consumption are off odours, slime formation and discolorations. Even though considerable research has been undertaken characterizing volatile organic compounds and microbial metabolic processes leading to meat spoilage, limited research has been done addressing slime formation (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015; Efsa Panel on Biological, 2016; Ercolini et al., 2006b). Slime formation in vacuum packed meat have been studied to some extent (Duskova, Kamenik, & Karpiskova, 2013; Dykes, Cloete, & Von Holy, 1994). However, surface slime formation on aerobically stored chilled meat has not been studied in detail. Slime is formed when bacteria grow on meat in as biofilms and excrete extracellular polymeric substances (Delaquis, Gariépy, & Montpetit, 1992; Jay, Vilai, & Hughes, 2003). The slime layer is composed of meat exudates, bacterial cells, extra cellular polymeric substances secreted by bacteria and hydrolysed muscle parts.

Biofilms are formed when planktonic bacteria attach to a surface or to themselves and embed themselves in a self-produced or an acquired EPS (Flemming, Wingender, Szewzyk, & SpringerLink (Online service), 2011). It is now believed that the biofilm mode of life is a staple for the majority of bacterial species (Fagerlind et al., 2012). Within a biofilm there are large numbers of bacterial cells packed in a small surface area. Disruption of biofilms during handling of spoiled meat can release aggregates of cells that can cross contaminate the production premises as well as other food products (Giaouris et al., 2014; Silagyi, Kim, Lo, & Wei, 2009). Furthermore, pre-formed biofilms can harbour certain pathogenic microorganisms which can aid them to sanitization treatments (Habimana, Heir, Langsrud, Asli, & Moretro, 2010; Puga, Dahdouh, SanJose, & Orgaz, 2018).

Pseudomonas fragi and *Pseudomonas lundensis* are two of the most abundant organisms found on meat causing spoilage during chilled aerobic storage (P. J. Delaquis & A. R. McCurdy, 1990; Ercolini et al., 2007; Jay et al., 2003). Of the two, *Pseudomonas fragi* is known as the most prominent bacterial species on aerobically stored chilled meat capable of outcompeting the rest of the spoilage *Pseudomonas* species (W. Chen et al., 2017; Ferrocino, Ercolini, Villani, Moorhead, & Griffiths, 2009). Despite the economic losses caused by spoilage, little information is available about *P. lundensis* and their growth on meat. These highly proteolytic psychrotrophic species withstands stressful environmental conditions better than other psychrotrophic spoilage bacteria and become the predominant organisms on meat.

Psychrotrophic spoilage pseudomonads readily form biofilms on meat when subjected to temperatures higher than the recommended refrigerated temperatures, that can occur during handling of meat. Even though much research has been done to comprehend the robustness of psychrotrophic pseudomonads, the role biofilm formation plays in their prominence over other species on meat is currently unknown. A thorough understanding of slime formation is necessary to comprehend the spoilage process as well as the role it plays in the success of psychrotrophic pseudomonads. To date, limited research has been done characterizing biofilm formation on meat using an *in situ* model systems. Little is known about the microstructural features of biofilms formed on the actual muscle surface. In order to fill this knowledge gap, the present research studied the biofilm formation characteristics of *Pseudomonas fragi* and *Pseudomonas lundensis* using an experimental model system that closely mimic the industry conditions. In this work, biofilm formation on chilled beef by selected strains of *P. fragi* and *P. lundensis* were characterised based on the biofilm's growth rate, structural arrangement and transformations on meat with time.

3.3 Materials and methods

***Bacterial strain selection*:** Five strains of *P. fragi* and 5 strains from *P. lundensis* were randomly selected based on a previous study which characterised genomic and metabolic traits of these isolates (Stanborough, Fegan, Powell, Singh, et al., 2018). Bacterial strains 1793,1794,1818,1832 & ATCC 27362 were selected as *P. fragi* strains while strains 1802, 1814, 1817, 1822 and ATCC 49968 were selected as *P. lundensis* strains. *Pseudomonas fragi* type strain 1832 is an environmental isolate and the rest of the strains are meat isolates (Table 3.1).

Table 3.1 A list of bacterial isolates used in this study along with their sources of isolation and level of volatile production

Bacterial Strain	Species	Source	Level of VOC production
1793	*P. fragi*	Beef steak	high
1794	*P. fragi*	Lamb fillets	very low
ATCC 4973/1832	*P. fragi*	Soil around Arctic ocean	low
1818	*P. fragi*	Beef steak	Moderate
ATCC 27362	*P. fragi*	Pork Sausage	No information is available on VOC production.
1801	*P. fragi*	Beef strips	high
1802	*P. lundensis*	Beef strips	high
1814	*P. lundensis*	Beef topside roast	high
1817	*P. lundensis*	Minced beef	low
1822	*P. lundensis*	Minced beef	High, Dimethyl sulphide in notable quantities.
ATCC 49968	*P. lundensis*	Prepacked beef	No information is available on its VOC production

***Meat sterilization*:** The beef 'eye round' cut was used throughout the series of experiments to minimize the variability that can arise from muscle's texture and composition. Fresh meat was purchased from the local butchers on the same morning of each experiment. The meat was cut in to 3 kg portions from large meats cuts at the butchers and was brought as chilled overwraps to the laboratory. The meat was kept at 3

°C in the laboratory and was processed within thirty minutes from the time of purchase. Since the interior tissues of healthy living animals are considered free from microorganisms, a surface sterilization method was applied. The muscle tissue was fully immersed in boiling water for 10 minutes. The drained muscle was placed inside a sterilised bio safety hood and the surface was flame sterilized for 30 seconds. Afterwards, the boiled, burnt exterior of the muscle was aseptically removed. The raw interior was used for sample preparation.

The meat was sliced with a sterilized stainless-steel deli slicer placed inside a bio safety hood to avoid contamination. The slicer was adjusted to obtain meat slices with 3 mm thicknesses which were further sectioned into 15* 15 mm pieces with a sterile scalpel. Each slice was placed separately in wells of a six well microtiter plate. To avoid drying of the muscle, 300 µl of 1X phosphate buffered saline of pH 7.4 was added to each well containing meat.

***Bacterial Cultures*:** Overnight cultures of the selected strains were prepared by inoculating single colony isolates into 5 ml of tryptone soy broth (TSB, Oxoid, Basingstoke, United Kingdom) and incubating at 25 °C for 18 hours in a shaking incubator at 180 rpm. After incubation, cultures were decimally diluted to inoculate the meat slices with approximately 10^3 CFU cm^{-2}. Surface sterilized control samples were treated with 100 µl of TSB. The microtiter plates were closed with the lid and incubated at 4 °C and 10 °C in a static incubator to allow biofilm formation. Temperatures at retail conditions were simulated at 4 °C while poor refrigeration and temperatures higher than recommended retail conditions were simulated at 10 °C. Sterilized uninoculated meat samples were also incubated under identical conditions.

***Biofilm Staining and Imaging*:** Meat samples were imaged on days 3, 5 and 7. At each time point, the samples were prepared for confocal laser scanning microscope (CLSM) imaging using a Leica SP5 (Leica Microsystems, Heidelberg, Germany) microscope. Live/Dead® BacLight™ Kit (L7012, Molecular Probes-Life Technologies, Eugene, OR, USA) was used to label the biofilms. Fluorescent stains were prepared by adding 5 µl of SYTO 9 and 5 µl of propidium iodide (PI) in 1 ml of Milli-Q water. A 100 µl of the

mixed dye mixture was added to each muscle slice. The stained samples were incubated in the dark at room temperature which is around 25 ℃ for 15-20 minutes. Samples were then removed from the microtiter plates using sterile forceps and placed on clean glass slides. A glass cover slip was gently placed on top of each muscle slice and samples were imaged under the 100x oil immersion objective.

Stained meat samples were excited with the 488 nm laser line from Argon laser at 20% intensity. The samples were scanned at a speed of 200 Hz and imaged at 1200 * 1200 resolutions. The emission wavelength was collected between 490-590 nm for SYTO 9 (displayed green) and between 600- 650 nm for PI (displayed red). Stacks of images (Z stacks) were acquired from six different locations on the meat surface which included the center, edges and in between. The biofilms were scanned from top to the muscle surface with a step size of 1 µm. The number of images in each stack varied according to the biofilm thickness.

TOTO 1 and SYTO 60 were used to observe the distribution of extra-cellular DNA (eDNA) in the biofilms (Okshevsky & Meyer, 2014). The stains were prepared by diluting 5 µl SYTO 60 in 1 ml of Milli-Q water and 10 ul of TOTO 1 in 1 ml PBS. A 100 µl of each stain solution was added onto each meat slice and incubated in the dark for 15-20 minutes prior to imaging. Stained samples were imaged with 633 nm and 514 nm laser lines for SYTO 60 and TOTO 1 respectively. The emission bands were collected at 660-680 nm regions for SYTO 60 and at 533-540 nm for TOTO 1.

***Image quantification using COMSTAT*:** The Z stacks were quantified with COMSTAT 2 application (Heydorn et al., 2000). The parameter bio-volume was selected to assess the overall biofilm biomass under the selected time and temperature combinations. Each image stack was separately thresholded manually by a single operator to remove the background noise (Heydorn et al., 2000).

***3D renderings with AVIZO*:** In order to obtain three dimensional views of biofilms to depict the cellular arrangement as well as the extent of muscle and of nuclei degradation, a commercially available visualization application, Avizo (Thermo Fisher Scientific) was used (Baudin, Cinquin, Sclavi, Pareau, & Lopes, 2017; Jin et al., 2017). The Z stacks were used to obtain volume renderings and ortho projections which show spatial and cellular arrangement of the biofilm on the muscle.

***Time Course Assay*:** In order to assess the transformations in biofilm structure and cellular arrangement with time, *P. fragi* and *P. lundensis*, biofilms were grown on meat for six days. To further assess the effects of chilled temperature on biofilm structures as well as on their transformation, chilled (10 °C) and ambient temperatures (25 °C) were selected. The samples were prepared as described previously. On each day from day 1 to day 6, the biofilms were stained with SYTO 9 and PI to and imaged with CLSM. Bacterial cell counts were obtained from biofilms grown on identical samples under identical conditions to validate the CLSM images as described below.

On each day, meat slices were removed from microtitre plates and placed separately inside falcon tubes containing 9 ml of maximum recovery diluent (MRD) (Oxoid, Basingstoke, UK). The corresponding wells of the microtitre plates were washed with 1 ml of MRD and pipetted to the 9 ml with the meat sample. The tube was vortex mixed for one minute and shaken at maximum speed using a mechanical flask shaker (Griffin and Tatlock Ltd, Birmingham, London) for five minutes. Afterwards, biofilms were disrupted with sonication by placing the tubes in an ultrasonic water bath (Ultrasonics. Pty. Australia), for five minutes. The tubes were then vortex mixed for 30 seconds. Tenfold serial dilutions were made and 100 µl of the dilutions were plated on Triptone Soy Agar (TSA, Oxoid, Basingstoke, UK). The plates were incubated at 25 °C for 48 hours. The CFU were determined for each strain at each time and temperature condition. The cell densities of the biofilms were calculated, and the results were expressed as CFU in a logarithmic scale per square centimetre of beef.

***Statistical analysis*:** Three independent biological replicates were performed for bio-volume and CFU number calculations. In addition, when calculating CFU numbers, three technical replicates were used for each strain grown at each time and temperature combination. The statistical significance in bio-volume and CFU counts between strains was compared with one-way ANOVA, using GraphPad® Prism® 7 (San Diego, CA, USA). All tests were performed with a confidence level of 95%.

3.4 Results:

Biofilm growth rate under chilled and temperature abuse conditions: The level of biofilm formation of the selected *Pseudomonas* strains on beef under refrigerated (4 °C) and temperature abuse (10 °C) conditions were assessed by calculating the total bio-volume produced by each strain at selected time and temperature combinations. The bio-volume is defined as the number of biomass pixels in all images of a Z stack multiplied by the voxel size and divided by the substratum area of the of the image stack (Heydorn et al., 2000). Thus, the unit of bio-volume is µm and it is an estimation of the total biofilm biomass of a sample.

The earliest stages of biofilm growth were visible in 3-day old samples incubated at 4 °C. Small aggregates of loosely attached cells to the muscle surface can be observed in CLSM images (Figure 3.1A). The bio-volume calculations clearly show the increase in average biofilm biomass from day 3 to day 7 when stored at 4 °C (Table 3.2). For example, the average bio-volume of *P. fragi* strain 1793 increased from 2.18 µm to 11.47 µm.

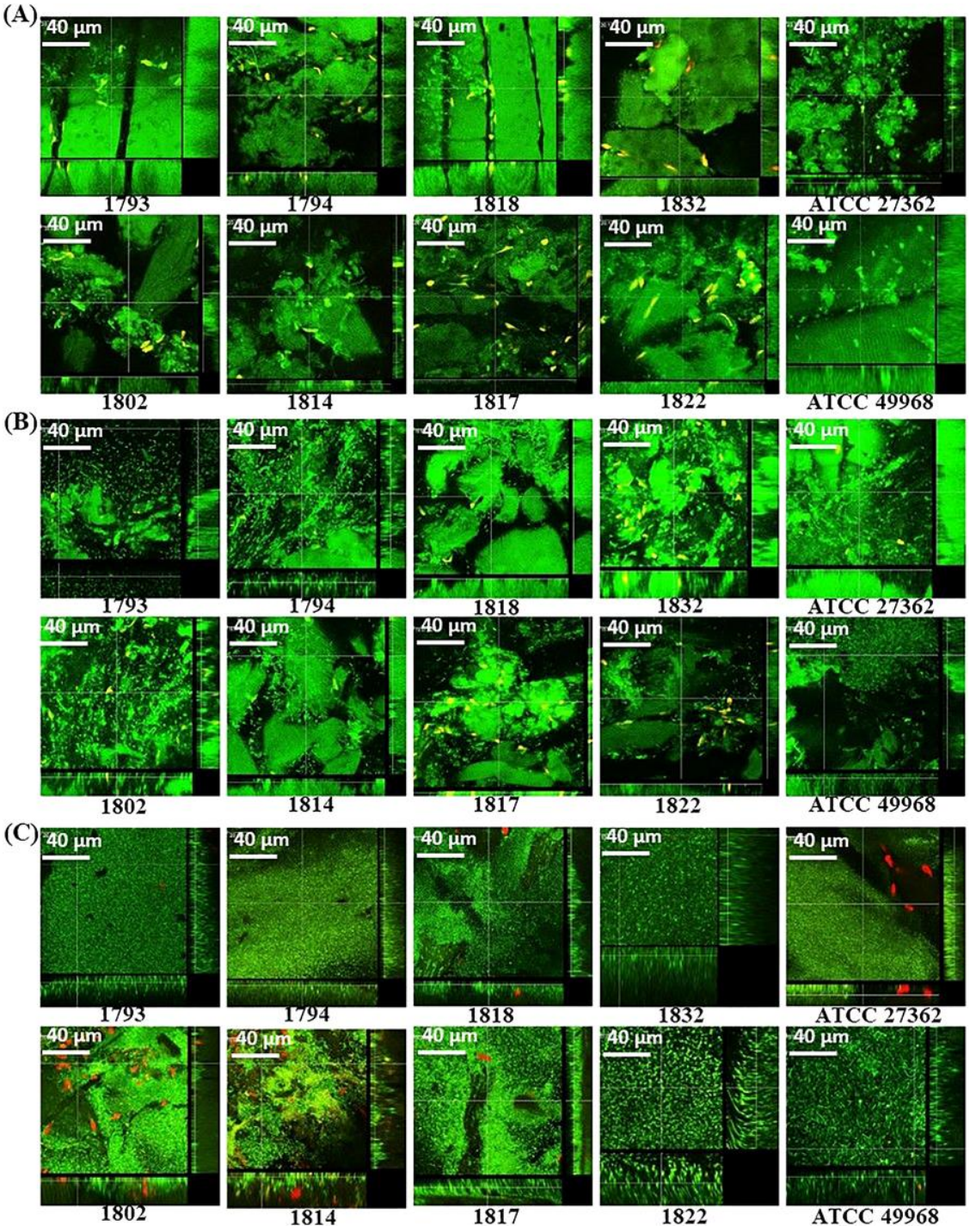

Figure 3.1: CLSM micrographs of biofilms of *P. fragi* (1793, 1794, 1818, 1832, ATCC 49968) and *P. lundensis* (1802, 1814, 1817, 1822, ATCC 49968) grown on beef at 4 °C for days 3 (A), 5 (B) and 7 (C). Biofilms were stained with SYTO 9 (green) and PI (red). Note the nuclei of the meat muscles also strain in red colour. CLSM images were taken under 100x magnification.

Table 3.2: The average bio-volumes of *P. fragi* and *P. lundensis* biofilms grown on meat at 4 °C and 10 °C for days 3, 5 and 7.

Strain	Day 3				Day 5				Day 7			
	4 ^{0}C		10 ^{0}C		4 ^{0}C		10 ^{0}C		4 ^{0}C		10 ^{0}C	
	Mean (µm)	SD	Mean (µm)	SD	Mean (µm)	SD	Mean (µm)	SD	Mean (µm)	SD	Mean (µm)	SD
1793	2.19	1.5	8.1	2.45	2.47	1.99	10.3	5.3	11.47	5.14	3.6	2.81
1794	1.5	1.4	9.2	4.12	2.42	1.61	10.9	5.39	11.52	4.41	4.29	3.05
1818	0.71	0.57	7.73	3.1	2.73	2.31	8.45	5.87	10.43	3.06	7.9	5.12
1832	1.3	0.92	8.34	4.49	2.42	1.78	9.8	5.19	11.11	4.94	5.98	4.39
ATCC 27362	0.97	0.56	7.96	3.05	2.73	2.01	9.3	4.76	11.07	3.47	4.59	2.74
1802	1.44	1.2	6.64	4.6	2.8	2.4	7.71	4.04	8.58	4.86	8.13	6.95
1814	1.16	0.7	7.62	3.75	1.78	1.52	5.79	3.25	9.49	4.98	7.92	5.76
1817	1.05	0.66	8.18	3.98	1.28	0.92	8.3	5.61	7.51	4.3	3.1	1.96
1822	0.9	0.75	7.37	4.78	2.88	2.01	7.2	4.61	10.76	3.95	4.54	2.87
ATCC 49968	1.28	0.74	8.01	4.2	1.9	1.6	6.31	4.59	7.9	5.07	4.48	3.55

Many planktonic bacteria were observed moving between muscle fibres and sometimes attaching to flocs of developing cellular aggregates by day 5 on meat samples incubated at 4 °C (Figure 3.1B), (appendix 1 & 2). By day 7, at 4 °C, dense biofilms can be seen in CLSM Z stacks of strains from both species and biofilms covered most of the muscle surface (Figure 3.1C). At the same time, moving planktonic bacteria could not be observed during this time and temperature combination. At this stage of biofilm growth, the highest average bio-volumes of 11.47 µm and 10.76 µm were calculated for *P. fragi* strain 1794 and *P. lundensis* strain 1822 respectively. As the biofilms spread over the muscle surface, the standard deviations have reduced compared to the average bio-volume (Table 3.2). Statistically significant differences could not be detected in average bio-volumes of any of the time and temperature combination calculated interspecies or intra-species. According to CLSM images and bio-volume calculations (Figure 3.3), the biofilm formation rate was considerably lower at 4 °C (Figure 3.1), than at 10 °C (Figure 3.2). The highest average bio-volume of 3-day old samples grown at 4 °C was 2.19 µm while the highest average bio-volume of 3-day old samples grown at 10 °C was 9.20 µm.

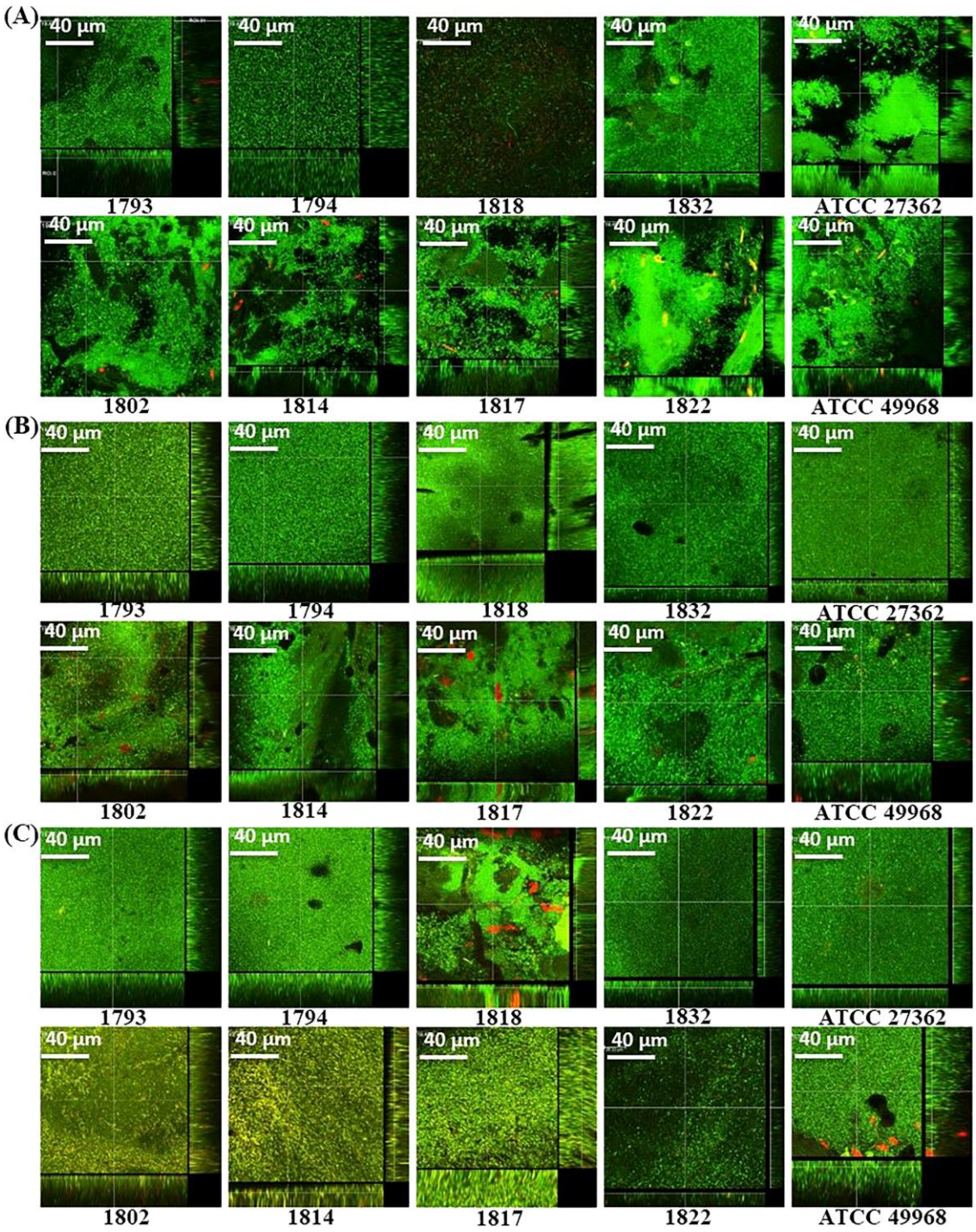

Figure 3.2: CLSM micrographs of biofilms of *P. fragi* (1793, 1794, 1818, 1832, ATCC 49968) and *P. lundensis* (1802, 1814, 1817, 1822, ATCC 49968) grown on beef at 10 °C for days 3 (A), 5 (B) and 7 (C). The biofilms were stained with SYTO 9 (green) and PI (red). Note the nuclei of the meat muscles also strain in red colour. CLSM images were taken under 100x magnification.

All the strains from both the species had formed confluent biofilms by day 3 in samples stored at 10 °C (Figure 3.2A) and the meat surface was still visible among emerging biofilm patches. Moving planktonic bacteria could be observed in some of the samples stored at 10 °C. The majority of cells in 3-day old biofilm grown at 10 °C were stained with the live stain, SYTO 9 (Figure 3.2A). Biofilm thickness increased with time and covered the exposed surfaces by day 5 (Figure 3.2B). No evidence of preferential colonization or specific micro-niches was detected.

As the biofilms continued to grow on the beef tissue, the average bio-volume also increased for strains from both the species grown at 4 °C (Figure 3.3C). However, the bio-volume had reduced by day 7 on biofilms of both the species grown at 10 °C (Figure 3.3F) and biofilm structures appeared distorted (Figure 3.2C). Most of the cells appeared in yellow color which indicates that the cells are taking up both the live and dead stains and are likely to be losing their viability.

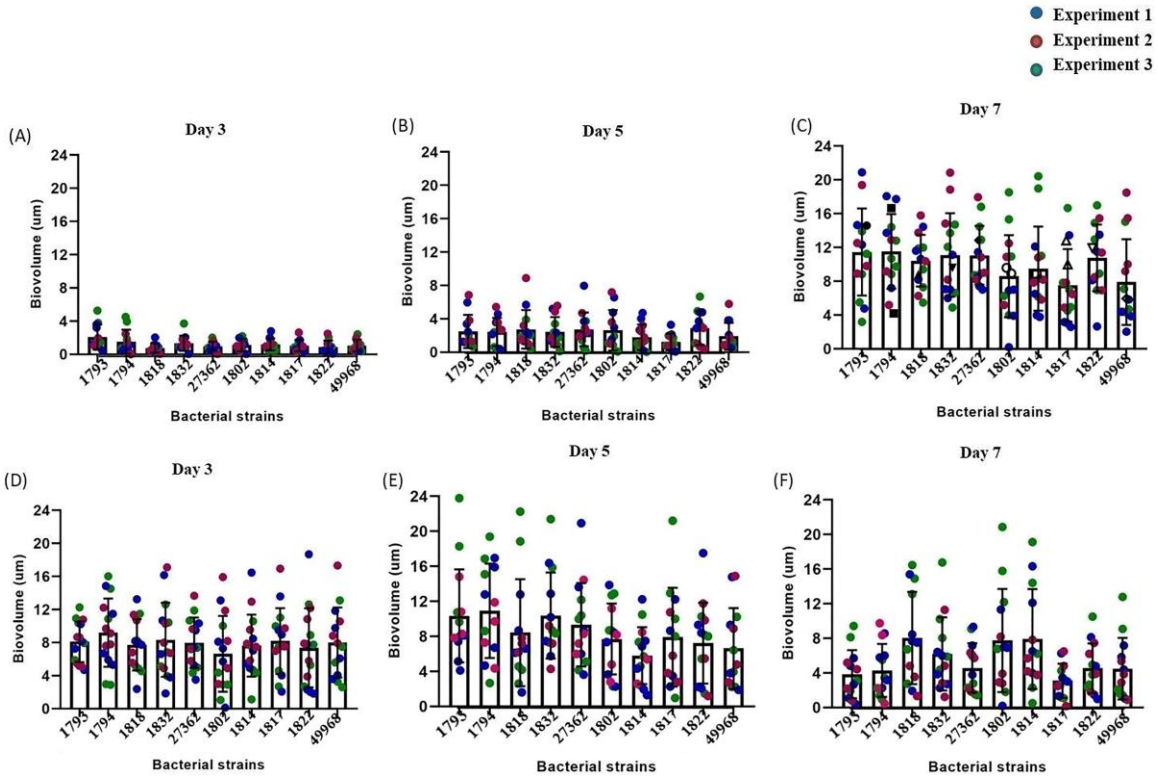

Figure 3.3: The average bio-volumes of *P. fragi* (1793, 1794, 1818, 1832, ATCC 27362) and *P. lundensis* (1802, 1814, 1817, 1822, ATCC 49968) biofilms grown on beef at 4 °C (A), (B) and (C) and at 10 °C (D), (E), (F) for 3, 5 and 7 days. Replicates from independent experiments 1, and 3 are coloured in red, blue and green, respectively. Each strain is represented by 15-18 data points. The error bars represent the variability of the data

Structural characterization of the biofilms: According to the x-y and y-z projections of CLSM Z stacks and ortho projections (Figure 3.4 and 3.5), *P. fragi* strains produced highly dense, flat biofilms. Within the biofilm, the bacterial cells were closely arranged with very limited intercellular gaps (Figures 3.4A-C). Due to its compactness, not much extracellular matrix material could be observed between cells. Also, the cellular arrangement was uniform from the top of the biofilm to the muscle surface with limited internal voids. The cells are more densely packed in inner layers than in upper layers within the biofilm (Figure 3.4A and B).

Due to their uniform growth, *P. fragi* strains produced lawn like biofilms with higher surface coverage of the muscle than *P. lundensis* strains (Figures 3.4A-C). When scanning the biofilms from top to the meat surface, not many meat nuclei are visible in dense *P. fragi* biofilms (Figures 3.5B and C). The CLSM Z stacks (Figure 3.4A-C), have shown that bacterial cells appear to take a round/circular form than rods within dense *P. fragi* biofilms.

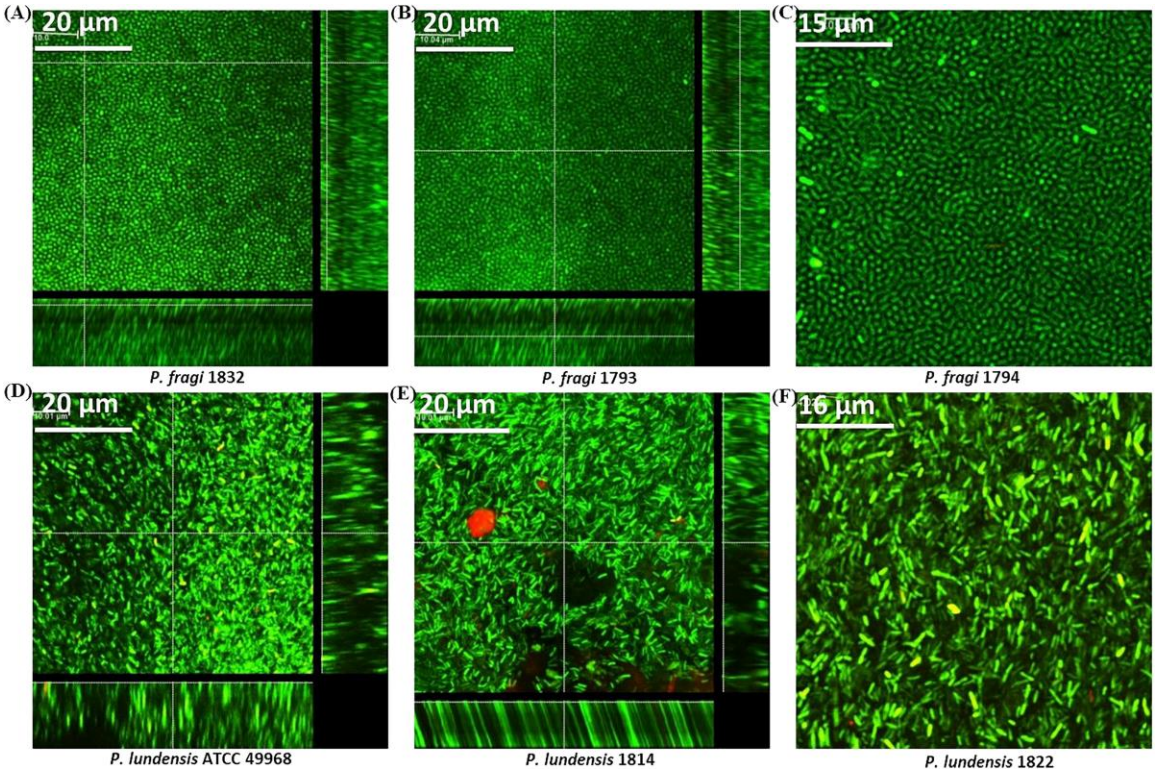

Figure 3.4: Representative CLSM micrographs of biofilms of *P. fragi* 1832 (A), 1793 (B), 1794 (C) and *P. lundensis* 1814 (D), ATCC 49968 (E) and 1822 (F) grown at 10 °C for 5 days. The x-y, x-z and y-z projections of the Z stacks show the cellular arrangement from top and side views respectively.

A greenish tint can be observed in meat slices inoculated with *P. lundensis* strains while this color was absent in samples inoculated with *P. fragi* strains. Also marked differences can be seen in biofilm microstructures of the two species. In contrast to compact biofilms formed by *P. fragi* strains, bacterial arrangement was disorganized in biofilms of *P. lundensis* strains with much intercellular spacing (Figures 3.4D-F). Layers within the biofilm were not uniform and many internal voids can be seen in Z stacks and 3D renderings. More matrix material and meat nuclei can be observed in biofilms produced by *P. lundensis* strains than *P. fragi* strains (Figure 3.4D-F) and (Figure 3.5D-F).

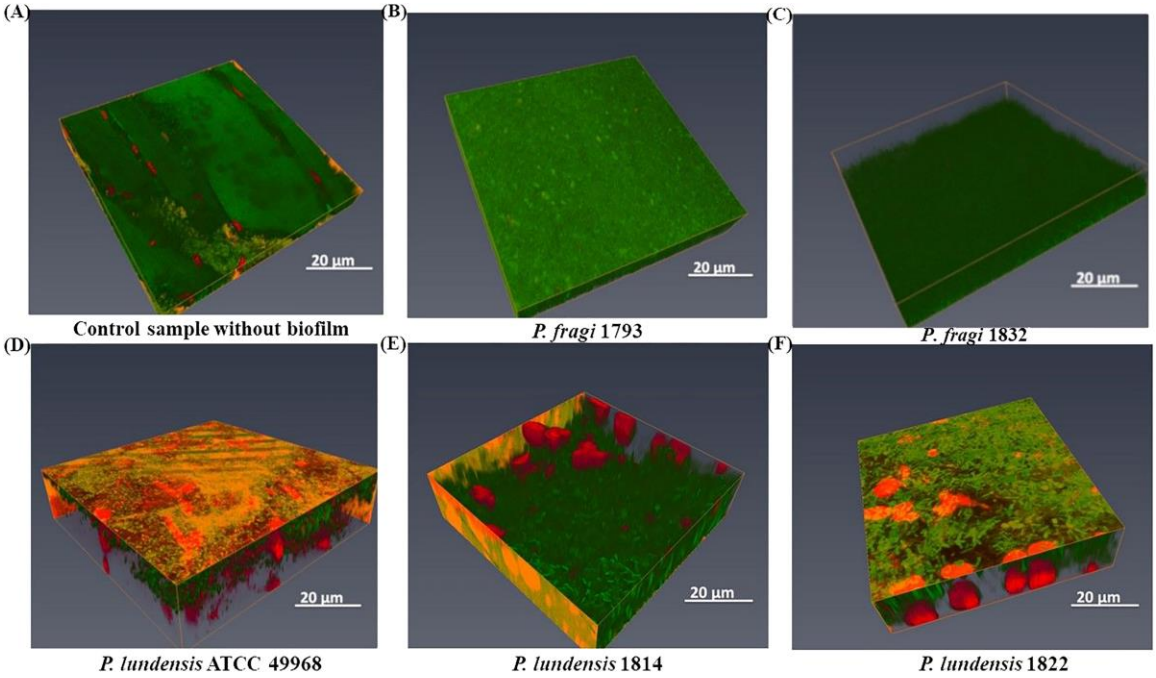

Figure 3.5: Three dimensional renderings obtained from Avizo representing the structural and spatial arrangement of biofilms on the beef muscle. *Pseudomonas fragi* (B), (C) and *P. lundensis* (D), (E), (F) biofilms were grown on meat at 10 °C for 5 days. The control sample without biofilm was also incubated at 10 °C for 5 days. The samples were stained with SYTO 9 (green) and PI (red). Meat nuclei are also stained in red.

A number of meat nuclei were clearly visible in yellow prior to biofilm development (Figure 3.1A). With time, meat nuclei appear in red, stained with PI (Figure 3.1C). Circular and elliptical voids in sizes similar to meat nuclei with clear margins can be

observed within mature biofilms formed by both species (Figure 3.6). Even though the rest of the area is densely packed with large number of bacteria, no cells can be seen within the circular voids (Figure 3.6).

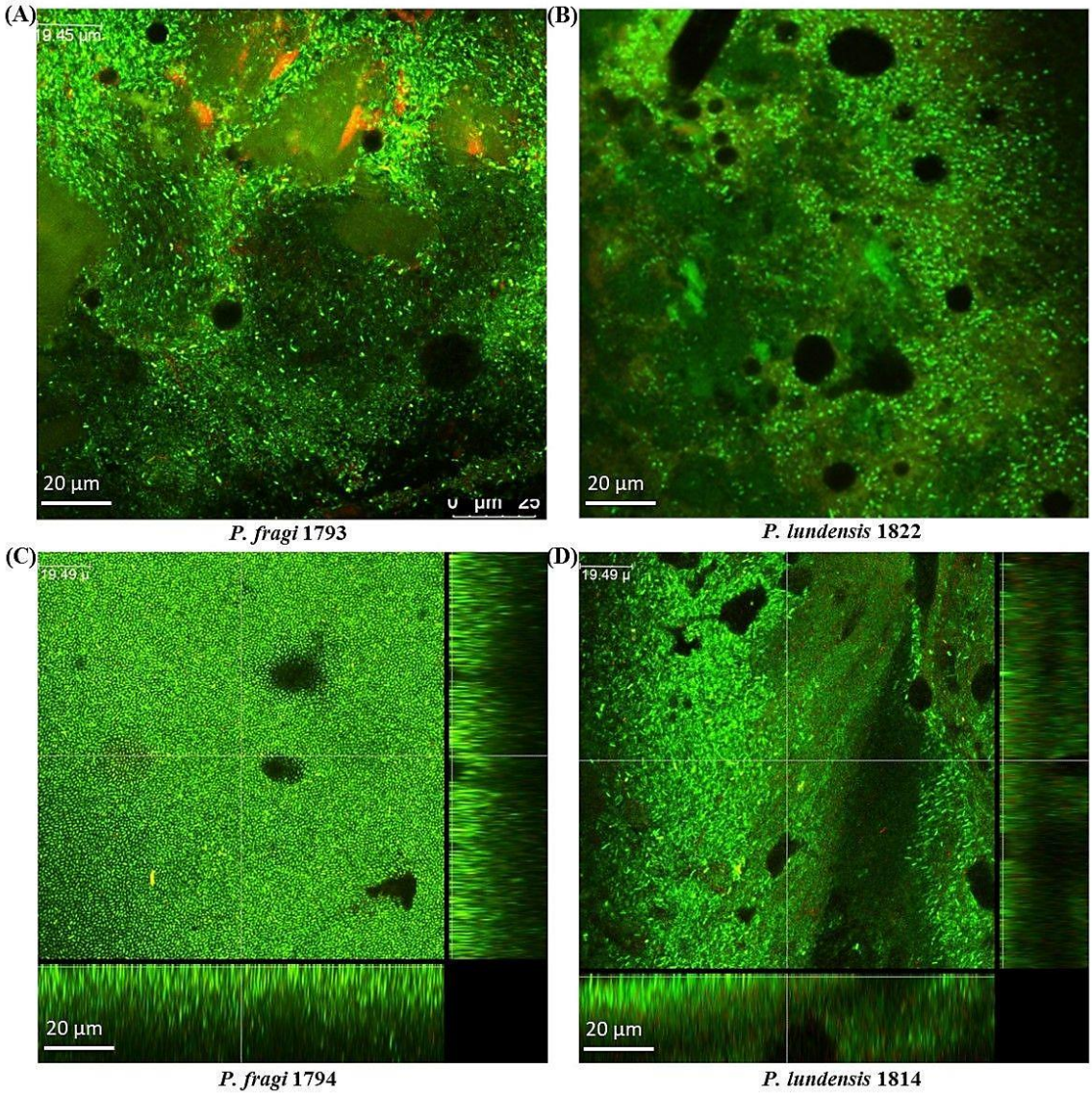

Figure 3.6: Circular voids with clear margins in mature biofilms of *P. fragi* (A), (C) and *P. lundensis* (B), (D). The biofilms are stained with SYTO 9 and PI. The voids appear empty and are not stained with the dyes.

Extra cellular DNA: Extra cellular DNA in the biofilm matrix of the selected strains were visualized by CLSM images of 7-day old biofilms. Day 3 and 5 old biofilms grown at 4 °C were not used for imaging as insufficient biofilm material is produced at those stages. According to the images, more eDNA appear to be present in biofilms produced by *P. lundensis* strains than *P. fragi* strains (Figure 3.7). Limited amount of eDNA can be seen within densely packed *P. fragi* biofilms and all the strains produced eDNA in similar structural arrangement. Much variability was observed in eDNA production between individual replicates and strains of *P. lundensis* biofilms. (Figure 3.7).

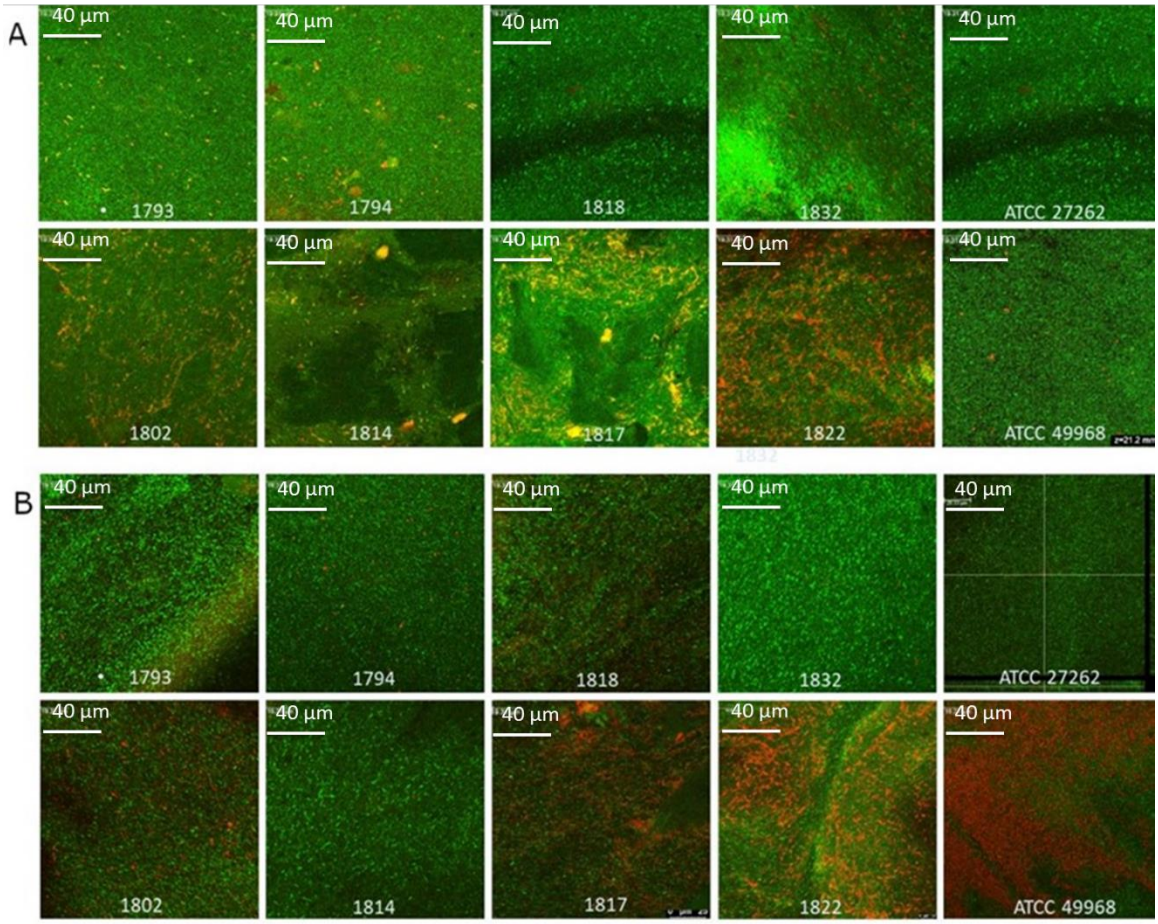

Figure 3.7: CLSM images of extracellular DNA of *P. fragi* (1793, 1794, 1818, 1832, ATCC 49968) and *P. lundensis* (1802, 1814, 1817, 1822, ATCC 49968) biofilms grown at 4 °C (A) and 10 °C (B) after seven days. The eDNA are stained with TOTO 1 (stained red) while the bacterial cells are counter stained with SYTO 60 (stained green).

Biofilm Transformation with time: To further comprehend the cause of the of bio-volume reduction and the distorted appearance of biofilms formed on day 7 at 10 °C, the biofilm cycle on meat was further studied in detail using two strains from each species. *Pseudomonas frag* 1793 and *P. lundensis* 1822 were selected as those strains grew slightly faster than the rest of the tested strains. In addition, the type strains of each species were selected as they are the points of reference that other strains are compared with.

On day 1, samples incubated at 25 °C had biofilms that did not fully cover the meat surface and majority of the bacterial cells appeared in green (Figure 3.9). The number of CFU in biofilms increased to approximately 10^8 CFU cm^{-2} (Figure 3.8), (Table 3.3). By day 2, the CFU numbers had increased to approximately 10^{10} CFU cm^{-2} and the meat surface was covered with a biofilm layer. The maximum population of around 10^{11} CFU cm^{-2} was reached around day 3 (Figure 3.8A). By day 3, the most of cells appeared in green with some areas appearing in yellow and the biofilm structures appeared distorted. By day 4, more dead cells were visible, and the biofilms structures were clearly broken (Figure 3.9). On days 5 and 6, the majority of cells in the biofilm had been dispersed and the muscle surface was clearly visible again. However, part of the biofilm population remained on the meat surface as live cells (Figure 3.9)

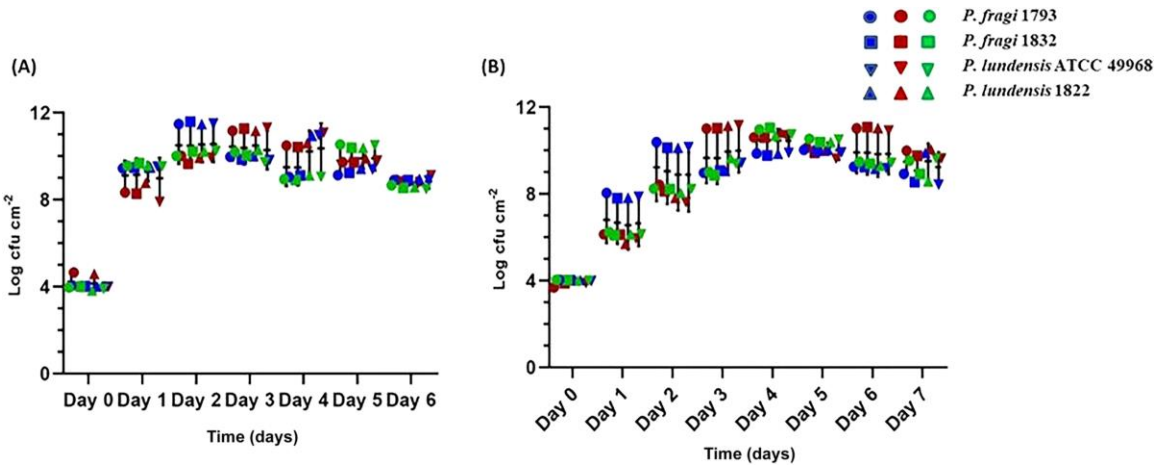

Figure 3.8: Cell counts of *P. fragi* 1793, 1832, and *P. lundensis* 1822, ATCC 49968 biofilms grown on meat at 25 °C (A) and 10 °C (B) from day zero to day six. Replicates from independent experiments 1, 2 and 3 are coloured in red, blue and green, respectively. The error bars represent the variability of data.

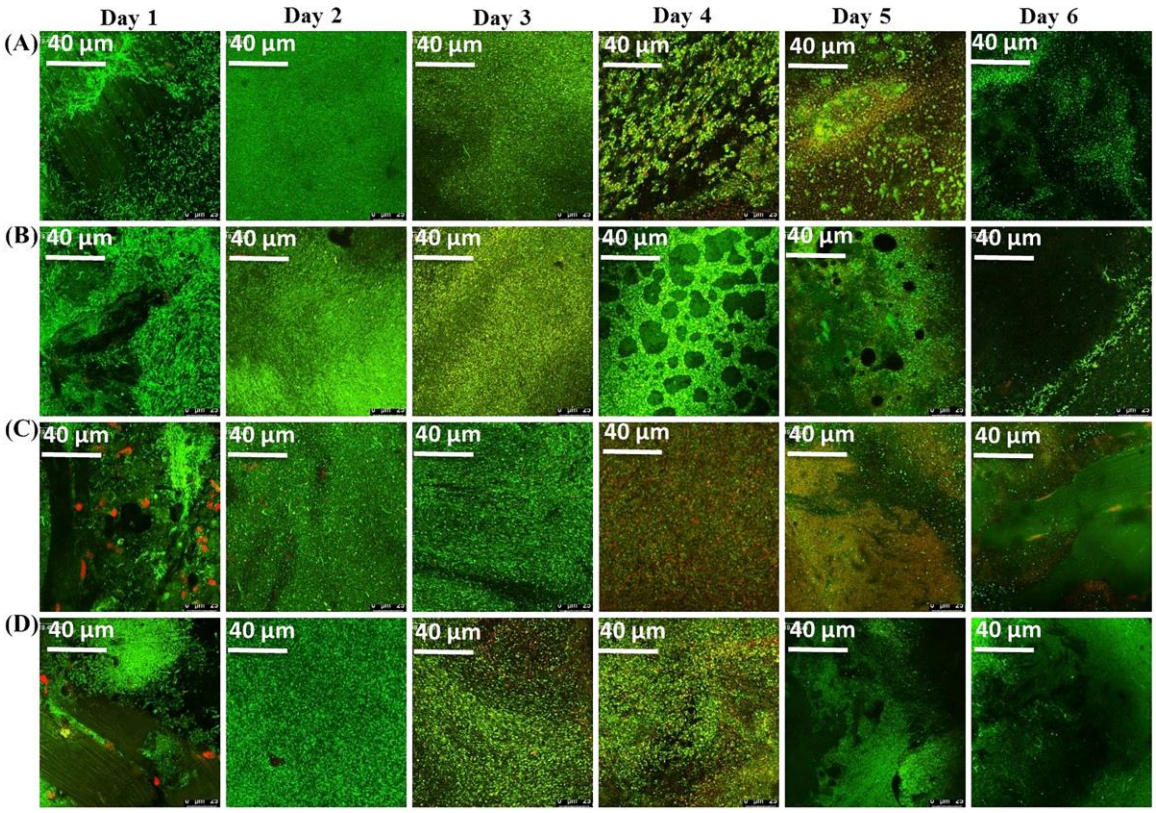

Figure 3.9: CLSM micrographs of biofilm cycle of *P. fragi* 1793 (A), 1832 (B) and *P. lundensis* 1822 (C), ATCC 49968 (D) grown meat incubated at 25 °C from day 1 to day 6. Live cells are stained with SYTO 9 (green) while dead cells and meat nucleic are stained with PI (red).

Table 3.3. The log CFU counts cm^{-2} of meat of *P. fragi* and *P. lundensis* biofilms grown at 25 ℃ from day zero to day six.

Days	**1793**			**1832**			**1822**			**ATCC 49968**		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
0	4.02	4.04	3.96	4.99	4.02	4	4.1	4.01	3.82	4.02	3.98	3.89
1	8.33	9.44	9.56	8.27	9.48	9.68	8.78	9.48	9.58	7.89	9.52	9.53
2	10.01	11.48	10.01	9.65	11.59	10.21	9.93	11.48	10.22	9.93	11.5	10.25
3	11.18	9.97	10.19	11.27	9.85	10.04	11.17	10	10.29	11.3	9.82	9.72
4	10.49	9.04	8.94	10.426	9.11	8.9	10.6	10.94	9.12	11.08	10.96	9.04
5	9.72	9.13	9.05	9.727	9.23	8.76	9.9	9.42	8.76	9.8	9.39	8.8
6	8.92	8.9	8.66	8.84	8.9	8.54	8.86	8.95	8.57	9.12	8.88	8.48

Table 3.4 The CFU counts of *P. fragi* and *P. lundensis* biofilms grown on meat at 10 ℃ from day zero to day six.

Days	**1793**			**1832**			**1822**			**ATCC 49968**		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
0	4.09	4.04	4.04	3.99	4.02	4.02	4.01	4.01	4.01	4.12	3.98	3.98
1	6.13	8.04	6.23	6.12	7.8	6.09	5.71	7.82	6.13	5.94	7.85	6.13
2	8.41	11.04	8.25	8.12	10.78	8.22	7.81	10.78	8.05	7.61	10.81	8.22
3	11.01	8.96	9	11.03	9.06	8.85	11.14	9.05	9.65	11.16	9.43	9.38
4	10.6	9.85	10.97	10.68	9.77	11.04	10.83	9.85	10.69	10.7	9.89	10.74
5	10.12	10.03	10.54	9.89	9.99	10.39	10	10.05	10.39	9.62	9.91	10.5
6	11.02	9.25	9.48	11.08	9.23	9.39	11.04	9.15	9.3	10.92	9.18	9.43
7	9.99						10.06			9.63		

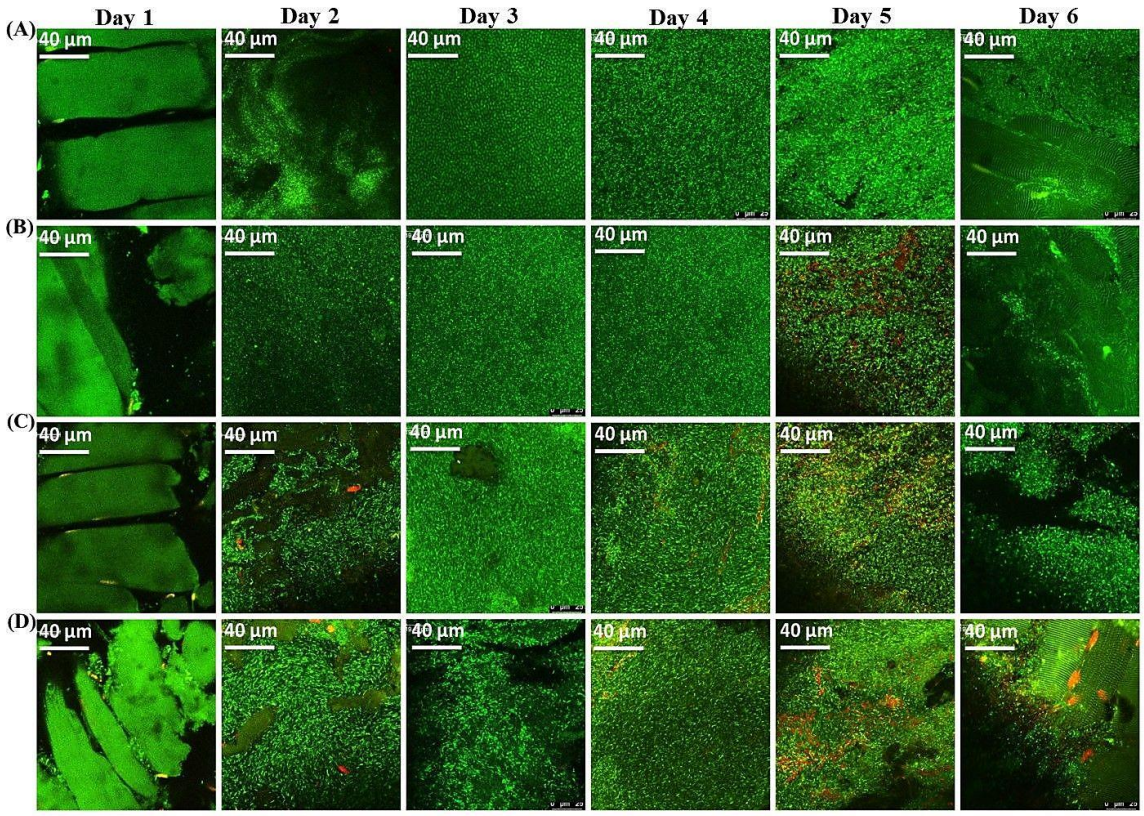

Figure 3.10: CLSM images of biofilm cycle of *P. fragi* (1793 & 1832) and *P. lundensis* (1822 & ATCC 49968) grown meat incubated at 10 °C from day 1 to day 6. Live cells are stained in SYTO 9 (green) while dead cells and meat nucleic are stained in PI (red).

Comparatively, biofilms grown at 10 °C had a slower growth rate but followed a similar pattern in biofilm initiation, maturation, dispersal and cell densities. Corresponding to the CFU counts, the CLSM images showed highly dense biofilms and the majority of the cells are seen as live cells until day 4 (Figure 3.10). The population maximum of around 10^{11} CFU cm^{-2} was reached around day 4 for 10 °C biofilms. The cell numbers were reproducible across the three biological replicates (Figure 3.8), (Table 3.4).

After population maximum (μ_{max}), the biofilm structures began to deteriorate. Even the compact biofilms of *P. fragi* strains appeared distorted after μmax. The results from CFU numbers and CLSM images appeared to show that after μmax has been reached the structures collapse and cell numbers decrease. The CLSM images from day zero to day six clearly illustrate the different stages of biofilm life cycle from initial development to maturation to dispersal.

3.5 Discussion

To date, most of research conducted on spoilage pseudomonads have used an artificial environment supplemented with essential nutrients to study meat spoilage bacteria. The environmental conditions within microtitre plates, glass vials or broth culture models are vastly different to the environment of a complex meat muscle. The metabolic pathways of microorganisms can vary depending on the external environmental conditions that they grow and as a result, metabolic by products can also differ (Drescher, Shen, Bassler, & Stone, 2013). Some studies have concluded that meat could be the ecological niche of *P. fragi* (Champomier-Vergès, Stintzi, & Meyer, 1996). Therefore, it is important to characterize their biofilm formation under *in-situ* conditions.

A surface sterilization technique was adopted in this meat model to sterilise the fresh, raw meat. It is known that intrinsic muscle tissue of healthy animals slaughtered under reasonably hygienic conditions is usually free of bacteria (Gill & Penney, 1977; Mackey & Derrick, 1979). Also, the bacteria on the meat surface do not secrete proteolytic enzymes until late exponential phase (Corry, 2006). Thus, the chemical composition of the fresh meat is intact at the beginning of the experiment. In this project the beef cut known as ‘eye round’ was used which is a skeletal muscle with minimal connective tissue. In order to closely resemble spoilage of aerobically stored retail meat, the samples were inoculated with cell numbers commonly reported to be present on retail meat which is approximately 10^3 CFU cm^{-2} (P. J. Delaquis & A. R. McCurdy, 1990; E. Drosinos & R. G. Board, 1995).

The greenish tint that was observed in meat slices inoculated with *P. lundensis* strains is most likely due to the pyocine pigment produced by members of this species (Molin & Ternstrom, 1986). This characteristic is not observed in *P. fragi* biofilms as it is a non-pigmented species (Champomier-Vergès et al., 1996). For CLSM imaging, staining the biofilms with nucleic acid dyes, SYTO 9 and PI aided the visualization of the biofilms as well as the muscle surface with muscle fibres and meat nuclei in juxtaposition with limited artefacts. Therefore, a qualitative estimate of the extent and nature of muscle degradation could be obtained.

Even though the metabolic reactions occur at a slower rate at 4 °C, based on the obtained data, it is clear that both the bacterial species can survive and multiply to become a visible layer of slime under refrigerated temperatures. Small aggregates developed into individual micro colonies which merged into a layer of slime on muscle surface. These observations are consistent with past studies where small micro colonies were observed after the irreversible attachment of planktonic bacteria which merged in to a uniform biofilm (Delaquis et al., 1992; Yada & Skura, 1982b). A large number of planktonic bacteria were visible moving among cellular aggregates during the early stages of biofilm formation. Planktonic bacteria were not observed in 7-day old biofilms grown at 4 °C or after day 3 of 10 °C grown biofilms. Biofilm grown after these time temperature combinations are more mature and mature biofilms do not contain many planktonic cells (Valentini & Filloux, 2016).

A key characteristic that is common to the biofilm mode of life is the high concentration of cyclic di-GMP (c-di-GMP), a secondary messenger which has been demonstrated to supresses flagella mediated swimming motility and promotes matrix production (Diane, Scott, Nicolas, Peter, & Staffan, 2011; Merighi, Lee, Hyodo, Hayakawa, & Lory). Mature biofilms contain high concentration of c-di-GMP. The absence of planktonic cells in the mature biofilms may be attributed to the activity of cyclic-di-GMP. Further studies measuring cyclic-di-GMP levels are necessary to study this hypothesis for meat biofilms formed by *P. fragi* and *P. lundensis.*

The results showed that *P. fragi* strains produced very thick biofilms. These observations are consistent with results of Delaquis et al. (1992) where dense biofilms were imaged with CLSM. When a biofilm thickness increases, the cells in the interior layers may have limited access of oxygen and nutrients which would presumably lead to cell death (Balaban, 2008; Rani et al., 2007). For biofilms grown on a meat surface, the nutrients are obtained via diffusion from bottom layers and the oxygen is obtained from top. Thus, the cells in innermost layers may have limited access to these commodities and they might have been expected to have reduced viability. In terms of localization of live and dead cells no such spatial distribution or 'central deadness' can be observed in the Z stacks of *P. fragi* and *P. lundensis* biofilms.

Bacteria grew over all the exposed surfaces of meat without forming micro-niches. No preferential colonization pattern can be observed on the muscle tissue. However, large circular and elliptical voids can be observed within mature biofilms of *P. fragi* and *P. lundensis* strains. Similar structures were reported by Delaquis et al. (1992), in CLSM images of *P. fragi* grown on porcine muscle. It was concluded that these voids were caused due to the difficulty of the fluorescent dye to penetrate the thick circular microcolonies of *P. fragi*. However, this may not be the case as similar structures were observed in this study when scanned from top to the muscle surface of mature biofilms of dense *P. fragi* and also in loosely arranged *P. lundensis* biofilms. During the intermediate stages of biofilm growth, meat nuclei of the skeletal muscle can be seen in red stained with PI, with similar sizes and shapes to these voids. It is highly likely that these voids are degraded meat nuclei or nuclei with degraded DNA. However, it is not clear why the voids are not filled with bacteria since the whole area surrounding the voids is densely packed with cells (Figure 3.6). This raises the intriguing possibility that despite the extensive proteolytic activity the bacteria are unable to rapidly degrade the nuclear membrane and penetrate the nucleus.

Pseudomonas fragi is equipped with many beneficial metabolic characteristics but the role biofilm formation plays in their robustness remained largely unstudied. According to this study, all the *P. fragi* strains produced highly dense, homogenous, lawn like biofilms with limited intercellular gaps. The cellular arrangement was uniform from top of the

biofilm to the muscle surface. This uniform lawn like growth, gave *P. fragi* strains a higher surface coverage of the muscle than *P. lundensis* strains. Due to this tight arrangement of cells, not much extracellular matrix material could be observed. Also compared to *P. lundensis* strains, *P. fragi* biofilms had less meat nuclei within the biofilm which is an indication that nuclei may have been fully degraded. The capacity to degrade the muscle may be higher *P. fragi* compared to *P. lundensis*. According to the past studies *P. fragi* caused the most extensive proteolysis when grown on meat (Hasegawa, Pearson, Price, Rampton, & Lechowich, 1970). However, separate experiments are necessary to quantitate the degradation rates to confirm this hypothesis.

The most striking feature of *P. fragi* biofilms is when imaged from above individual bacterial cells appear to take a circular shape than rods (Figure 3.4). This is probably due to rod shaped bacteria vertically orienting themselves as if 'standing up' in the biofilm. A small degree of variation was observed in the intensity of orientation between the selected *P. fragi* strains and individual experiments. In some samples the orientation was more prominent in terms of cell number and in the level of rotation. However, the cellular orientation was clearly visible in each sample of *P. fragi* biofilms and the results were reproducible. Some rod shaped bacteria have been demonstrated to take a coccoid form under cold conditions (Roszak & Colwell, 1987; Trueba, Spronsen, Traas, & Woldringh, 1982). However, when *P. fragi* planktonic cells grown at 4 ^{0}C were imaged with CLSM, the shape of the cells was clearly visible as rods. Also rod-shaped bacteria can be observed in immature *P. fragi* biofilms during early stages. Therefore, it is unlikely that a change in cell shape is an effect of temperature. Furthermore, scanning the biofilms structures from top to the muscle surface showed that, verticalization was more prominent at the centre of the biofilms than at the upper surfaces. The majority of the cells at the periphery aligned radially and remained horizontal to the substrate.

Similar biofilm structures to those seen in this study were observed in biofilms of pathogenic bacterium *Vibrio cholerae*. High resolution CLSM images have shown that the cells are strongly vertically ordered, especially at the core of the cell clusters. (Beroz et al., 2018; Drescher et al., 2016; J. Yan, Sharo, Stone, Wingreen, & Bassler, 2016). The main cause of nematic ordering of *Vibrio cholerae* cells is considered to be the

exponential increase in cell mass which intensifies the pressure within the biofilm (Volfson, Cookson, Hasty, & Tsimring, 2008). Verticalization occurs when effective surface pressures created by rapid cell growth exceeds cell to surface adhesion which create a series of localized mechanical instabilities on the cellular scale (Beroz et al., 2018). Also the composition of the biofilm matrix plays a key role in determining the cellular architecture of a biofilm (Mann & Wozniak, 2012). Similar reasons may affect *P. fragi* biofilms where a rapid increase in cell numbers may cause mechanical instabilities or the matrix composition may lead to nematic ordering. Further studies are necessary to confirm these hypotheses.

The dense arrangements of bacterial cells has several advantages in the stability and evolution of a bacterial species in a community. J. Yan et al. (2016) have found that this type of cellular arrangement, provides superior mechanical properties that give resistant to shear forces, and are more stable (Hobley, Harkins, MacPhee, Stanley-Wall, & Albers, 2015). A key determinant that governs invasion resistance in a biofilm is its compactness (Carey et al., 2015; J. Yan, Nadell, Stone, Wingreen, & Bassler, 2017). The matrix production requires a lot of energy and it is expensive for the bacteria. Thus, the biofilm interior is a valuable commodity where non matrix producing bacteria can take advantage of. Studies have found that *Listeria monocytogenes* invades preformed *P. fluorescence* biofilms and induces matrix over production (Puga et al 2018). Since there are extremely limited intercellular gaps, in *P. fragi* biofilms they are difficult to be invaded by other microorganisms. This helps to exclude cheaters from breaching the biofilm interior. These attributes may likely contribute in *P. fragi* becoming the predominant organism in long term stored meat. Past studies have found that *P. fluorescens* species was dominant at the initial stages of animal slaughter but *P. fragi* becomes predominant during long term storage (P. J. Delaquis & A. R. McCurdy, 1990; Lebert et al., 1998).

eDNA network: Currently no information is available about eDNA in meat biofilms of *P. fragi* and *P. lundensis*. The main objective of this work was to determine if a general correlation exist between amount of eDNA and bacterial species as well as between temperatures levels. Extracellular DNA are released to the biofilm matrix via cell lysis

and active secretion of membrane vesicles (Whitchurch, Tolker-Nielsen, Ragas, & Mattick, 2002a). The role of eDNA appear to vary depending on the bacterial species (Das, Sharma, Busscher, van der Mei, & Krom, 2010). Overall, very small amount of eDNA could be observed in densely packed biofilms of *P. fragi*. The lack of eDNA does not necessarily mean *P. fragi* strains produce weak biofilms (Okshevsky & Meyer, 2015).

The importance of eDNA for structural stability of a biofilm cannot be judged from the amount of eDNA accumulated in the matrix (Okshevsky & Meyer, 2015). At the same time, the cellular architecture of *P. fragi* does allow to exhibit DNA within the biofilm. Strain level differences were observed in the amount of eDNA in the tested *P. lundensis* biofilms. No specific correlation could be detected between the amount of eDNA and selected temperature levels. This could be due to the complex nature of biofilm mode of life compared to planktonic mode where some degree of variation is unavoidable in biofilms grown on complex surfaces (Swearingen et al., 2016). Further experiments focused specifically on the detection and quantitation of eDNA are required given the demonstrated challenges of such measurements.

Time course assay: During biofilm characterization of ten strains, a decline in the biomass and distortions in biofilm structures were observed in day 7 old biofilms grown at 10 °C. Seven day old biofilms grown at 4 °C did not show a decline most likely due to the fact that the biofilms were not mature enough at that time and temperature combination. On days 4 and 5 of 25 °C samples, the majority of the cells appeared yellow which indicates that both SYTO 9 and PI are being penetrated into the cells. This is an indication that the cells are losing their viability.

Meat samples incubated at 25 °C with 10^3 CFU cm^{-2} on day zero increased up to 10^{11} CFU cm^{-2} by day 3. After the population maximum was reached, the biofilm structure began to collapse and bacteria in the biofilm began to disperse. The four tested strains had approximately similar maximum population levels in all the biological replicates. Since biofilms grown under chilled and ambient temperature conditions followed a similar pattern in structural deterioration, population numbers and dispersal, it is clear that this phenomenon is not a response to low temperature.

During biofilm dispersal, the lysed cells provide nutrients to dispersing cells which become planktonic (Purevdorj-Gage, Costerton, & Stoodley, 2005). After dispersal, the CLSM images showed that part of the bacterial population remained on the surface meat as live cells. Due to heterogeneous nature of the cells in different layers of a biofilm, only a subpopulation will undergo cell lysis and some remain on as live cells (Diane et al., 2011).

Even though both *P. fragi* and *P. lundensis* are highly proteolytic spoilage organisms which had continuous access to nutrients via proteolysis, the bacterial population did not grow after a certain population level. The cause for dispersal of biofilms formed on meat by proteolytic spoilage pseudomonads is currently unknown. Since the sample muscle slice was intact and remained whole, access to nutrients was uninterrupted. Carbon starvation induced dispersal has been observed in flow cell grown biofilms of *P. putida* which is another meat spoilage bacterium and taxonomically closely related to *P. fragi* (Gjermansen, Ragas, Sternberg, Molin, & Tolker-Nielsen, 2005).

The spoilage characteristics of meat become detectable when the bacterial populations reach approximately 10^7-10^8 CFU cm^{-2} (Casaburi et al., 2015; Corry, 2006; Nychas, Skandamis, Tassou, & Koutsoumanis, 2008). By that time bacteria have utilized all the glucose and other simple carbohydrates available in the muscle tissue. Afterwards bacteria secrete proteolytic enzymes and degrade muscle proteins. During proteolysis metabolic by products including nitrogenous compounds such as amino acids, ammonia and molecular nitrogen are released. The concentrations of these nitrogenous compounds tend to increase with time as the bacterial population grows. A marked production of ammonia was detected when *P. fragi* reach the climax population on chilled meat (E. Drosinos & R. G. Board, 1995). Genes homologues to nitrite reductase gene of *P. fluorescence* F113 were identified in *P. fragi* strain 1793 and 1794. However, such genes were not found in any of the *P. lundensis* strains that were used in this work (Stanborough, Fegan, Powell, Singh, et al., 2018). Amino acid signals are also known causes of biofilm dispersal. D Amino acids have been demonstrated to prevent biofilm formation by *Staphylococcus aureus* and *P. aeruginosa* (Kolodkin-Gal et al., 2010). Also the increase in free amino acid is high during spoilage under aerobic conditions than

under modified atmospheric conditions (Nychas et al., 2008). A dispersal of this sort is an active mechanism as a passive dispersal such as erosion could not occur in the selected experimental setup. Dispersal of biofilms formed on spoiled meat can be a potential source of cross contamination of industry premises and other meat products.

In many bacterial species quorum sensing plays an important role in biofilm formation and dispersal. The signaling molecules of *P. fragi* and *P. lundensis* are currently unknown and further studies are necessary to identify the role of quorum sensing in meat grown biofilm dispersal. Biofilm dispersal could be triggered from a change in nutrient availability during rapid growth and/or the release of nitrogenous byproducts due to proteolysis. Further knowledge in biofilm dispersal can may be beneficial in the development of methods to slow or reduce reducing slime formation on meat.

Experimental reproducibility: Biofilms have a considerable amount of variability and independent rounds of biofilm experiments do not result in absolute reproducibility (Heydorn et al., 2000). However, by controlling the main factors influencing biofilm formation, such as, temperature, incubation conditions, history of the cultures and the type of meat, the experimental reproducibility can be improved considerably. The key observations of this experiment including cellular arrangement, patterns in biofilm cycle and the population numbers for *P. fragi* and *P. lundensis* were highly reproducible. A limitation of this model is the use of a cover slip which limits a number of parameters that can be used from COMSTAT due to distortions that may be caused to the biofilm.

3.6 Conclusions:

This study found that *P. fragi* strains produce highly dense, compact biofilms and due to this dense nature of cellular arrangement in bacterial cells reorient themselves within the biofilm. There are no significant differences in structures of biofilms grown at chilled and ambient temperatures in both the species. Despite having access to nutrients, biofilms formed on meat by proteolytic *Pseudomonas fragi* and *Pseudomonas lundensis* disperse after reaching population maximum.

3.7 References

Balaban, N. (2008). *Control of Biofilm Infections by Signal Manipulation Springer Series on Biofilms*, Berlin, Heidelberg: Springer Berlin Heidelberg

Baudin, M., Cinquin, B., Sclavi, B., Pareau, D., & Lopes, F. (2017). Understanding the fundamental mechanisms of biofilms development and dispersal: BIAM (Biofilm Intensity and Architecture Measurement), a new tool for studying biofilms as a function of their architecture and fluorescence intensity. *Journal of Microbiological Methods,140*, 47-57. doi:10.1016/j.mimet.2017.06.021

Beroz, F., Yan, J., Meir, Y., Sabass, B., Stone, H. A., Bassler, B. L., & Wingreen, N. S. (2018). Verticalization of bacterial biofilms. *Nature Physics,954-960.doi:* 10.1038/s41567-018-0170-4

Carey, D. N., Knut, D., Ned, S. W., & Bonnie, L. B. (2015). Extracellular matrix structure governs invasion resistance in bacterial biofilms. *The ISME Journal, 9*(8). doi:10.1038/ismej.2014.246

Casaburi, A., Piombino, P., Nychas, G.-J., Villani, F., & Ercolini, D. (2015). Bacterial populations and the volatilome associated to meat spoilage. *Food Microbiology, 45*(Pt A), 83-102. doi:10.1016/j.fm.2014.02.002

Champomier-Vergès, M. C., Stintzi, A., & Meyer, J. M. (1996). Acquisition of iron by the non-siderophore-producing *Pseudomonas fragi. Microbiology, 142*(5), 1191-1199. doi:10.1099/13500872-142-5-1191

Chen, W., Hu, H., Zhang, C., Huang, F., Zhang, D., & Zhang, H. (2017). Adaptation response of Pseudomonas fragi on refrigerated solid matrix to a moderate electric field. *BMC Microbiology, 17*(1). doi:10.1186/s12866-017-0945-2

Corry, J. E. L. (2006). Spoilage organisms of red meat and poultry. Microbial analysis of red meat, poultry and eggs.Woodhead publishing

Das, T., Sharma, P. K., Busscher, H. J., van der Mei, H. C., & Krom, B. P. (2010). Role of Extracellular DNA in Initial Bacterial Adhesion and Surface Aggregation. *Applied and Environmental Microbiology, 76*(10), 3405. doi:10.1128/AEM.03119-09

Delaquis, P. J., Gariépy, C., & Montpetit, D. (1992). Confocal scanning laser microscopy of porcine muscle colonized by meat spoilage bacteria. *Food Microbiology, 9*(2), 147-153. doi:10.1016/0740-0020(92)80021-U

Delaquis, P. J., & McCurdy, A. R. (1990). Colonization of beef muscle surfaces by *Pseudomonas fluorescens* and *Pseudomonas fragi. Journal of Food Science* (4), 898-902. doi: 10.1111/j.1365-2621.1990.tb01560.x

Diane, M., Scott, A. R., Nicolas, B., Peter, D. S., & Staffan, K. (2011). Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nature Reviews Microbiology, 10*(1), 39. doi:10.1038/nrmicro2695

Drescher, K., Dunkel, J., Nadell, C. D., van Teeffelen, S., Grnja, I., Wingreen, N. S., Bassler, B. L. (2016). Architectural transitions in Vibrio cholerae biofilms at single-cell resolution. *Proceedings of the National Academy of Sciences of the United States of America, 113*(14), E2066. doi:10.1073/pnas.1601702113

Drescher, K., Shen, Y., Bassler, B. L., & Stone, H. A. (2013). Biofilm streamers cause catastrophic disruption of flow with consequences for environmental and medical systems. *Proceedings of the National Academy of Sciences of the United States of America, 110*(11), 4345. doi:10.1073/pnas.1300321110

Drosinos, E., & Board, R. G. (1995). Microbial and physiochemical attributes of minced lamb- sources of contaminations with pseudomonads. *Food Microbiology, 12*(3), 189-197.

Duskova, M., Kamenik, J., & Karpiskova, R. (2013). *Weissella viridescens* in meat products - a review. *Acta Veterinaria. BRNO, 82*(3), 237-241. doi:10.2754/avb201382030237

Dykes, G. A., Cloete, T. E., & Von Holy, A. (1994). Identification of *Leuconostoc* species associated with the spoilage of vacuum-packaged Vienna sausages by DNA-DNA hybridization. *Food Microbiology, 11*(4), 271-274. doi:10.1006/fmic.1994.1030

Efsa Panel on Biological, H. (2016). Growth of spoilage bacteria during storage and transport of meat. *EFSA Journal, 14*(6), n/a-n/a. doi:10.2903/j.efsa.2016.4523

Ercolini, D., Russo, F., Blaiotta, G., Pepe, O., Mauriello, G., & Villani, F. (2007). Simultaneous detection of *Pseudomonas fragi*, *P. lundensis*, and *P. putida* from

meat by use of a multiplex PCR assay targeting the carA gene. *Applied and Environmental Microbiology, 73*(7), 2354.

Ercolini, D., Russo, F., Torrieri, E., Masi, P., & Villani, F. (2006). Changes in the spoilage-related microbiota of beef during refrigerated storage under different packaging conditions. *Applied and Environmental Microbiology, 72*(7), 4663. doi:10.1128/AEM.00468-06

Fagerlind, M. G., Webb, J. S., Barraud, N., McDougald, D., Jansson, A., Nilsson, P., Rice, S. A. (2012). Dynamic modelling of cell death during biofilm development. *Journal of Theoritical Biology.295,* 23-36. doi:10.1016/j.jtbi.2011.10.007

Ferrocino, I., Ercolini, D., Villani, F., Moorhead, S. M., & Griffiths, M.W. (2009). *Pseudomonas fragi* strains isolated from meat do not produce N-acyl homoserine lactones as signal molecules. *Journal of Food Protection, 72*(12), 2597-2601.

Flemming, H.-C., Wingender, J., Szewzyk, U., & SpringerLink (Online service). (2011). *Biofilm Highlights Springer Series on Biofilms,* 243-273.Springer, Berlin.

Giaouris, E., Heir, E., Hébraud, M., Chorianopoulos, N., Langsrud, S., Møretrø, T., Nychas, G.-J. (2014). Attachment and biofilm formation by foodborne bacteria in meat processing environments: Causes, implications, role of bacterial interactions and control by alternative novel methods. *Meat Science, 97*(3), 298-309. doi:10.1016/j.meatsci.2013.05.023

Gill, C. O., & Penney, N. (1977). Penetration of bacteria into meat. *Applied and Environmental Microbiology*, *33*(6), 1284. doi:10.1128/AEM.33.6.1284-1286.1977

Gjermansen, M., Ragas, P., Sternberg, C., Molin, S., & Tolker-Nielsen, T. (2005). Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms. *Environmental Microbiology*, *7*(6), 894-904. doi:10.1111/j.1462-2920.2005.00775.x

Habimana, O., Heir, E., Langsrud, S., Asli, A. W., & Moretro, T. (2010). Enhanced surface colonization by *Escherichia coli* O157:H7 in biofilms formed by an *Acinetobacter calcoaceticus* isolate from meat-processing environments. *Applied and Environmental Microbiology, 76*(13), 4557. doi:10.1128/AEM.02707-09

Hasegawa, T., Pearson, A. M., Price, J. F., Rampton, J. H., & Lechowich, R. V. (1970). Effecet of microbial growth uponsarcoplasmic and urea soluble proteins from muscle. *Journal of Food Science, 35*(6), 720-724. doi:10.1111/j.1365-2621.1970.tb01979.x

Heydorn, A., Nielsen, A., Hentzer, M., Sternberg, C., Givskov, M., Ersboell, B., & Molin, S. (2000). Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology, 146*(10), 2395-2407. doi:10.1099/00221287-146-10-2395

Hobley, L., Harkins, C., MacPhee, C. E., Stanley-Wall, N. R., & Albers, S.-V. (2015). Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes. *FEMS Microbiology Reviews, 39*(5), 649-669. doi:10.1093/femsre/fuv015

Jay, J. M., Vilai, J. P., & Hughes, M. E. (2003). Profile and activity of the bacterial biota of ground beef held from freshness to spoilage at 5–7 °C. *International Journal of Food Microbiology, 81*(2), 105-111. doi:10.1016/S0168-1605(02)00189-7

Jin, X., Anderson, T. H., Dongyeop, K., Domenick, T. Z., Hyun, K., & Geelsu, H. (2017). Biofilm three-dimensional architecture influences in situ pH distribution pattern on the human enamel surface. *International Journal of Oral Science, 9*(2). doi:10.1038/ijos.2017.8

Kolodkin-Gal, I., Romero, D., Cao, S., Clardy, J., Kolter, R., & Losick, R. (2010). D-Amino Acids Trigger Biofilm Disassembly. *Science, 328*(5978), 627-629.

Lebert, I., Begot, C., & Lebert, A. (1998). Growth of *Pseudomonas fluorescens* and *Pseudomonas fragi* in a meat medium as affected by pH (5.8–7.0), water activity (0.97–1.00) and temperature (7–25°C). *International Journal of Food Microbiology, 39*(1), 53-60. doi:10.1016/S0168-1605(97)00116-5

Mackey, B. M., & Derrick, C. M. (1979). Contamination of the deep tissues of carcasses by bacteria present on the slaughter instruments or in the gut. *Journal of Applied Bacteriology, 46*(2), 355-366. doi:10.1111/j.1365-2672.1979.tb00832.x

Mann, E. E., & Wozniak, D. J. (2012). *Pseudomonas* biofilm matrix composition and niche biology, *FEMS Microbiology Reviews,* (36), 893-916.doi: 10.1111/j.1574-6976.2011.00322.x

Merighi, M., Lee, V. T., Hyodo, M., Hayakawa, Y., & Lory, S. Nychas, G.-J. E., Skandamis, P. N., Tassou, C. C., & Koutsoumanis, K. P. (2008). Meat spoilage during distribution. *Meat Science, 78*(1), 77-89. doi:10.1016/j.meatsci.2007.06.020

Okshevsky, M., & Meyer, R. L. (2014). Evaluation of fluorescent stains for visualizing extracellular DNA in biofilms. *Journal of Microbiological Methods, 105*, 102-104. doi:10.1016/j.mimet.2014.07.010

Okshevsky, M., & Meyer, R. L. (2015). The role of extracellular DNA in the establishment, maintenance and perpetuation of bacterial biofilms. *Critical Reviews in Microbiology*, (41) 341-352. doi: 10.3109/1040841X.2013.841639

Puga, C. H., Dahdouh, E., SanJose, C., & Orgaz, B. (2018). *Listeria monocytogenes* colonizes *Pseudomonas fluorescens* biofilms and induces matrix over-production. *Frontiers in Microbiology, 9*. doi:10.3389/fmicb.2018.01706

Purevdorj-Gage, B., Costerton, W. J., & Stoodley, P. (2005). Phenotypic differentiation and seeding dispersal in non-mucoid and mucoid *Pseudomonas aeruginosa* biofilms. *Microbiology (Reading, England), 151*(Pt 5), 1569. doi:10.1099/mic.0.27536-0

Rani, S. A., Pitts, B., Beyenal, H., Veluchamy, R. A., Lewandowski, Z., Davison, W. M., Stewart, P. S. (2007). Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states. *The Journal of Bacteriology, 189*(11), 4223. doi:10.1128/JB.00107-07

Roszak, D. B., & Colwell, R. R. (1987). Survival strategies of bacteria in the natural environment. *Microbiology and Molecular Biology Reviews, 51*(3), 365.

Silagyi, K., Kim, S.-H., Lo, Y. M., & Wei, C.-i. (2009). Production of biofilm and quorum sensing by *Escherichia col*i O157:H7 and its transfer from contact surfaces to meat, poultry, ready-to-eat deli, and produce products. *Food Microbilogy.* (*26)*, 514-519.

Stanborough, T., Fegan, N., Powell, S. M., Singh, T., Tamplin, M., & Chandry, P. S. (2018). Genomic and metabolic characterization of spoilage-associated *Pseudomonas* species. *International Journal of Food Microbiology, 268*, 61-72. doi:10.1016/j.ijfoodmicro.2018.01.005

Swearingen, M. C., Mehta, A., Mehta, A., Nistico, L., Hill, P. J., Falzarano, A. R., Bjarnsholt, T. (2016). A novel technique using potassium permanganate and reflectance confocal microscopy to image biofilm extracellular polymeric matrix reveals non-eDNA networks in *Pseudomonas aeruginosa* biofilms. *FEMS Pathogens and Disease*, *74*(1). doi:10.1093/femspd/ftv104

Trueba, F., Spronsen, E., Traas, J., & Woldringh, C. (1982). Effects of temperature on the size and shape of *Escherichia coli* cells. *Archives of Microbiology*, *131*(3), 235-240. doi:10.1007/BF00405885

Valentini, M., & Filloux, A. (2016). Biofilms and Cyclic di-GMP (c-di-GMP) signaling: lessons from *Pseudomonas aeruginosa* and other bacteria. *The Journal of biological chemistry*, *291*(24), 12547-12555. doi:10.1074/jbc.R115.711507

Volfson, D., Cookson, S., Hasty, J., & Tsimring, L. S. (2008). Biomechanical ordering of dense cell populations. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(40), 15346. doi:10.1073/pnas.0706805105

Whitchurch, C. B., Tolker-Nielsen, T., Ragas, P. C., & Mattick, J. S. (2002). Extracellular DNA required for bacterial biofilm formation. *Science (New York, N.Y.)*, *295*(5559), 1487. doi:10.1126/science.295.5559.1487

Yada, R. Y., & Skura, B. J. (1982). Scanning electron microscope study of *Pseudomonas fragi* on intact and sarcoplasm-depleted bovine *longissimus dorsi* muscle. *Applied and Environmental Microbiology*, *43*(4), 905.

Yan, J., Nadell, C., Stone, H., Wingreen, N., & Bassler, B. (2017). Extracellular-matrix-mediated osmotic pressure drives *Vibrio cholerae* biofilm expansion and cheater exclusion. *Nature Communication*, *8*(1), 327-327. doi:10.1038/s41467-017-00401-1

Yan, J., Sharo, A. G., Stone, H. A., Wingreen, N. S., & Bassler, B. L. (2016). *Vibrio cholerae* biofilm growth program and architecture revealed by single-cell live imaging. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(36), E5337. doi:10.1073/pnas.1611494113

CHAPTER 4

Characterization of the biofilm matrix composition of psychrotrophic, meat spoilage pseudomonads

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4.1 Abstract

Psychrotrophic *Pseudomonas* species are the key spoilage bacteria of aerobically stored chilled meat. These organisms readily form biofilms on meat under refrigerated conditions leading to consumer rejection and associated economic losses. Limited information is available on the matrix composition of the biofilms formed by these bacteria. We quantified and characterized the main components of the matrix of mono-species biofilms of selected *Pseudomonas fragi* and *Pseudomonas lundensis* strains using chemical analysis and Raman spectroscopy. The biofilms were grown at 10 °C and 25 °C on nitro-cellulose membranes placed on surface sterilized beef cuts. Extra-cellular polymeric substances of the matrix were extracted in soluble and bound forms and were chemically assessed for total carbohydrates, proteins and extra-cellular DNA. Both *Pseudomonas* species showed a significant increase in total carbohydrates and total proteins when grown at 10 °C as compared to 25 °C. Extra-cellular DNA did not show a strong correlation with growth temperature. Raman spectra were obtained from planktonic bacteria and membrane grown biofilms at 10 °C and 25 °C. Higher levels of guanine were detected in planktonic cells as compared to biofilm cells. This study suggests that psychrotrophic *Pseudomonas* species may respond to cold stress by increasing extra-cellular polymer secretions.

4.2 Introduction

Meat is a rich source of nutrients with high water activity which makes it a highly perishable food commodity (Doulgeraki et al., 2012). Psychrotrophic pseudomonads are the main cause of organoleptic degradation of aerobically stored chilled meat (Ercolini, Russo, Torrieri, Masi, & Villani, 2006). These organisms are metabolically diverse and can withstand the stressful environmental conditions of chilled storage as well as competition from other psychrotrophic organisms on meat (Gram et al., 2002; Wickramasinghe et al., 2019). A key characteristic of psychrotrophic pseudomonads is that they readily form biofilms under chilled storage (Wickramasinghe et al., 2019). When these biofilms combine with meat exudates, this leads to slime formation which is an important quality defect which leads to consumer rejection of meat.
Although considerable research has been undertaken on planktonic spoilage pseudomonads in broth culture models, limited information is available about their biofilm formation as well as how biofilms contribute to their predominance on meat. Biofilms are formed when bacterial cells attach themselves irreversibly to a surface or to each other and embed in a self-produced and/or an acquired exo-polymeric matrix (Nicolas Barraud et al., 2015; Flemming, Neu, & Wozniak, 2007). These sessile groups of bacteria exhibit different phenotypic characteristics as compared to their planktonic counterparts (Donlan, 2002).

The exo-polymeric matrix of biofilms protects the bacteria against harmful environmental conditions such desiccation, radiation, predation and antimicrobial compounds (Carey et al., 2015; Luyan et al., 2009). The matrix immobilizes microorganisms and aids in quorum sensing, horizontal gene transfer and enzymatic reactions (Sutherland, 2001). It also aids in cellular arrangement, and provides mechanical stability which affects the overall structural arrangement of the biofilm (Flemming et al., 2007; Sutherland, 2001). The biofilm matrix is therefore more than an inert material and warrants study in detail.

In order to minimize and control biofilm formation, a thorough understanding of the matrix components and their proportions is essential. The biofilm matrix is typically composed of water, extra-cellular polymeric substances (EPS), extra-cellular DNA, lipids and extra-cellular vesicles (Adav & Lee, 2008;Flemming & Wingender 2010)). EPS are high molecular weight substances and are often be divided into two main fractions known as soluble EPS (SEPS) and bound EPS (BEPS) (Chen et al., 2013).

Pseudomonas fragi and *Pseudomonas lundensis* are two important species that cause spoilage of chilled meat globally (Wickramasinghe et al., 2019). Since biofilms provide many benefits to protect the residing bacteria from harmful environmental conditions, it is likely that the ability to form biofilms is advantageous to psychrotrophic *Pseudomonas* spp. To date, limited information is available on the mechanisms of biofilm formation by these organisms and its associated structure and matrix composition.

Since the environmental conditions are known to affect the metabolism of biofilms, it is important to find out if there are differences in the matrix composition between biofilms formed at chilled and ambient temperature conditions. A key objective of this research was to assess if psychrotrophic *P. fragi* and *P. lundensis* respond to low temperature by changing their matrix composition or quantity. For this reason 10 °C was selected to mimic low but temperature abuse conditions that can result during handling of chilled meat (Nychas et al., 2008; Wang et al., 2017). The ambient temperature which has the best optimal growth for these two species is reported to be 25 °C (Delaquis et al., 1992; Molin et al., 1986).

This study characterizes the key extra-cellular polymeric compounds of biofilm matrix of *P.s fragi* and *P. lundensis* strains when grown under lower (10 °C) and ambient temperature (25 °C) conditions using chemical and spectroscopic methods. It further investigates differences in the chemical composition between bacterial modes of growth by comparing the Raman spectral profiles of planktonic and biofilm cultures of these species.

4.3 Materials and methods

***Bacterial culture preparation*:** Two strains each of *P. fragi* (1793 and 1832) and *P. lundensis* (1822 and ATCC 49968) were selected for this study as being representative of the species based on previous research on biofilm formation by these and other strains (Wickramasinghe et al., 2019). Specifically, *P. fragi* 1793 and *P. lundensis* 1822 had high growth rates (Wickramasinghe et al., 2019). The type strains of each species (*P. fragi* ATCC 4973 (1832) and *P. lundensis* ATCC 49968) were selected as they are points of reference for other strains that may be investigated.

***Preparation of biofilms on meat*:** Fresh beef 'eye round' cuts were purchased from local butchers and transported to the laboratory chilled (3 °C) within 20 minutes of purchase. The water activity of fresh beef was measured using a Novasina LabSwift-water activity meter and pH of the muscle was measured using a PHM210 standard pH Meter for each biological replicate to assure consistency. Meat was surface sterilized by immersing in boiling water for ten minutes. The cooked exterior was aseptically removed inside a sterilized laminar flow hood and the uncooked, raw interior was used for further experiments. The beef was sectioned into slices of 4 mm thickness using a sterilized stainless-steel deli slicer. The slices were sectioned aseptically to fit a 55 cm^2 petri plate. Nitro-cellulose membranes with an area of 55 cm^2 and pore size of 0.2 µm (PALL product ID: S80209) were sterilized by keeping each side under ultraviolet light for 15 minutes. Each beef slice was placed inside a petri dish and a nitro cellulose membrane was placed on the meat and patted gently to stick uniformly to the top surface of the muscle.

Overnight cultures of each bacterial strain were prepared by inoculating a single colony of the selected strains into 5 ml of tryptone soy broth (TSB, Oxoid, Basingstoke, United Kingdom) and incubating for 18-20 hours in at 25 °C at 180 rpm in a shaking incubator. Tenfold serial dilutions were made in TSB and 1 ml from the 10^4 CFU ml^{-1} dilution was added to each membrane placed on the beef muscle and spread evenly on the membrane using sterile plastic spreaders. The petri plates were covered with lids and incubated at 10 °C and 25 °C to allow biofilm formation.

***Selection of the extraction time point*:** In order to select matrix extraction time point at which biofilms were at a similar level of maturity after growth at two different temperatures bacterial counts were determined. The counts in biofilms formed on membranes placed on meat were determined from day 1 to day 7. The cell counts were determined by dissolving the matrix in maximum recovery diluent (MRD) (Oxoid, Basingstoke, UK) as described in earlier studies (Wickramasinghe et al., 2019) and plating on *Pseudomonas* isolation agar medium (Oxoid, Basingstoke, United Kingdom).

***Extraction of the matrix*:** On days 5 and 6.5 (determined to be the extraction time points) the meat slices incubated at 25 °C and 10 °C, respectively, were removed from the incubator. The biofilms which were formed on the membranes were collected gently with a sterile cell scraper and the wet weight of the biofilm was measured. The biofilms were placed separately in 50 ml Falcon tubes containing 10 ml of MiliQ water. Then the tubes were vortexed for 1 minute at maximum speed and shaken using a mechanical flask shaker (Griffin and Tatlock Ltd, Birmingham, London) at maximum speed for five minutes to disperse the biofilm uniformly in water. The biofilms were then sonicated in an ultrasound water bath (Ultrasonics. Pty. Australia) for five minutes to break the aggregates and then vortexed again for 30 seconds. Each tube was centrifuged at 2000 g for 15 minutes at 4 °C using Sigma6-6k centrifuge. The supernatant was collected as the soluble EPS and filter sterilized using 0.2 µm pore sized membrane filters (Figure 4.1)

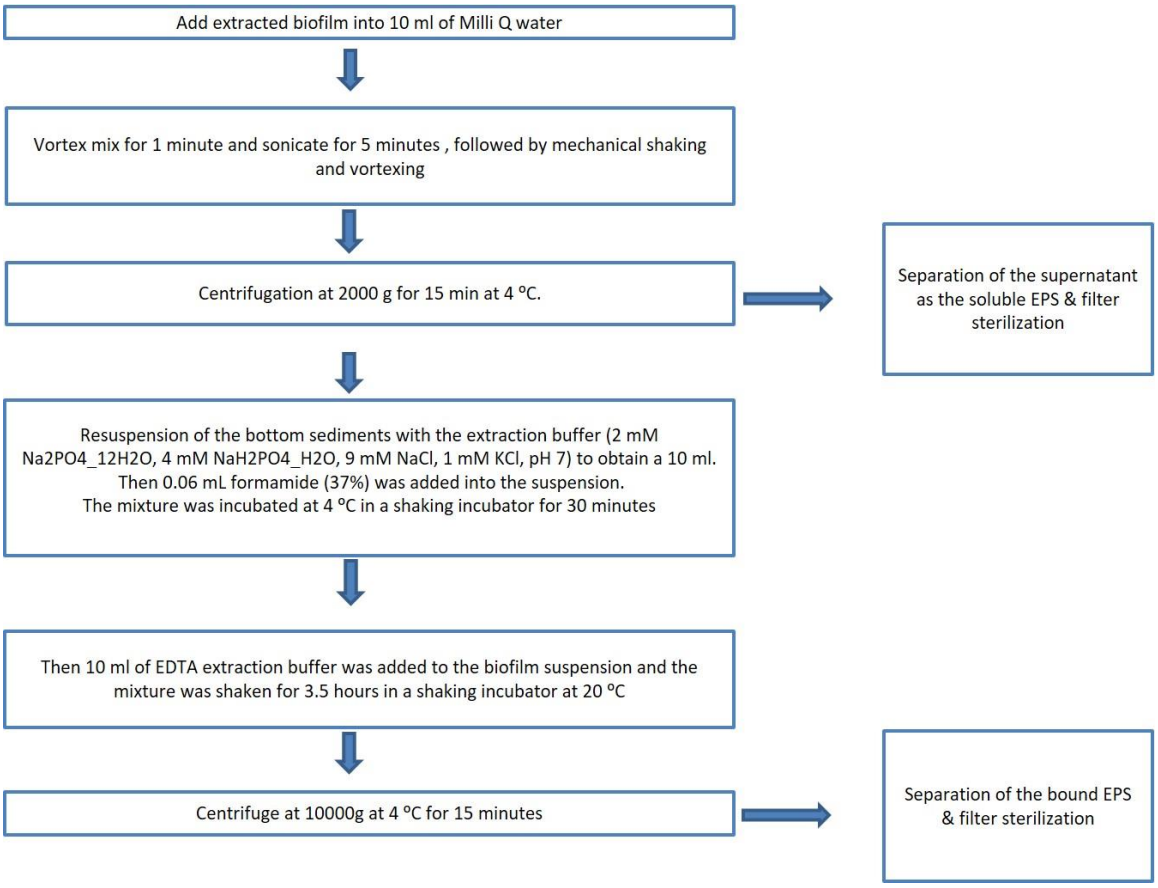

Figure 4.1. The chemical extraction process of the biofilm matrix EPS in soluble and bound forms

The bottom sediment remaining in the tubes was dissolved in 10 ml of extraction buffer (2 mM $Na_2PO_4.12H_2O$, 4 mM $NaH_2PO_4.H_2O$, 9 mM NaCl, 1 mM KCl, pH 7) and a 60 µl aliquot of 40% formamide was added. The mixture was incubated at 4 °C in a shaking incubator at 180 RPM for 30 minutes (Figure 4.1). Subsequently, 10 ml of ethylenediamine tetraacetic acid (EDTA) extraction buffer was added to the biofilm suspension and the mixture was shaken for a further 3.5 hours in a shaking incubator at 20 °C at 180 RMP (Liang et al., 2010). The flasks were vortex mixed for 30 seconds and centrifuged at 10000 *g for 20 minutes. The supernatant was collected as bound EPS and filter sterilized using 0.2 µm pore sized membrane filters (Liang et al., 2010). The extracted samples were kept at 3 °C until further analysis. Samples were not frozen to avoid alteration of the chemical composition. Four individual biological replicates were

carried out under identical conditions. The soluble and bound EPS were used for chemical quantification of key matrix EPS components. In order to normalize the EPS concentration across samples of different wet weight, the results were divided by the wet weight of the corresponding biofilm. The results are presented as ug/ml per gram of biofilm wet weight.

***Protein content determination*:** The protein concentrations of the extracted soluble and bound EPS were analyzed using the Qubit protein assay kit (ThermoFisher Scientific- Q33211) according to manufacturer's instructions. Three technical replicates were carried out for each sample. Protein concentration was normalized by dividing values by the corresponding wet weight of the samples.

***Carbohydrate content determination*:** Total carbohydrates in the extracted soluble and bound EPS of each sample were quantified using the total carbohydrate assay kit (Sigma, MAK104-1KT) according manufacturer's instructions. The assay was prepared in 96 well plates with a series of glucose standards. Three technical replicates were carried out for each standard and sample. The concentration of the total carbohydrates in each sample was calculated using the standard curve. The amount of total carbohydrates in each sample was normalized by dividing values by the wet weight of the corresponding biofilm.

***Extra-cellular DNA concentration determination*:** The Qubit™ dsDNA Assay Kit (Molecular Probes, Europe- Q33230) was used to determine the eDNA concentration in soluble and bound EPS samples according to manufacturer instructions. The eDNA concentration in each sample was normalized by dividing values by the corresponding wet weight of the biofilm.

***Planktonic cell Raman spectra acquisition*:** Overnight cultures of strains were prepared as previously described. The cultures were centrifuged at 10000 g for 5 minutes. The supernatant was removed, and the collected precipitates were washed twice with MiliQ water to remove traces of broth media. A 10 ul aliquot of the washed culture was placed on a calcium fluoride (CaF_2) microscope slide and air dried for 10 minutes. The dried culture was analyzed using a confocal Raman spectroscope (Renishaw inVia, Renishaw

plc.,Wotton-under-Edge, UK). Specifically, a Raman spectrometer equipped with a Leica microscope plus a deep depletion charge-coupled device detector, 2400 lines per mm grating and a holographic notch filter with slit size of 65 µm was used. As previously described by Hlaing et al., 2016. the incident laser power was adjusted to 1.5 mW of 514 nm radiation from the Argon laser. An estimated spatial resolution on the order of 0.8 µm was used for acquiring the spectra from each sample. The system was calibrated and monitored using a silicon reference (520.5 cm^{-1}) before the measurements. A single bacterial cell was focused for each measurement using a 20x microscope objective (NA = 0.4 in air). The accumulation time for each acquisition was 10 s and single accumulation was collected for a single measurement over the confocal region containing the selected cell. The same pre-processing steps were carried out for obtaining the Raman spectra for planktonic and biofilm samples.

Biofilm Raman spectra acquisition: Raman spectra were obtained from biofilms grown on membranes incubated at 25 °C and 10 °C on days 5 and 6.5. The membranes were carefully removed with sterile forceps and gently washed with MilliQ water to remove any planktonic bacteria. The biofilms were then air dried for 30 minutes. The membranes were placed on a CaF_2 cube and Raman spectra were obtained as described above.

***Raman data processing*:** The spectra were collected in the 2000 to 500 cm^{-1} range that covers the fingerprint region of most biological materials (Nawaz et al., 2010). The cosmic peaks in the obtained spectra were removed using WiRE 3.4 Raman software integrated in the Renishaw inVia Raman spectroscopy system. The spectra intensities were normalized using total intensity normalization to remove sample-to-sample variations and the background was subtracted (Kramer, 1998). Raman spectrum of nitrocellulose membrane provided a consistent background signal at 846 cm^{-1} and 1282 cm^{-1}. To recover the spectra from the bacterial cells, the peak intensities of the bacterial spectra collected from an intact biofilm isolated on the membrane were normalized by dividing with the intensity of the nitrocellulose membrane signal at 1282 cm^{-1} after background subtraction.

The normalization process was also performed on the background subtracted membrane spectra. The spectrum from a single cell of intact bacterial biofilms was then recovered by subtracting the normalized nitrocellulose membrane spectrum from the normalized spectrum of bacterial cell together with membrane.

The normalized Raman spectra were then mean-centered to reposition the centroid of the data at the origin (Kramer, 1998). The mean-centered data were analyzed by calculating the principal components (PCs), creating score plots. Each spectrum was plotted as a separate point in a multidimensional space. The characteristic peaks from the loading plots were determined using existing literature for cellular components (Hlaing et al., 2016). These characteristic Raman peak assignments are associated with abundant cellular components in the substance.

***Statistical analysis*:** For chemical analysis, four biological replicates were obtained and for each biological replicate three technical replicates were tested. Data analysis was performed using GraphPad Prism 5 software (GraphPad Software, San Diego, California USA, www.graphpad.com). Statistical differences for a single time points were evaluated through one-way ANOVA, with a confidence level of 95% ($P < 0.05$). The statistical significance for multiple groups was determined with multiple t tests. Results are presented as the mean ± the standard deviation (SD), and a P value of <0.05 was considered statistically significant.

For Raman spectroscopy, ten spectra were obtained from each of the three biological replicates of planktonic and biofilm samples. A commercially available software, MATLAB (version 7.10.0. Natick, Massachusetts: The MathWorks Inc., 2010) was used for processing the Raman data. A multivariate statistical method of principal component analysis (PCA) was applied to detect differences and similarities within the biofilm and planktonic spectral data sets

4.4 Results

***Cell counts in biofilms*:** For biofilm matrix studies to be comparable between the two different temperatures, biofilm components were extracted at approximately similar levels of maturity. As bacterial growth rate within the biofilm was dependent on temperature, the level of maturity was determined by the number of cells in *P. fragi* and *P. lundensis* biofilms formed on nitro-cellulose membranes at 10 °C and 25 °C over 7 days. Based on the data, day 5 was selected for biofilms grown at 25 °C and day 6.5 for biofilms grown at 10 °C. At these time points the numbers of bacteria in the biofilms for each of the strains weresimilar as presented in Table 4.1.

Table 4.1. Cell counts in mono-species biofilms of the *P. fragi* and *P. lundensis* strains grown at 25 °C and 10 °C on nitro-cellulose membranes placed on meat

Bacterial strain	Log CFU cm^{-2} at 25 °C on day 5	Log CFU cm^{-2} at 10 °C on day 6.5
***P. fragi* 1793**	11.5	11.2
***P. fragi* 1832**	11.3	11.5
***P. lundensis* 1822**	12.1	11.4
***P. lundensis* ATCC 49968**	11.9	10.6

***Matrix protein content*:** At both the growth temperatures, the highest content of total protein was detected in the matrix of the two *P. lundensis* strains as compared with thethe *P. fragi* strains. When formed at 25 °C the total protein content of the *P. lundensis* ATCC 49968 matrix was 1644 µg/ml/g and when formed at 10 °C it was significantly higher ($P=0.019$) at 2635 µg ml^{-1} g^{-1}, a 1.6-fold increase (Figure 4.2). When formed at 25 °C the total protein content of the *P. lundensis* 1822 was 1013 µg ml^{-1} g^{-1} and when formed at 10 °C it was significantly higher ($P<0.001$) at 1867 µg ml^{-1} g^{-1}, a 1.84 fold increase

(Figure 4.2). For *P. lundensis* at the lower temperature there was greater protein content in the soluble EPS than in the bound EPS, while at the higher temperature the levels of protein were similar (Figure 4.3). When formed at 25 °C the total protein content of the *P. fragi* 1793 matrix was 568 µg ml^{-1} g^{-1} and when formed at 10 °C it was significantly higher (P=0.001) at 1397 µg ml^{-1} g^{-1}, a 2.45 fold increase (Figure 4.2).

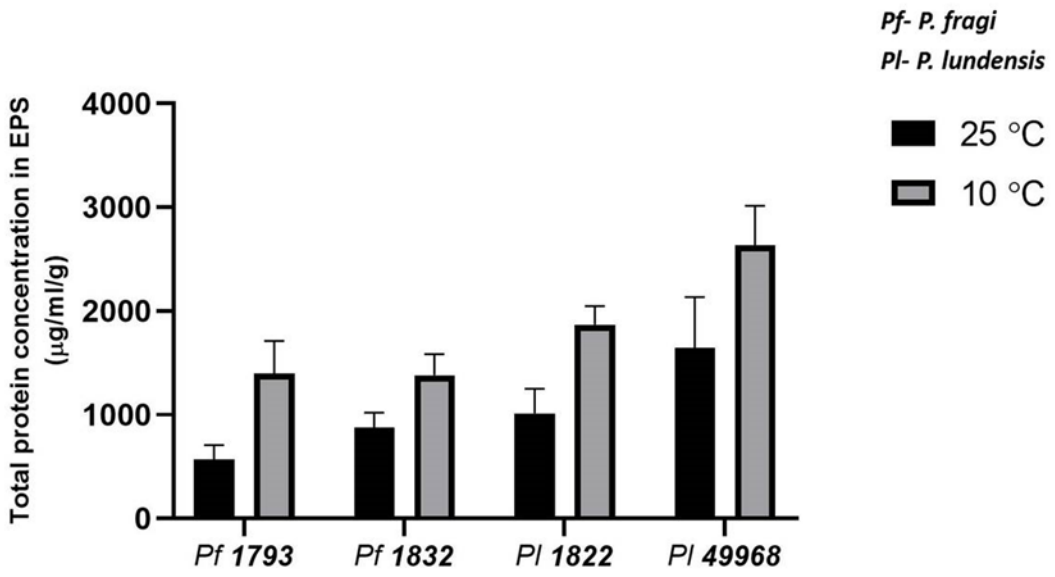

Figure 4.2. The total protein content of the matrix EPS. The protein content of soluble and bound fractions of extracted EPS of *P. fragi* (1793 and 1832) and *P. lundensis* (1822 and ATCC 49968) biofilms formed on nitro-cellulose membrane placed on meat at 10 °C and 25 °C. The error bars show the standard deviations from four biological replicates. Statistical differences were evaluated through one-way ANOVA, with a confidence level of 95% ($P < 0.05$).When formed at 25 °C the total protein content of the *P. fragi* 1832 matrix was 877 µg ml^{-1} g^{-1} and when formed at 10 °C it was significantly higher (P=0.001) at 1382 µg ml^{-1} g^{-1}, a 1.57 fold increase (Figure 4.2). By comparison to the *P. lundensis* strains, the *P. fragi* strains produced a lower quantity of proteins in both soluble and bound EPS fractions. However, the matrix of both *P. fragi* strains had a relatively higher total protein under low temperature in both soluble as well as bound EPS than at ambient temperature (Figure 4.3).

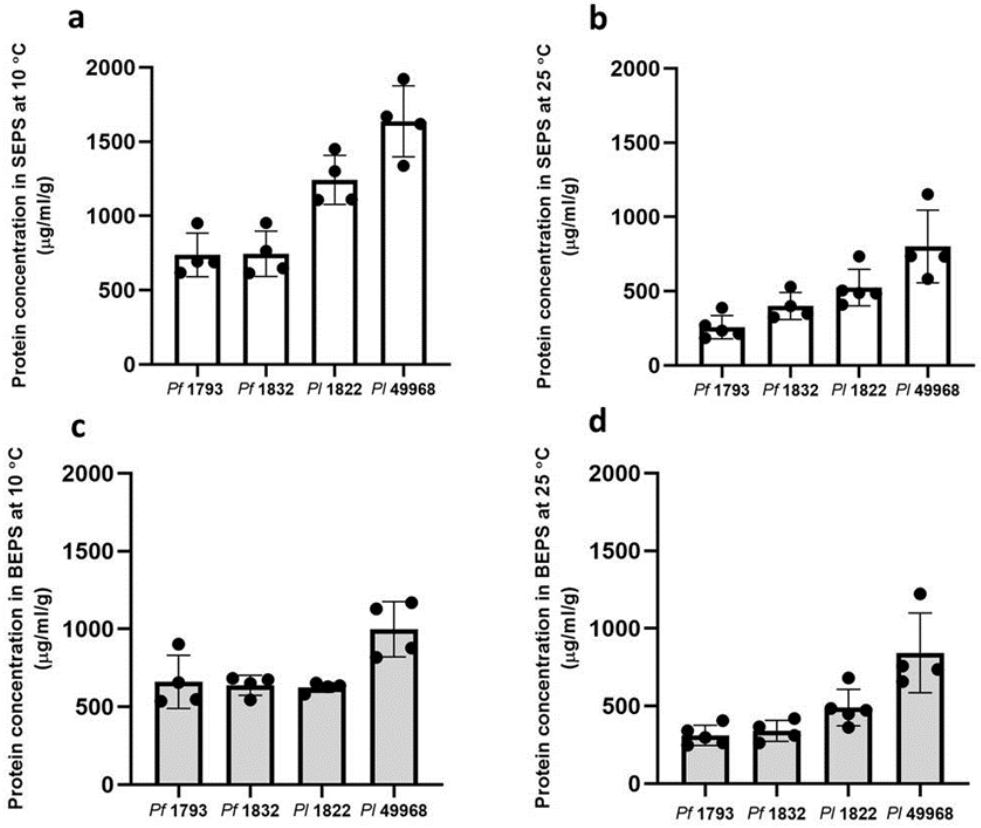

Figure 4.3. The protein content of the soluble and bound fractions of matrix EPS. The protein content of four biological replicates of *P. fragi* (1793, 1832) and *P. lundensis* (1822, ATCC 49968) biofilms formed on nitro-cellulose membrane placed on meat at 10 °C (A and C) and 25 °C (B and D). Error bars show the standard deviations from four biological replicates. Statistical differences were evaluated through one-way ANOVA, with a confidence level of 95% ($P < 0.05$).

***Matrix carbohydrate content*:** At both the growth temperatures, the highest content of total carbohydrate was detected in the matrix of the two *P. fagi* strains as compared to the *P. lundensis* strains. When formed at 25 °C the total carbohydrate content of the *P. fragi* 1793 matrix was 535 µg ml^{-1} g^{-1} and when formed at 10 °C it was significantly higher (P=0.000) at 1140 µg ml^{-1} g^{-1}, a 2.1 fold increase (Figure 4.4). When formed at 25 °C the total carbohydrate content of the *P. fragi* 1832 matrix was 579 µg ml^{-1} g^{-1} and when formed at 10 °C it was significantly higher (P=0.018) at 851 µg ml^{-1} g^{-1}, a 1.5 fold increase (Figure 4.4). When formed at 25 °C the total carbohydrate content of the *P. lundensis* ATCC 49968 matrix was 245 µg ml^{-1} g^{-1} and when formed at 10 °C it was significantly higher (P=0.011) at 511 µg ml^{-1} g^{-1}, a 2.1 fold increase (Figure 4.4).

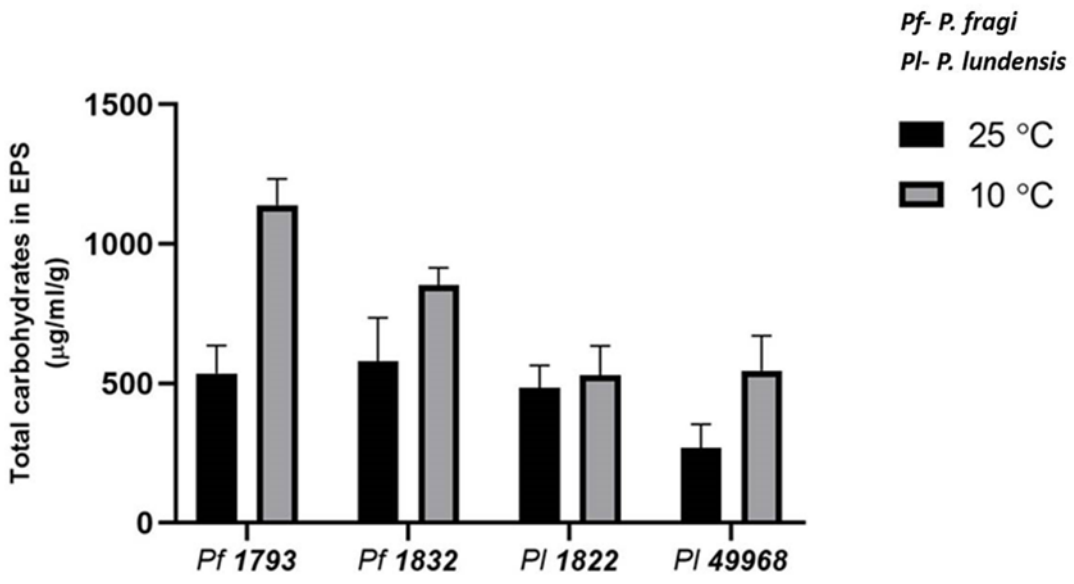

Figure 4.4. The total carbohydrate content of the matrix EPS. The carbohydrate content of soluble and bound fractions of extracted EPS of *P. fragi* (1793 and 1832) and *P. lundensis* (1822 and ATCC 49968) biofilms formed on nitro-cellulose membrane placed on meat at 10 °C and 25 °C. The error bars show the standard deviations from four biological replicates. Statistical differences were evaluated through one-way ANOVA, with a confidence level of 95% ($P < 0.05$).

The total carbohydrate content of the *P. lundensis* 1822 matrix produced at different temperatures were not a significantly different (P=0.485) from each other. Bacterial strains of both species had higher levels of total carbohydrates in the extracted matrix EPS when biofilms formed under low temperature as compared to ambient temperature. Furthermore, the *P. lundensis* strains had higher amounts of carbohydrates in the soluble fraction of the matrix EPS compared to the bound fraction (Figure 4.5).

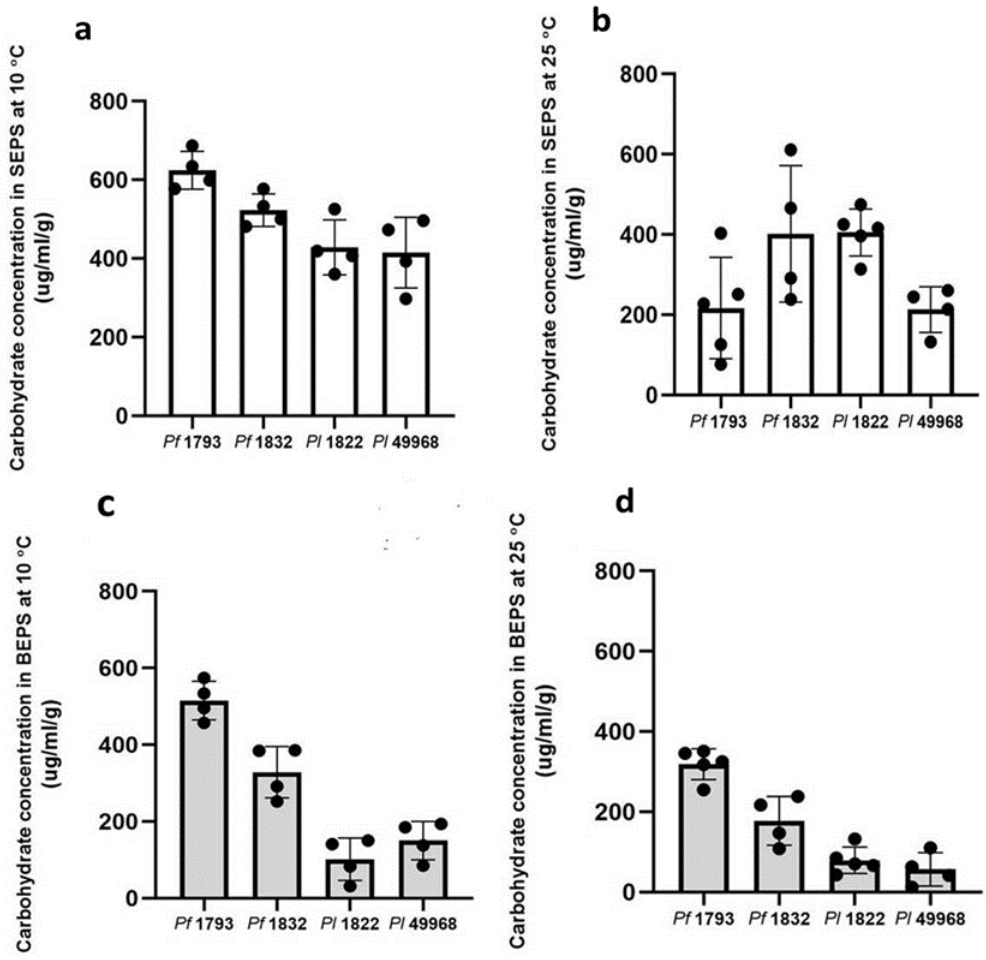

Figure 4.5. The carbohydrate content of the soluble and bound fractions of matrix EPS. The carbohydrate content of four biological replicates of *P. fragi* (1793, 1832) and *P. lundensis* (1822, ATCC 49968) biofilms formed on nitro-cellulose membrane placed on

meat at 10 °C (A and C) and 25 °C (B and D). Error bars show the standard deviations from four biological replicates. Statistical differences were evaluated through one-way ANOVA, with a confidence level of 95% ($P < 0.05$).

The total protein to total carbohydrate ratio was higher in *P. fragi* strains1793,1832 and in *P. lundensis* 1822 when formed under low temperature conditions compared to 25 °C (Table 4.2).

Table 4.2. Total protein to carbohydrate ratios of the biofilm matrix of the *P. fragi* and *P. lundensis* strains grown at 10 °C and 25 °C.

Temperature	*P. fragi* 1793	*P. fragi* 1832	*P. lundensis* 1822	*P. lundensis* ATCC 49968
10 °C	1.2	1.6	3.5	4.7
25 °C	1.1	1.3	2.1	6.1

***Matrix extra-cellular DNA (eDNA) content*:** A clear association between the matrix eDNA content and bacterial species, or between matrix eDNA content and temperature level was not apparent in this study. When formed at 25 °C the matrix eDNA content of the *P. lundensis* ATCC 49968 matrix was 47 µg/ml/g and when formed at 10 °C it was significantly higher ($P<0.000$) at 622 µg/ml/g, a 13.2 fold increase (Figure 4.7). Interestingly, this strain had the lowest total eDNA content in the matrix out of the four strains when grown at ambient temperature. When formed at 25 °C the matrix eDNA content of the *P. fragi* 1793 matrix was 51 µg/ml/g and when formed at 10 °C it was significantly higher ($P=0.004$) at 142 µg/ml/g, a 2.9 fold increase (Figure 4.7). Difference in eDNA content could not be detected at the different temperatures for the *P. fragi* 1832 matrix ($P= 0.342$) or the *P. lundensis* 1822 matrix ($P= 0.954$). The eDNA content in both SEPS and BEPS increased by several folds across the biological replicates (Figure 4.6).

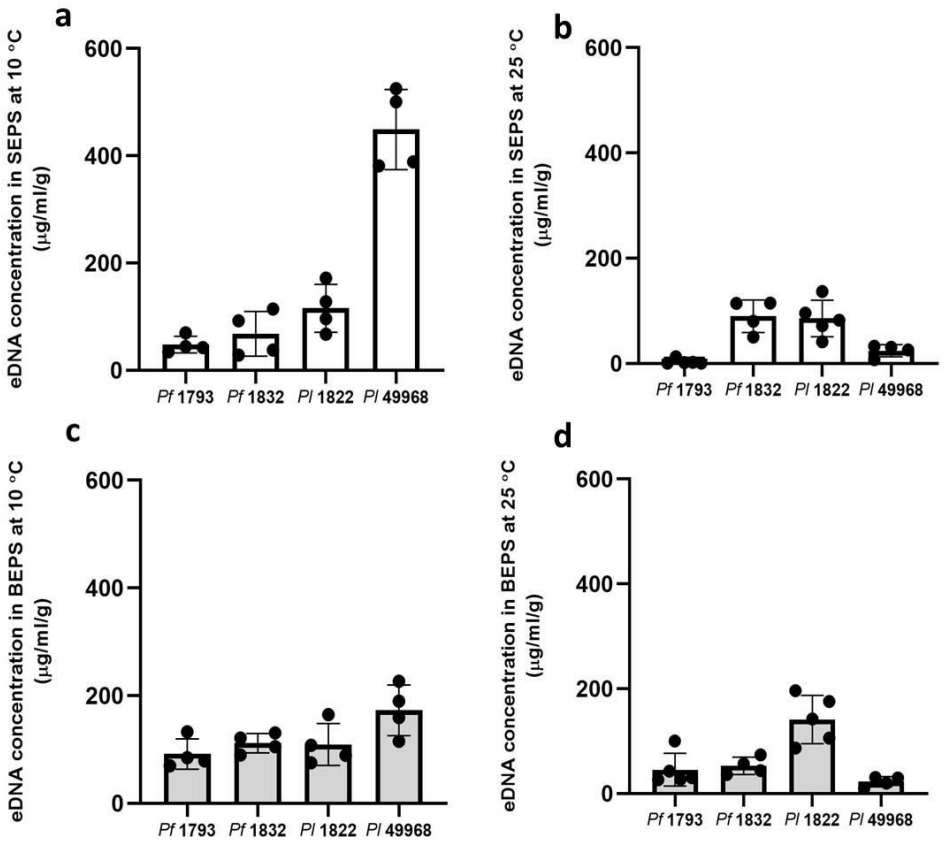

Figure 4.6. The eDNA content of the matrix EPS. The eDNA content of soluble and bound fractions of extracted EPS of *P. fragi* (1793 and 1832) and *P. lundensis* (1822 and ATCC 49968) biofilms formed on nitro-cellulose membrane placed on meat at 10 °C and 25 °C. Error bars show the standard deviations from four biological replicates. Statistical differences were evaluated through one-way ANOVA, with a confidence level of 95% ($P < 0.05$).

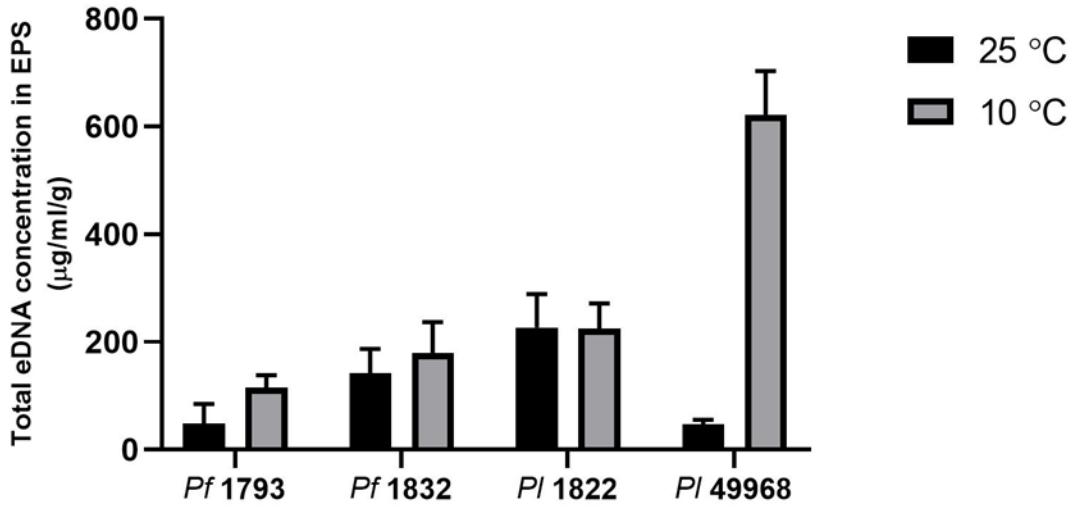

Figure 4.7 The total carbohydrate content of the matrix EPS. The carbohydrate content of soluble and bound fractions of extracted EPS of *P. fragi* (1793 and 1832) and *P. lundensis* (1822 and ATCC 49968) biofilms formed on nitro-cellulose membrane placed on meat at 10 °C and 25 °C. Error bars show the standard deviations from four biological replicates. Statistical differences were evaluated through one-way ANOVA, with a confidence level of 95% ($P < 0.05$).

***Raman spectroscopy*:** Averaged, intensity-normalised Raman spectra of planktonic bacteria grown at 25 °C and biofilms formed on nitro-cellulose membranes placed on meat at 10 °C and 25 °C were assessed for differences in spectral intensity. Strain level differences can be seen in the intensities of planktonic and biofilm Raman spectra (Figure 4.8). The spectra of the four different strains showed many similar peaks that can be assigned to cellular constituents associated with DNA/RNA, proteins, lipids, carbohydrates, based on previous studies as summarized in Table 4.3. Since Raman spectroscopy analysis is a phenotypic method, all related environmental factors (i.e. growth media and conditions) as well as physiological states of cells can influence in identifying the Raman peaks between different species, or even within the same species or strains (Lorenz, Wichmann, Stöckel, Rösch, & Popp, 2017). Moreover, it has been reported that certain bacterial species produce biocomponents which may contribute as

specific Raman-spectral fingerprints to them (Stöckel, Stanca, Helbig, Rösch, & Popp, 2015).

The Raman spectra of planktonic samples had more prominent peaks than the Raman spectra of biofilm samples grown under both temperatures (Figure 4.8). Prominent peaks of biofilm and planktonic samples are listed in Table 4.3. The sharp peaks in the planktonic spectra can be assigned to proteins (Amide III and II bands found at 1230 cm-^{1} and 1545 cm-^{1}) whereas the CH_3CH_2 twisting mode of collagen/lipid can be found at 1313 cm^{-1}(Table 4.3). The bands at 1400 cm-^{1} and at 1171-4 cm^{-1} were related to N-H plane deformation and tyrosine phenyl alanine CH bend, respectively (Table 4.3). The most prominent peak found in the planktonic Raman spectra of all the strains was guanine (1361-5 cm^{-1}).

The spectral peaks of biofilm samples were comparatively less sharp. The key peaks which were particularly prominent in biofilm samples were 606-668 cm^{-1} assigned to T, G (ring breathing), 700-90 cm^{-1} assigned to A, T, U, C (ring breathing modes in the DNA/RNA bases), 811 cm^{-1} assigned to PO_2^- str. RNA and 974 cm^{-1} assigned to ribose vibration, which is one of the distinct RNA modes. Compared to other biofilm samples, pronounced differences can be seen in the spectral peaks of biofilms of *P. fragi* 1832 and biofilms of both *P. lundensis* strains grown at 10 °C. High levels of carbohydrates and phenylalanine were detected at 1148 cm^{-1} and 1595 cm^{-1} respectively (Figure 4.8).

Table 4.3. Selected Raman frequencies and their peak assignments for the spectra shown in Figures 7.

Wave number (cm^{-1})	Peak assignment
DNA/RNA	
668 (606-68)	T, G (ring breathing)
726, 746	A, T (ring breathing mode of DNA/RNA bases)
781	U, T, C (ring breathing modes in the DNA/RNA bases)
785	U, T, C (ring breathing), PO_2^- *str*. of DNA backbone
811	PO_2^- str. RNA
621-46	C-C twisting mode of phenylalanine (proteins)
974	Ribose vibration, one of the distinct RNA modes
1070–90	Symmetric PO_2^- *str* of DNA (represents more DNA in cell)
Protein/ Lipid/ Carbohydrate/ Polysaccharide	
840–60	Polysaccharide structure
1002	Phenylalanine (ring breathing, symmetric)
1105, 1149	Carbohydrates peak for solutions, solids
1171-74	Tyrosine, phenylalanine, C-H bend (protein)
1230–300	Amide III (arising from coupling of C-N stretching & N-H bonding)
1313	CH_3CH_2 twisting mode of collagen/lipid
1325–30	CH_3CH_2 wagging mode in purine bases of nucleic acids
1361-5	Guanine
1400	NH in-plane deformation
1460	CH_2/CH_3 deformation of lipids & collagen
1506-15	Cytosine
1544-5	Amide II
1586-8	Phenylalanine, hydroxyproline
1622	Tryptophan
1655–80	T, G, C (ring breathing modes of the DNA/RNA bases), amide I (protein)

Abbreviations: *A*, adenine; *C*, cytosine; *G*, guanine; *PO_2^-*, phosphate; *str*, stretching; *T*, thymine; *U*, uracil. Assignments are based on studies in the references.

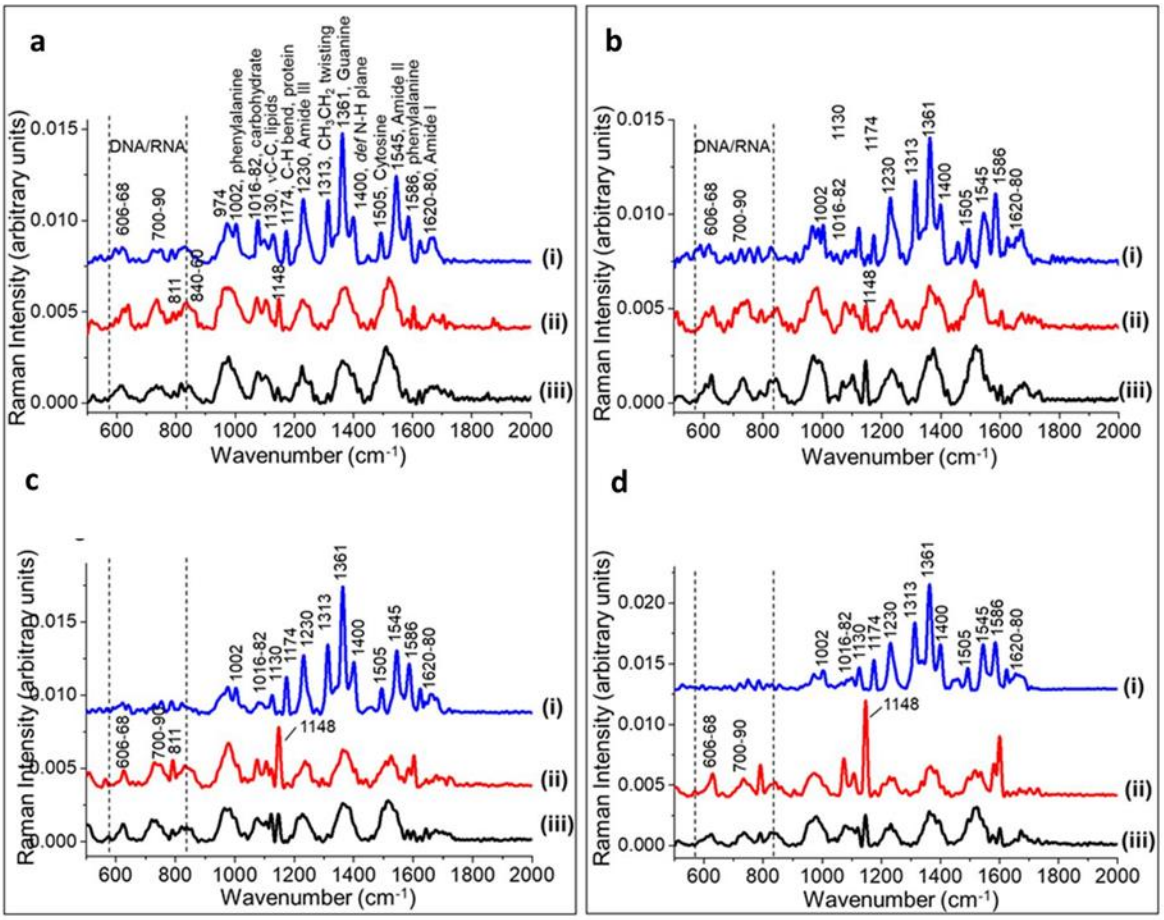

Figure 4.8. Averaged, intensity-normalized and background subtracted Raman spectra from planktonic cells (i) and biofilms of 10 °C (ii) and 25 °C (iii) of the four bacterial strains (a *P. fragi* 1793, b. *P. lundensis* 1822, c *P. fragi* 1832, d. *P. lundensis* 49968). The dominant peaks for DNA/RNA, proteins and carbohydrate are shown with the peak assignments listed in Table 4.3

***PCA analysis of Raman spectra*:** Principal component analysis was carried out for averaged, intensity normalised Raman spectra of planktonic cells and biofilms of all four strains. Scatter plots of the first and second principal components (PC1 and PC2) show a significant separation between planktonic cells and biofilm samples for each bacterial strain (Figure 4.9a). The first principal component (PC1) accounted for about 45% and 49% of the variance between planktonic and biofilm samples for *P. fragi* strains 1793 and 1832, respectively (Figure 4.9a). The PC1 for *P. lundensis* accounted for 61% and 58% of the variance between planktonic and biofilm samples of strains 1822 and ATCC 49968, respectively (Figure 4.9a). The chemical changes responsible for the variations between planktonic and biofilm spectra can be observed in the PCA loadings plots (Figure 4.9b).

The first principal component loading plot (Figure 4.9b) shows peaks at 1180 cm^{-1}, 1600 cm^{-1} (associated with proteins), 1310 cm^{-1} (associated with carbohydrates), 1500 cm^{-1} (associated with cytosine) and 1600 (associated with phenylalanine and tyrosine). The peaks which could be used as the chemical fingerprint regions to differentiate biofilm and planktonic samples are associated with carbohydrates, proteins (phenylalanine and tyrosine), DNA/RNA synthesis (cytosine) and lipids. The loading plot has a similar trend for all four bacterial strains.

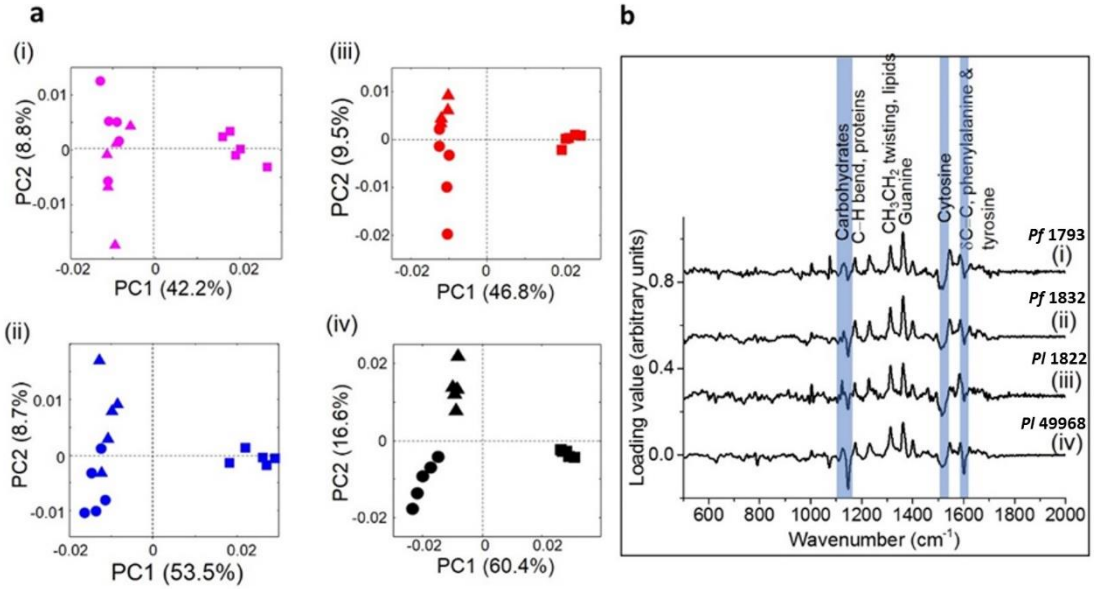

Figure 4.9. (a) Scatter plots of principal component analysis of the Raman spectra from planktonic cells and biofilms of the four bacterial strains: (i) *P. fragi* 1793, (ii) *P. fragi 1832*, (iii) *P. lundensis* 1822 and (iv) *P. lundensis ATCC* 49968. (*square*, planktonic cells; *circle*, biofilms grown at 10 °C; *triangle*, biofilms grown at 25 °C). (**b).** The loading plots**.** The corresponding PC1 loading plots of each strain exhibit the spectral differences of each comparison.

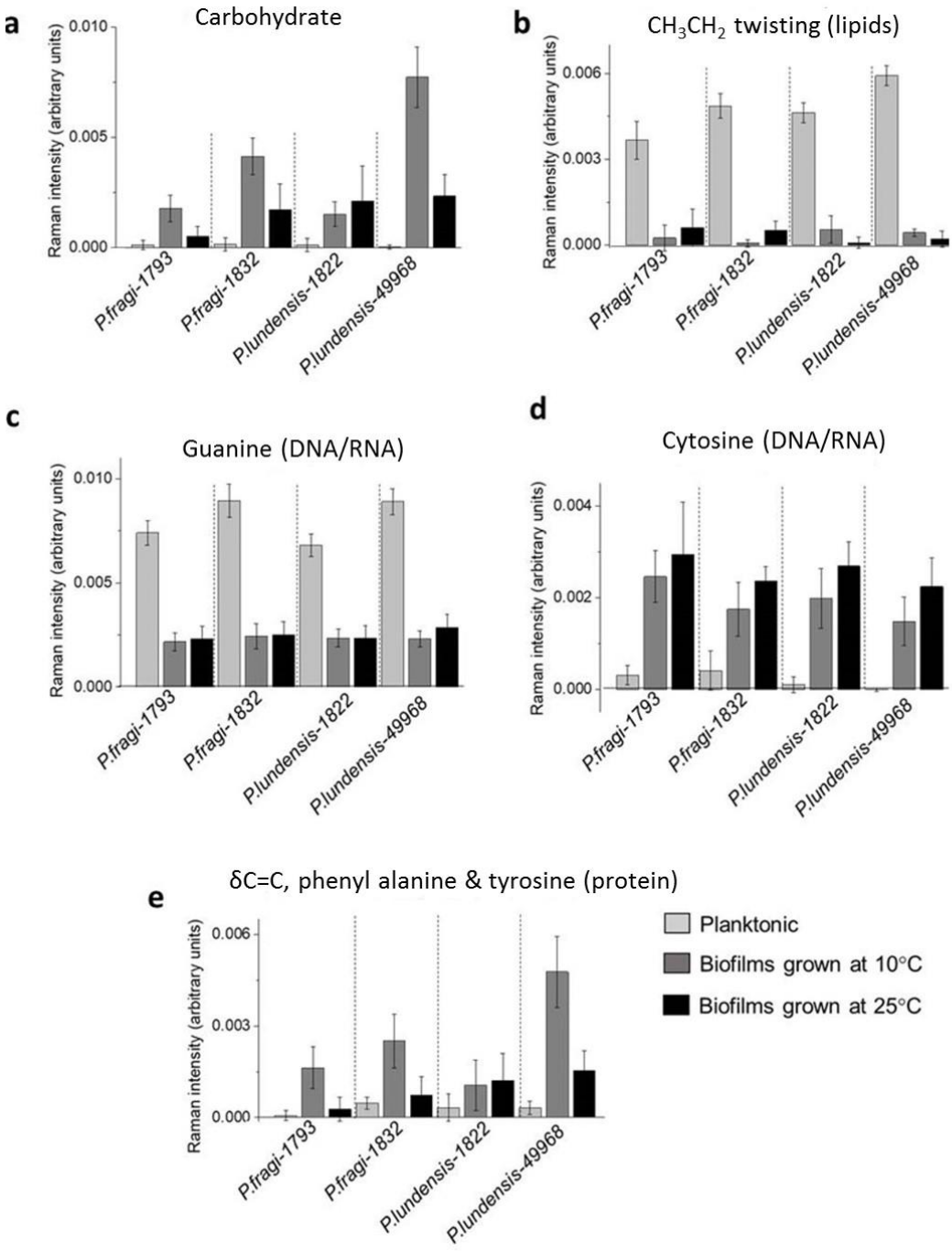

Figure 4.10. Specific peak analysis of the Raman spectra of planktonic and biofilms of the four bacterial strains. Univariate analysis was performed on the normalised intensity of carbohydrate, protein/lipid, and DNA/RNA structure-specific peaks in the Raman spectra taken from planktonic and biofilm samples. The error bars represet the variation within replicates.

The specific peaks were selected from the loading plots and the average and the standard deviations of biological replicates of Raman spectra were calculated (Figure 4.10). Specific peak analysis from the loading plots showed that the carbohydrate concentration was higher in biofilm samples of all four strains compared planktonic samples. Also, the carbohydrate content was higher in all biofilms formed at 10 °C compared to biofilms formed at 25 °C. According to the results, the protein concentration was high in biofilms of *P. fragi*, 1793 and 1832, and in *P. lundensis* ATCC 49968 grown at 10 °C. Cytosine was higher in biofilms samples grown at 25 °C as compared to 10 °C for *P. fragi* 1732 and 1832, and for *P. lundensis* ATCC 49968. The lipid content was higher in planktonic bacteria of *P. fragi* 1832 and in both *P. lundensis* strains. However, for *P. fragi* 1793, the lipid content was higher in biofilms grown at 25 °C as compared to planktonic bacteria.

4.4 Discussion

Biofilms are a dominant form of microbial life and are difficult to be completely eradicated (Nadell, Xavier, & Foster, 2009). The biofilm mode of life provides many advantages to the residing bacteria. The majority of past research studying biofilms of spoilage pseudomonads used broth culture models or abiotic surfaces (Ercolini et al., 2010; Ferrocino et al., 2009; Liu et al., 2015). Biofilms are formed in environments that are vastly different to one another and it is important to characterize them *in-situ*. The metabolic activities and by-products of microorganisms vary greatly based on the environmental conditions they are grown (Drescher et al., 2013). The production of EPS is affected by the carbon source (Miqueleto, Dolosic, Pozzi, Foresti, & Zaiat, 2010), growth conditions and environmental conditions (Avella et al., 2010). The model system used in this study closely resembles spoilage conditions on meat muscle under industry applicable conditions.

This study assessed the key matrix constituents and quantity of *P. fragi* and *P. lundensis* biofilms formed under chilled and ambient temperature conditions to assess their response to low temperature. The experimental model used in this study allowed the biofilms to be formed on porous nitro-cellulose membranes placed on surface sterilized meat. The pore size of the membranes allowed adequate access of nutrients and water from meat but prevented the substances of meat being collected during extraction. According to the CFU calculations, biofilms formed under 10 °C and 25 °C reached an approximately similar CFU counts on days 5 and 6.5. Previous studies characterizing the biofilm matrix of *P. fragi* grown directly on chicken breast pieces under modified atmospheric conditions reported substantially higher levels of protein in the matrix compared to other studies (Wang et al., 2017). It was concluded that this could be due to proteins collected from the meat interfering with the matrix composition.

This model may somewhat reduce the rate of bacterial growth due to a lack of direct contact with the meat surface. However, by monitoring bacterial cell counts we previously demonstrated that these biofilms follow a similar pattern to biofilms that are formed directly on the meat muscle (Wickramasinghe et al., 2019). Various EPS extraction methods consisting of physical, chemical or a combination of these means are available in literature. It has been found that different extraction methods can yield different results (Liang et al., 2010; Liu & Fang, 2002). The EDTA extraction method has been shown to be an efficient extraction method with minimal cell lysis (Di Martino, 2018; Liang et al., 2010; Sheng, Yu, & Yu, 2005). The biofilm mechanical breakdown process used in this method prior to chemical extraction via vortex mixing and sonicating increases the efficiency of EPS extraction (McSwain, Irvine, Hausner, & Wilderer, 2005). Total carbohydrate extracts contain charged and uncharged polysaccharides and EDTA is known to chelate metal ions that form the linkages between carbohydrates and increase the extraction efficiency (Liang et al., 2010). The formamide used in this extraction process enhances the efficiency of bound EPS extraction (Chen et al., 2017) and decrease the contamination by intracellular substances (Adav & Lee, 2008).

The EPS were extracted in bound and soluble forms to obtain a better representation of the polymer distribution. According to past research done on EPS, soluble and bound forms are distributed in different layers. Soluble EPS are distributed in outside layers in aqueous phase are weakly adhered to cells and easily dissolved in solutions (Choi et al., 2001). Bound EPS closely accumulate on the outside of cells. Tightly bound EPS strongly support the mechanical stability of biofilms (Guangyin & Youcai, 2017). Therefore, changes in SEPS and BEPS quantity can affect changes in the structure of the biofilm**.**

In this study, the key components of matrix EPS of spoilage pseudomonads were assessed. It was demonstrated that the psychrotrophic *Pseudomonas* spp. studied here responded to low temperature conditions by altering their matrix composition. The difference in total carbohydrate content was significantly higher in biofilms formed at low temperature for both *P. fragi* strains and for *P. lundensis* ATCC 49968. An increase in total protein content when biofilms were grown at 10 °C as compared to 25 °C was statistically significant for all the four strains. Previous studies have found that bio-flocculation of microbes increased with increasing protein content of the matrix (Shi et al., 2017). Some studies have also reported an increase in EPS in biofilms due to increased biomass (Choi et al., 2001). In this study the increase in EPS secretion under low temperature was not correlated to an increase in the biomass as the biofilms were extracted at approximately similar maturity and cell count levels.

Low temperatures can be considered stressful for microorganisms and stressful environmental conditions are known to increase EPS production (Chen et al., 2017; Mei, Huang, Liu, He, & Fang, 2016). The increased EPS production seen in our studies are in agreement with past research which quantified the biofilm matrix of *P. lundensis* meat isolates grown in microtiter plates (Chen et al., 2013). In that study, a higher biofilm production was detected by crystal violet staining when biofilms were grown at lower temperatures (Liu et al., 2015). This study also reported significant differences between the maximum amounts of biofilm produced at 30, 10 and 4 °C. Similar results were observed for *Pseudomonas putida* which is another psychrotrophic meat spoilage organism. When grown as biofilms on polyvinyl chloride coupons under low and ambient

temperatures, and stained with crystal violet, higher levels of biofilms formed at lower temperatures (Morimatsu, Eguchi, Hamanaka, Tanaka, & Uchino, 2012). The EPS and total carbohydrate production of *P. putida* biofilms increased with increasing matrix stress due to water loss (Chang et al., 2007). Biofilms formed under high shear stress conditions grow to be more dense and high hydrodynamic shear forces appear to promote production of excessive cellular polysaccharides (Ohashi & Harada, 1994). Based on the results of our study and past studies it appears that the production of EPS increases under stressful environmental conditions. Apart from providing protection against stressful environmental conditions, EPS also acts as a carbon/energy reservoir during biological processes (Guangyin & Youcai, 2017).

An interesting observation of this study is that marked differences can be seen in the levels of carbohydrate and protein between the *P. fragi* and *P. lundensis* biofilm matrix despite the close taxonomic distance between these species (Ercolini et al., 2007). *Pseudomonas fragi* strains had a higher proportion of total carbohydrates and a comparatively lower proportion of total proteins in matrix EPS compared with *P. lundensis*. It has been established that the carbohydrates in the matrix play an important role in a biofilms structure and stability. A previous study by Tay and Liu (2001) established that the disappearance of aerobic sludge granules was closely related to a reduction in cellular polysaccharides and the polysaccharides help to stabilize the biofilm. The high concentration of carbohydrate may therefore help *P. fragi* strains to produce mechanically stable biofilms. Also *P. fragi* had higher carbohydrate content in bound EPS compared to *P. lundensis* strains at 25 °C which indicates the *P. fragi* matrix can be mechanically stronger than *P. lundensis* even at ambient temperatures.

Previous results in chapter 03, *P. fragi* and *P. lundensis* biofilm growth directly on sterile beef muscle detected significant differences in microstructural and cellular arrangement between these two species. Confocal laser scanning microscopy images of fluorescently stained biofilms showed that *P. fragi* produced highly dense, compact, flat biofilms of vertically oriented cells with limited intercellular gaps. In contrast, *P. lundensis* produced biofilms with loosely arranged cells with considerable intercellular gaps and voids. Studies on *Vibrio cholerae* biofilms which have a similar cellular arrangement to *P. fragi*,

have shown that one of the matrix proteins, namely RbmA, aids in binding cells closely together (Drescher et al., 2016). Such matrix material helped to form biofilms with tightly coherent cells which limited the entry of foreign cells into the biofilm and promoted invasion resistance (Carey et al., 2015; Drescher et al., 2016). It can be hypothesized that the cellular arrangement of *P. fragi* strains could be a result of the matrix composition. Studies have also found that *P. fragi* becomes the predominant bacterial species on long term stored chilled meat (Lebert et al., 1998). The cellular arrangement and the matrix composition may aid in their long-term survival.

Extra-cellular DNA is an important component of the biofilm matrix. Extra-cellular DNA is released to the matrix via cellular disruption and/or through membrane vesicles (Whitchurch, Tolker-Nielsen, Ragas, & Mattick, 2002). It can be hypothesized that the significantly higher levels of eDNA and protein detected in the low temperature grown *P. lundensis* ATCC 49968 strain could also be due cellular disruption and cell death as a result of the lower temperature**.** However, lysed cells are not the only source of eDNA, and further studies are necessary to assess if the DNA are genomic DNA released due to cell death or secreted actively.

In this study, there was not a strong correlation between matrix eDNA production and temperature levels. *Pseudomonas fragi* strains 1793 and 1832 and *P. lundensis* ATCC 49968 had a higher eDNA content when grown under low temperature while *P. lundensis* 1822 had a slightly lower eDNA content under low temperature. These results are in agreement with our previous studies, where no significant difference could be detected in the levels of eDNA production between several *P. fragi* and *P. lundensis* strains formed at different temperatures (Wickramasinghe et al., 2019). Strain level differences in eDNA production are apparent for *P. fragi* and *P. lundensis* rather than species level differences.

In this study the total quantity of carbohydrates, proteins and eDNA in the matrix EPS were assessed by chemical analysis. A detailed analysis of the types of carbohydrates and

types proteins in the biofilm matrix EPS is important to identify specific functions of these polymers. However, in complex eco-systems such as biofilms, polysaccharides form bonds with proteins and other substances and form complex chemical complexes which make the identification of these compounds difficult. Also, due to the diversity in sugar monomers and linkages, it is difficult to isolate and characterize specific polysaccharides from total carbohydrates in environmental samples (Flemming et al., 2007). Therefore, chemical analysis should be combined with other analytical methods when studying matrix composition.

An advantage of Raman spectroscopy is that an overall estimation of the chemical components present in a biological substance and their relative abundances can be determined. It is a non-invasive, laser based technology which detects inelastic scattering of the monochromatic light and each chemical vibration is assigned to a specific Raman wavenumber (Votteler, Carvajal Berrio, Pudlas, Walles, & Schenke-Layland, 2012). Staining the matrix components with specific fluorescent dyes can also provide an estimation of the chemical structure in a biofilm (Chen, Lee, & Tay, 2007). However, such methods have limitations due to the limited availability of specific dyes as well as the limitations in dye penetration (Chao & Zhang, 2012). Apart from assessing the total carbohydrates, total proteins and eDNA, the Raman spectra provides a view of the overall chemical composition and its intensities in biofilm and planktonic samples.

This study used Raman spectroscopy to detect biochemical differences between planktonic and biofilm bacteria (Beier, Quivey, & Berger, 2012; Wang et al., 2013). Since media residues were washed off the planktonic bacteria, the Raman spectra obtained from them can be used as a reference to observe the chemical changes that occurred during biofilm formation. Theoretically, the Raman peak intensity is directly proportional to the concentration of the represented chemical constituents (Chan & Lieu, 2009).

Based on the Raman spectral intensity, it is clear that guanine was present in high levels in planktonic samples as compared to biofilm samples. The exact reason for such a difference is currently unknown. However, a universal trait of all biofilms is the response

to secondary messenger cyclic dimeric guanosine monophosphate (cyclic di-GMP) (Baudin et al., 2017; McDougauldet al., 2011). A high cyclic di-GMP level necessary for biofilm formation while a decrease in cyclic di-GMP levels can lead to biofilm dispersal. Guanosine is a nucleoside derived from guanine and ribose. Guanine at biofilm stage could be used for the development of cyclic di-GMP. Thus, the detection of low levels of guanine in biofilms samples could likely be that it is utilized for cyclic-di-GMP formation.

According to the results of the loading plots, biofilms contained high levels of phenyl alanine. Some studies have observed an increase in biofilm formation in *Pseudomonas aeruginosa* in the presence of certain amino acids including phenylalanine (Bernier, Ha, Khan, Merritt, & O'toole, 2011). According to the Raman spectra, biofilms contain high level of carbohydrates compared to planktonic bacteria. At the same time biofilms grown at 10 °C had high spectral intensity of carbohydrates compared to 25 °C grown biofilms. Past studies have reported that Raman spectra of *Klebsiella pneumonia, Escherichia coli*, and *P. aeruginosa* biofilms contained a larger amount of polysaccharides compared to the planktonic cells (Kusić et al., 2015). However, the same study reported lower spectral peaks for proteins and nucleic acids in biofilms.

The results of Raman spectroscopy correlate with the results of chemical analysis where high carbohydrate and protein contents were detected in low temperature stored samples. The spectral peaks of biofilm samples were less prominent compared to planktonic spectra. This maybe due to the presence of large amount of complex matrix material surrounding the cells. Biofilms are highly complex and consist of a heterogeneous mixture of biomolecules that contribute to the collected Raman spectra. Therefore, its spectral pattern can be complex (Heydorn et al., 2000).

The chemical analysis results showed differences in EPS composition between the tested *P. fragi* and *P. lundensis* strains as described above. However, species specific differences in matrix carbohydrate and protein could not be detected using Raman spectroscopy. Also, the differences in the matrix EPS composition between the two

temperatures were clearer for all the strains in the chemical analysis data compared to spectroscopic data. These differences are likely caused by the differences in data acquisition. For the chemical analysis, the bacterial cells were separated from the biofilm matrix and EPS were measured from the liquid. For the Raman spectroscopy, the spectra were obtained by focusing on single cells and their immediate surroundings of the membrane grown biofilms (Hlaing et al., 2016). In order to confirm whether the differences in carbohydrate and protein composition are species specific, more bacterial strains need to be assessed. A greater understanding of the composition of the biofilm matrix *in situ* determined in this study may contribute to establishing mechanisms to disrupt biofilms formation on meat and extend shelf-life.

4.6 Conclusion

Previous studies have tested chemical compounds, including heavy metals such as mercury or copper and proteases, that target and degrade EPS of the biofilm (Teitzel & Parsek, 2003). However, heavy metals and large amounts of proteases are not suitable for the control of biofilms formed on meat. Other studies have applied glycosidases, proteases and DNases to and their combinations to degrade the extracellular matrix (Algburi, Comito, Kashtanov, Dicks, & Chikindas, 2017). The effectiveness of these enzymes can depend on environmental conditions and their effect on meat is unknown. In order to select a suitable approach to target the biofilm matrix on meat, a detailed knowledge of the main matrix components and their proportions under practical industry conditions is important. This study provides an insight into the main matrix components of meat spoilage pseudomonads and their proportions and changes induced during chilled storage. This knowledge is useful for the development of biofilm degrading compounds for food material.

4.7 References

Adav, S. S., & Lee, D.-J. (2008). Extraction of extracellular polymeric substances from aerobic granule with compact interior structure. *Journal of Hazardous Materials, 154*(1), 1120-1126. doi:10.1016/j.jhazmat.2007.11.058

Algburi, A., Comito, N., Kashtanov, D., Dicks, L., & Chikindas, M. (2017). Control of Biofilm Formation: Antibiotics and Beyond. *Applied Environmental Microbiology., 83*(3). doi:10.1128/AEM.02508-16

Avella, A. C., Delgado, L. F., Görner, T., Albasi, C., Galmiche, M., & de Donato, P. (2010). Effect of cytostatic drug presence on extracellular polymeric substances formation in municipal wastewater treated by membrane bioreactor. *Bioresource Technology, 101*(2), 518-526. doi:10.1016/j.biortech.2009.08.057

Barraud, N., Kjelleberg, S., & Rice, S. A. (2015). Dispersal from microbial biofilms. *Microbiology spectrum, 3*(6). doi:10.1128/microbiolspec.MB-0015-2014

Baudin, M., Cinquin, B., Sclavi, B., Pareau, D., & Lopes, F. (2017). Understanding the fundamental mechanisms of biofilms development and dispersal: BIAM (Biofilm Intensity and Architecture Measurement), a new tool for studying biofilms as a function of their architecture and fluorescence intensity. *Journal of Microbiological Methods, 140*, 47-57. doi:10.1016/j.mimet.2017.06.021

Beier, B., Quivey, R., & Berger, A. (2012). Raman microspectroscopy for species identification and mapping within bacterial biofilms. *AMB Express, 2*(1), 1-6. doi:10.1186/2191-0855-2-35

Bernier, S. P., Ha, D.-G., Khan, W., Merritt, J. H., & O'toole, G. A. (2011). Modulation of Pseudomonas aeruginosa surface-associated group behaviors by individual amino acids through c-di-GMP signaling. *Research in Microbiology, 162*(7), 680-688. doi:10.1016/j.resmic.2011.04.014

Carey, D. N., Knut, D., Ned, S. W., & Bonnie, L. B. (2015). Extracellular matrix structure governs invasion resistance in bacterial biofilms. *The ISME Journal, 9*(8). doi:10.1038/ismej.2014.246

Chan, J. W., & Lieu, D. K. (2009). Label-free biochemical characterization of stem cells using vibrational spectroscopy. *Journal of Biophotonics, 2*(11), 656-668. doi:10.1002/jbio.200910041

Chang, W.-S., van de Mortel, M., Nielsen, L., Nino de Guzman, G., Li, X., & Halverson, L. J. (2007). Alginate production by *Pseudomonas putida* creates a hydrated microenvironment and contributes to biofilm architecture and stress tolerance under water-limiting conditions. *The Journal of Bacteriology, 189*(22), 8290.

Chao, Y., & Zhang, T. (2012). Surface-enhanced Raman scattering (SERS) revealing chemical variation during biofilm formation: from initial attachment to mature biofilm. *Analytical and Bioanalytical Chemistry, 404*(5), 1465-1475. doi:10.1007/s00216-012-6225-y

Chen, M., Lee, D., & Tay, J. (2007). Distribution of extracellular polymeric substances in aerobic granules. *Applied Microbiology and Biotechnology, 73*(6), 1463-1469. doi:10.1007/s00253-006-0617-x

Chen, W., Hu, H., Zhang, C., Huang, F., Zhang, D., & Zhang, H. (2017). Adaptation response of *Pseudomonas fragi* on refrigerated solid matrix to a moderate electric field. *BMC Microbiology*, *17*(1). doi:10.1186/s12866-017-0945-2

Chen, Y.-P., Zhang, P., Guo, J.-S., Fang, F., Gao, X., & Li, C. (2013). Functional groups characteristics of EPS in biofilm growing on different carriers. *Chemosphere*, *92*(6), 633-638. doi:10.1016/j.chemosphere.2013.01.059

Choi, E., Yun, Z., Park, Y., Lee, H., Jeong, H., & Kim, K. (2001). Extracellular polymeric substances in relation to nutrient removal from a sequential batch biofilm reactor. *Water Science & Technology*, *43*(6), 185-185. doi:10.2166/wst.2001.0371

Delaquis, P. J., Gariépy, C., & Montpetit, D. (1992). Confocal scanning laser microscopy of porcine muscle colonized by meat spoilage bacteria. *Food Microbiology, 9*(2), 147-153. doi:10.1016/0740-0020(92)80021-U

Di Martino, P. (2018). Extracellular polymeric substances, a key element in understanding biofilm phenotype. *AIMS Microbiology, 4*(2), 274-288. doi:10.3934/microbiol.2018.2.274

Diane, M., Scott, A. R., Nicolas, B., Peter, D. S., & Staffan, K. (2011). Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nature Reviews Microbiology*, *10*(1), 39. doi:10.1038/nrmicro2695

Donlan, R. M. (2002). Biofilms: microbial life on surfaces. *Emerging infectious diseases*, *8*(9), 881-890. doi:10.3201/eid0809.020063

Doulgeraki, A. I., Ercolini, D., Villani, F., & Nychas, G.-J. E. (2012). Spoilage microbiota associated to the storage of raw meat in different conditions. *International Journal of Food Microbiology*, *157*(2), 130-141. doi:10.1016/j.ijfoodmicro.2012.05.020

Drescher, K., Dunkel, J., Nadell, C. D., van Teeffelen, S., Grnja, I., Wingreen, N. S., Bassler, B. L. (2016). Architectural transitions in *Vibrio cholerae* biofilms at single-cell resolution. *Proceedings of the National Academy of Sciences of the United States of America, 113*(14), E2066. doi:10.1073/pnas.1601702113

Drescher, K., Shen, Y., Bassler, B. L., & Stone, H. A. (2013). Biofilm streamers cause catastrophic disruption of flow with consequences for environmental and medical systems. *Proceedings of the National Academy of Sciences of the United States of America, 110*(11), 4345. doi:10.1073/pnas.1300321110

Ercolini, D., Casaburi, A., Nasi, A., Ferrocino, I., Di Monaco, R., Ferranti, P., Villani, F. (2010). Different molecular types of *Pseudomonas fragi* have the same overall behaviour as meat spoilers. *International Journal of Food Microbiology*, *142*(1), 120-131. doi:10.1016/j.ijfoodmicro.2010.06.012

Ercolini, D., Russo, F., Blaiotta, G., Pepe, O., Mauriello, G., & Villani, F. (2007). Simultaneous detection of *Pseudomonas fragi, P. lundensis,* and *P. putida* from meat by use of a multiplex PCR assay targeting the *carA* Gene. *Applied and Environmental Microbiology*, *73*(7), 2354.

Ercolini, D., Russo, F., Torrieri, E., Masi, P., & Villani, F. (2006). Changes in the spoilage- related microbiota of beef during refrigerated storage under different packaging conditions. *Applied and environmental microbiology*, *72*(7), 4663. doi:10.1128/AEM.00468-06

Ferrocino, I., Ercolini, D., Villani, F., Moorhead, S. M., & Griffiths, M. W. (2009). *Pseudomonas fragi* strains isolated from meat do not produce N-acyl homoserine lactones as signal molecules. *Journal of Food Protection, 72*(12), 2597-2601.

Flemming, H.-C., Neu, T. R., & Wozniak, D. J. (2007). The EPS Matrix: The "House of Biofilm Cells". *The Journal of Bacteriology, 189*(22), 7945. doi:10.1128/JB.00858-07

Gram, L., Ravn, L., Rasch, M., Bruhn, J. B., Christensen, A. B., & Givskov, M. (2002). Food spoilage—interactions between food spoilage bacteria. *International Journal of Food Microbiology, 78*(1), 79-97. doi:10.1016/S0168-1605(02)00233-7

Guangyin, Z., & Youcai, Z. (2017). Chapter Five - Harvest of bioenergy from sewage sludge by anaerobic digestion. In Z. Guangyin & Z. Youcai (Eds.), *Pollution Control and Resource Recovery for Sewage Sludge*. 181-273, Butterworth-Heinemann.

Hans-Curt, F., & Jost, W. (2010). The biofilm matrix. *Nature Reviews Microbiology, 8*(9), 623. doi:10.1038/nrmicro2415

Heydorn, A., Nielsen, A., Hentzer, M., Sternberg, C., Givskov, M., Ersboell, B., & Molin, S. (2000). Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology, 146*(10), 2395-2407. doi:10.1099/00221287-146-10-2395

Hlaing, M. M., Dunn, M., Stoddart, P. R., & McArthur, S. L. (2016). Raman spectroscopic identification of single bacterial cells at different stages of their lifecycle. *Vibrational Spectroscopy, 86*(C), 81-89. doi:10.1016/j.vibspec.2016.06.008

Kramer, R. (1998). Chemometric techniques for quantitative analysis. New York: New York: Marcel Dekker. Baton Rouge: CRC Press

Kusić, D., Kampe, B., Ramoji, A., Neugebauer, U., Rösch, P., & Popp, J. (2015). Raman spectroscopic differentiation of planktonic bacteria and biofilms. *Analytical and Bioanalytical Chemistry, 407*(22), 6803-6813. doi:10.1007/s00216-015-8851-7

Lebert, I., Begot, C., & Lebert, A. (1998). Growth of *Pseudomonas fluorescens* and *Pseudomonas fragi* in a meat medium as affected by pH (5.8–7.0), water activity

(0.97–1.00) and temperature (7–25°C). *International Journal of Food Microbiology, 39*(1), 53-60. doi:10.1016/S0168-1605(97)00116-5

Liang, Z., Li, W., Yang, S., & Du, P. (2010). Extraction and structural characteristics of extracellular polymeric substances (EPS), pellets in autotrophic nitrifying biofilm and activated sludge. *Chemosphere, 81*(5), 626-632. doi:10.1016/j.chemosphere.2010.03.043

Liu, H., & Fang, H. H. P. (2002). Extraction of extracellular polymeric substances (EPS) of sludges. *Journal of Biotechnology, 95*(3), 249-256. doi:10.1016/S0168-1656(02)00025-1

Liu, Y. J., Xie, J., Zhao, L. J., Qian, Y. F., Zhao, Y., & Liu, X. (2015). Biofilm formation characteristics of *Pseudomonas lundensis* isolated from meat. *Journal of Food Science, 80*(12), M2904-M2910. doi:10.1111/1750-3841.13142

Lorenz, B., Wichmann, C., Stöckel, S., Rösch, P., & Popp, J. (2017). Cultivation-free Raman spectroscopic investigations of bacteria. *Trends in Microbiology, 25*(5), 413-424. doi:10.1016/j.tim.2017.01.002

Luyan, M., Matthew, C., Haiping, L., Matthew, R. P., Kenneth, B., & Daniel, J. W. (2009). Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. *PLoS Pathogens, 5*(3), e1000354. doi:10.1371/journal.ppat.1000354

McSwain, B. S., Irvine, R. L., Hausner, M., & Wilderer, P. A. (2005). Composition and distribution of extracellular polymeric substances in aerobic flocs and granular sludge. *Applied and environmental microbiology, 71*(2), 1051. doi:10.1128/AEM.71.2.1051-1057.2005

Mei, Y.-Z., Huang, P.-W., Liu, Y., He, W., & Fang, W.-W. (2016). Cold stress promoting a psychrotolerant bacterium *Pseudomonas fragi* P121 producing trehaloase. *World Journal of Microbiology and Biotechnology, 32*(8), 1-9. doi:10.1007/s11274-016-2097-1

Miqueleto, A. P., Dolosic, C. C., Pozzi, E., Foresti, E., & Zaiat, M. (2010). Influence of carbon sources and C/N ratio on EPS production in anaerobic sequencing batch biofilm reactors for wastewater treatment. *Bioresource Technology, 101*(4), 1324-1330. doi:10.1016/j.biortech.2009.09.026

Molin, G., Ternstrom, A., & Ursing, J. (1986). *Pseudomonas lundensis*, a new bacterial species isolated from meat. *International Journal of Systematic Bacteriology, 36*(2), 339-342. doi:10.1099/00207713-36-2-339

Morimatsu, K., Eguchi, K., Hamanaka, D., Tanaka, F., & Uchino, T. (2012). Effects of temperature and nutrient conditions on biofilm formation of *Pseudomonas putida. Food Science and Technology Research, 18*(6), 879-883.

Nadell, C. D., Xavier, J., & Foster, K. (2009). The sociobiology of biofilms *Fems Microbiololgy Reviews.* (33) 206-224.

Nawaz, H., Bonnier, F., Knief, P., Howe, O., Lyng, F. M., Meade, A. D., & Byrne, H. J. (2010). Evaluation of the potential of Raman microspectroscopy for prediction of chemotherapeutic response to cisplatin in lung adenocarcinoma. *Analyst, 135*(12), 3070-3076. doi:10.1039/c0an00541j

Nychas, G.-J. E., Skandamis, P. N., Tassou, C. C., & Koutsoumanis, K. P. (2008). Meat spoilage during distribution. *Meat Science, 78*(1), 77-89. doi:10.1016/j.meatsci.2007.06.020

Ohashi, A., & Harada, H. (1994). Adhesion strength of biofilm developed in an attached-growth reactor. *Water Science and Technology, 29*(10-11), 281-288. doi:10.2166/wst.1994.0771

Sandeep Kaur, S., Gopaljee, J., & Prakash Chandra, M. (2019). Enzymatic degradation of biofilm by metalloprotease from microbacterium sp. SKS10. *Frontiers in Bioengineering and Biotechnology, 7*. doi:10.3389/fbioe.2019.00192

Sheng, G.-P., Yu, H.-Q., & Yu, Z. (2005). Extraction of extracellular polymeric substances from the photosynthetic bacterium *Rhodopseudomonas acidophila. Applied Microbiology and Biotechnology, 67*(1), 125-130. doi:10.1007/s00253-004-1704-5

Shi, Y., Huang, J., Zeng, G., Gu, Y., Chen, Y., Hu, Y., Shi, L. (2017). Exploiting extracellular polymeric substances (EPS) controlling strategies for performance enhancement of biological wastewater treatments: An overview. *Chemosphere, 180*, 396-411. doi:10.1016/j.chemosphere.2017.04.042

Stöckel, S., Stanca, A., Helbig, J., Rösch, P., & Popp, J. (2015). Raman spectroscopic monitoring of the growth of pigmented and non-pigmented mycobacteria.

Analytical and Bioanalytical Chemistry, *407*(29), 8919-8923. doi:10.1007/s00216-015-9031-5

Sutherland, I. W. (2001). The biofilm matrix -an immobilized but dynamic microbial environment. *Trends in Microbiology*, *9*(5), 222-227. doi:10.1016/S0966-842X(01)02012-1

Tay, J. H., Liu, Q. S., & Liu, Y. (2001). The role of cellular polysaccharides in the formation and stability of aerobic granules. *Letters in Applied Microbiology*, *33*(3), 222-226. doi:10.1046/j.1472-765x.2001.00986.

Teitzel, G. M., & Parsek, M. R. (2003). Heavy Metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology, 69*(4), 2313. doi:10.1128/AEM.69.4.2313-2320.2003

Votteler, M., Carvajal Berrio, D. A., Pudlas, M., Walles, H., & Schenke-Layland, K. (2012). Non-contact, label-free monitoring of cells and extracellular matrix using Raman Spectroscopy. *Journal of Visualized Experiments*(63). doi:10.3791/3977

Wang, G.-y., Li, M., Ma, F., Wang, H.-h., Xu, X.-l., & Zhou, G.-h. (2017). Physicochemical properties of *Pseudomonas fragi* isolates response to modified atmosphere packaging. *FEMS Microbiology Letters, 364*(11). doi:10.1093/femsle/fnx106

Wang, G. Y., Ma, F., Wang, H. H., Xu, X. L., & Zhou, G. H. (2017). Characterization of extracellular polymeric substances produced by *Pseudomonas fragi* under Air and modified atmosphere Packaging. *Journal of Food Science, 82*(9), 2151-2157. doi:10.1111/1750-3841.13832

Wang, H., Ding, S., Wang, G., Xu, X., & Zhou, G. (2013). *In situ* characterization and analysis of *Salmonella* biofilm formation under meat processing environments using a combined microscopic and spectroscopic approach. *International Journal of Food Microbiology, 167*(3), 293-302. doi:10.1016/j.ijfoodmicro.2013.10.005

Whitchurch, C. B., Tolker-Nielsen, T., Ragas, P. C., & Mattick, J. S. (2002). Extracellular DNA required for bacterial biofilm formation. *Science (New York, N.Y.), 295*(5559), 1487-1487. doi:10.1126/science.295.5559.1487

Wickramasinghe, N. N., Ravensdale, J., Coorey, R., Chandry, S. P., & Dykes, G. A. (2019). The Predominance of psychrotrophic pseudomonads on aerobically stored

chilled red meat. *Comprehensive Reviews in Food Science and Food Safety, 18*(5), 1622-1635. doi:10.1111/1541-4337.12483

Wickramasinghe, N. N., Ravensdale, J. T., Coorey, R., Dykes, G. A., & Scott Chandry, P. (2019). characterisation of biofilms formed by psychrotrophic meat spoilage pseudomonads. *Biofouling*, 1. doi:10.1080/08927014.2019.1669021

CHAPTER 5

Transcriptional profiling of biofilms formed on chilled beef by psychrotrophic meat spoilage bacterium, *Pseudomonas fragi* 1793

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5.1 Abstract

Pseudomonas fragi is the predominant bacterial species associated with spoiled aerobically stored chilled meat worldwide. It readily forms biofilms on meat under refrigeration used in the meat industry. Biofilm growth leads to slime development on meat which in turn becomes a major quality defect. In this research, RNA sequencing was carried out for the main stages of *P. fragi* strain 1793 grown on aerobically stored meat at 10 °C. RNA was extracted at different stages of the biofilm cycle namely initiation, maturation and dispersal. At the same time, the biofilm growth was assessed by fluorescent staining and imaging using confocal laser scanning microscopy. The results of RNA sequencing were verified by qRT-PCR using twelve genes that were most significantly up and down regulated at each stage. Differential expression analysis at biofilm maturation revealed 332 significantly upregulated genes and 37 downregulated genes relative to initiation. Differential expression analysis at biofilm dispersal reveled 658 upregulated and 275 downregulated genes relative to initiation. During biofilm maturation and dispersal, genes coding for Flp family type IVb pilin, ribosome modulation factor and creatininase were the most upregulated genes while genes encoding for iron uptake systems and taurine transport were significantly down regulated. The results show that protein synthesis and cellular multiplication cease when the biofilm population maximum has reached. The results of this study may have potential applications in the control of biofilms on meat tissue.

5.2 Introduction

Meat is a rich source of nutrients with high water activity and is a highly perishable food commodity (Ercolini, Russo, Torrieri, Masi, & Villani, 2006). Spoilage of commercial meat by microorganisms causes considerable financial losses to the meat industry (Nychas, Skandamis, Tassou, & Koutsoumanis, 2008). To minimize spoilage, the industry uses chilled temperature conditions for meat storage and transportation. However, psychrotrophic bacterial species can still grow and cause spoilage under low temperature conditions (Ercolini et al., 2006). Psychrotrophic *Pseudomonas* species are the key spoilage organisms in aerobically stored chilled meat (Ercolini et al., 2007). These species are equipped with many metabolic traits which aid them in withstanding the harsh environmental conditions under low temperature storage and in overcoming competition from other psychrotrophic spoilage organisms (Wickramasinghe, Ravensdale, Coorey, Chandry, & Dykes, 2019).

Among the psychrotrophic spoilage pseudomonads species, *Pseudomonas fragi* is the most frequently encountered spoilage species on meat including beef, chicken, pork, lamb and fish, worldwide (Ferrocino, Ercolini, Villani, Moorhead, & Griffiths, 2009; Lebert, Begot, & Lebert, 1998). *Pseudomonas fragi* readily form biofilms on meat under refrigerated temperature conditions used in the meat industry (Wickramasinghe et al., 2019). When biofilms combine with meat exudate it results in slime formation which is a key quality defect that leads to consumer rejection of meat. Biofilms are formed when planktonic bacteria attach to a surface or to each other and embed themselves in a self-produced or an acquired exo-polymeric matrix (Flemming et al., 2016). Biofilms protect the residing bacteria from harsh environmental conditions including desiccation, radiation, predation and antimicrobial compounds (McDougald et al., 2011). It is likely that biofilm formation provides *P. fragi* with a competitive advantage which allows them to survive stressful environmental conditions and become the predominant spoilage flora on long-term stored chilled meat. Biofilms undergo key distinct stages in their life which include irreversible attachment, initiation, maturation and dispersal (Seckbach, 2010). Each of these stages are controlled by specific sets of genes which regulates different metabolic functions.

Planktonic *P. fragi* are well studied in broth culture models but limited information is available on its biofilms formed on muscle tissue. When studying the characteristics of bacterial biofilms, it is important to study them *in-situ* or using industry applicable experimental model systems. The metabolic reactions, rates and by-products as well as expression of genes can vary greatly based on the environmental conditions that they are grown under (Ercolini, Russo, Nasi, Ferranti, & Villani, 2009). Therefore, it is important to design experimental model systems that closely mimic the practical spoilage conditions in the meat industry.

In this study, we investigated the gene expression using transcriptome profiling of *P. fragi* biofilms formed on beef muscles, aerobically stored under abuse temperature conditions common in the meat industry. We evaluated differentially expressed genes at each of three key stages of the biofilm cycle, namely biofilm initiation, maturation and dispersal. This transcriptional study provides information on the key genes which aid *P. fragi* biofilms to successfully grow on chilled meat. To the best of our knowledge, the data presented in this study provide the first report of the molecular basis of *P. fragi* growth on chilled beef

5.3 Material and methods

***Biofilm growth on meat*:** Based on our previous work, *P. fragi* strain 1793 was selected due to its rapid and dense biofilm formation on meat (Wickramasinghe et al., 2019). Beef 'eye round' cut was used throughout the series of experiments to minimize the variability that can arise from the composition of different cuts. The beef was purchased from local butchers as 3 kg cuts and brought chilled to the laboratory within 20 minutes. The meat was kept at 3 °C until processing.

Since the interior tissues of healthy living animals are considered free from microorganisms (Gill & Penney, 1977; Mackey & Derrick, 1979), a surface sterilization method was applied. The beef was surface sterilized by immersing the meat in boiling water for 10 minutes. Meat was removed from water, the cooked exterior was aseptically excised and was sliced into 3 mm thick cuts using a sterilized, stainless steel deli-slicer placed inside a laminar flow hood. The meat slices were further sectioned into circular shapes with a diameter of 10 cm and were placed in petri plates.

Overnight cultures of *P. fragi* 1793 were prepared by inoculating a single colony into 5 ml of tryptone soy broth (TSB, Oxoid, Basingstoke, United Kingdom) and incubating it at 25 °C for 18 hours in a shaking incubator at 180 rpm. Then the cultures were decimally diluted, and the meat slices were inoculated with approximately 10^4 CFU cm^{-2}. Then the petri plates were covered with lids and meat slices were incubated at 10 °C in a static incubator. Biofilms were extracted from meat as described below at 48, 76 and 115 hours, which correlate to time points of biofilm initiation, maturation and dispersal (Wickramasinghe, Ravensdale, Coorey, Dykes, et al., 2019).

***Biofilm staining and imaging*:** To assess the structural and cellular transformations over time of biofilms formed on meat, meat samples incubated at 10 °C were imaged from day one to day six. At each time point, the samples were prepared for Confocal Laser Scanning Microscope (CLSM) imaging using a Leica SP5 (Leica Microsystems, Heidelberg, Germany) microscope. Live/Dead® BacLight™ Kit (L7012, Molecular Probes-Life Technologies, Eugene, OR, USA) was used to stain the biofilms. The stained samples were incubated in the dark at around 25 °C and placed on glass slides. A cover slip was gently placed on top of each muscle slice and samples were imaged under the 100x oil immersion objective.

Stained meat samples were excited with the 488 nm laser line from an Argon laser at 20% intensity. The samples were scanned at 200 Hz speed and imaged using 1200 * 1200 resolutions. The emission wavelength was collected between 490-590 nm for SYTO 9 (displayed green for live cells) and between 600- 650 nm for PI (displayed red for dead cells). Images (Z stacks) were acquired from six different locations on the meat surface which included the centre, edges and in between.

***RNA extraction process*:** Samples for RNA sequencing were taken at biofilm initiation (48 h), maturation (76 h) and dispersal (115 h) stages. The time points of extraction were selected based on biofilm cell numbers calculated in previous cell cycle studies (Wickramasinghe et al., 2019). RNA was extracted with the RNeasy minikit (Qiagen, MD, cat no-74104) using the protocol for disruption of bacteria grown on solid media, according to manufacturer's instructions. A stabilization mixture of the RNA protect bacteria (Qiagen, MD) and TSB was added to the meat surface with the biofilm. Then the biofilm was gently extracted using a sterile cell scraper and pipetted into a centrifuge tube. The tube was vortex mixed and then centrifuged at 5000 g for 10 minutes.

Additional changes were made to the manufacturer's protocol for enzymatic lysis and proteinase K digestion, which included the addition of 15 µl of Proteinase K (Qiagen, MD) and an increase in the amount of lysozyme to 15 mg ml^{-1} in the TE buffer. Then the biofilm was mechanically disrupted using a tissue lyser (SpeedMill) for 5 min at maximum speed. Genomic DNA was removed using on column DNase digestion with DNAse digestion kit (Qiagen, MD, cat no 79254). After extraction, total RNA yield was quantified using a Nanodrop (ND-1000, ThermoFisher Scientific). The RNA integrity of each extract was determined by 16S and 23S rRNA peak examination using a Tape station 2100 (Agilent, Santa Clara, CA).

Since the biofilms were grown on the beef muscle, bacterial RNA may be contaminated with bovine RNA. For this reason, during rRNA depletion, both bacterial and bovine rRNA were removed. The rRNA removal, cDNA synthesis, library preparation and sequencing of the samples was carried out by Next Generation Sequencing facility of Western Sydney University, Australia. The library type was Zymo-Seq Ribofree total RNA. The sequencing was carried out on Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA, USA) and 126 bp length paired-end reads were generated (appendix 3).

***Analysis of sequenced data*:** The quality of the sequenced reads was assessed using FastQC (Galaxy Version 0.72+galaxy1) function in Galaxy (https://usegalaxy.org.au/). As the sequencing reads are of high quality with high per base sequence quality and without any adaptor contamination, trimming of the data was not carried out. The reads were mapped to the *P. fragi* 1793 reference genome (Gene bank accession no NQKS00000000) with the Burrows-Wheeler Aligner (version 0.7.17) using the BWA-MEM algorithm. The mapped reads, which were in BAM format, were visualised using Jbrowse genome browser (version 1.16.4) to determine the efficiency of mapping. Mapped reads were used in subsequent analyses. To estimate the number of reads overlapping with each gene in the reference genome, the SAM/BAM to count matrix of Galaxy was used.

Differential expression was examined using the Voom tool (Galaxy Version 0.28) with a differential count model which provided statistical routines to identify differentially expressed genes (DEGs) between the stages (Law, Chen, Shi, & Smyth, 2014). The gene expression of *P. fragi* in the three stages of the biofilm: (Initiation vs Maturation), (Initiation vs Dispersal), and (Maturation vs Dispersal) were compared pairwise using the DEGUST tool (http://degust.erc.monash.edu/). Genes with a False Discovery Rate (FDR)/adjusted p-value <0.01 and of log2-fold change (log FC) higher or lower than 2.0 were identified as being significantly differentially expressed. The log2 Fold Change of gene expression shows the fold change in the treatment relative to the control and values in the "Control" are considered as zero. Volcano plots and MA plots were created with Graphpad Prism software (version 7 for Windows, La Jolla California USA) to compare each stage of the biofilm cycle.

***Clusters of orthologous groups (COG) function categorization*:** The DGEs of each stage were classified through homology with protein functions determined from Clusters of orthologous groups (COG) database. The COG functional categories for the DEGs were obtained by annotating the protein sequences of the reference *P. fragi* 1793 genome using the Eggnog mapper v4.5.1 (http://eggnogdb.embl.de) and genes were placed into COG functional categories. The output of Eggnog provided GO terms, KEGG KO and Seed orthologs of the genes. The gene locus tags, log2 FC (fold change), FDR, COG categories and gene product description of the highest fifteen differentially expressed genes are provided in Tables 2-5.

***DEG verification using quantitative reverse transcriptase PCR (qRT-PCR)*:** The RNA samples that were used for sequencing were also used for quantitative reverse transcriptase PCR (qRT-PCR) to determine if gene expression was consistent between the two approaches. Dilutions were made from the stock RNA to obtain approximately similar amount (800 ng) of RNA for reverse transcription. After that, cDNA was synthesised from RNA of three biological replicates using the iScript cDNA Synthesis Kit (Bio-Rad Hercules, CA, cat no 1708890) according to manufacturer's instructions. Control reactions were performed without reverse transcriptase (RT). Twelve significantly up and down regulated genes (*P* value < 0.01) at each stage of RNA seq reaction were selected for verification by qRT-PCR.

Gene specific primers were designed for selected for the most up and down regulated genes at each stage. The primers were designed using NCBI primer blast and the primers used in this study are listed in appendix 5. The 16S rRNA gene was used for normalization within samples. From the cDNA synthesized, qPCR was conducted in a 96 well qPCR plate with three technical replicates for each reaction. No template controls and no RT control were used as control reactions to assess the purity of the reagents and genomic DNA contamination.

Real-time PCR was performed using Aria Max (Agilent) real time PCR system under following reaction conditions: 95 °C for 3 min, 45 cycles consisting of 95 °C for 10 s and 60 °C for 30 s, and 72 °C for 30 s. Melt curve analysis (55 to 81 °C, 0.5 °C increments for 30 s) was performed to ensure the specificity of primers. The ΔΔCt method was used to analyze the relative expression fold change of the targeted genes (Livak & Schmittgen, 2001).The expression level of each target gene was compared relative to the 16S rRNA internal control gene.

5.4 Results

***Biofilm morphology*:** The CLSM images on day 1 (24 hours), showed extremely limited biofilm formation on meat (Figure 5.1A). The muscle surface and fibres were clearly visible. Small flocs of biofilm can be seen on day 2 (48 hours), and many planktonic bacteria were visible moving between flocs of growing biofilms (Figure 5.1B). By day 3 (76 hours), confluent biofilms had started to form on meat and the majority of the cells are live and can be seen in green (Figure 5.1C). By this time, the number of CFU in biofilms increased from 10^4 to $\sim 10^8$ CFU cm^{-2} (Wickramasinghe, Ravensdale, Coorey, Dykes, et al., 2019). The maximum population of 10^{11} CFU cm^{-2} was reached around day 4 (100 hours) (Figure 5.1D). By day 5 (124 hours), most cells appeared in yellow and dead cells can also be seen, while the biofilm structure appeared distorted. By day 6 (148 hours), more dead cells were visible in red, and the biofilms structures were clearly degraded (Figure 5.1E). By day 7, the majority of cells in the biofilm had been dispersed and the muscle surface was clearly visible again. However, part of the biofilm population remained on the meat surface as live cells.

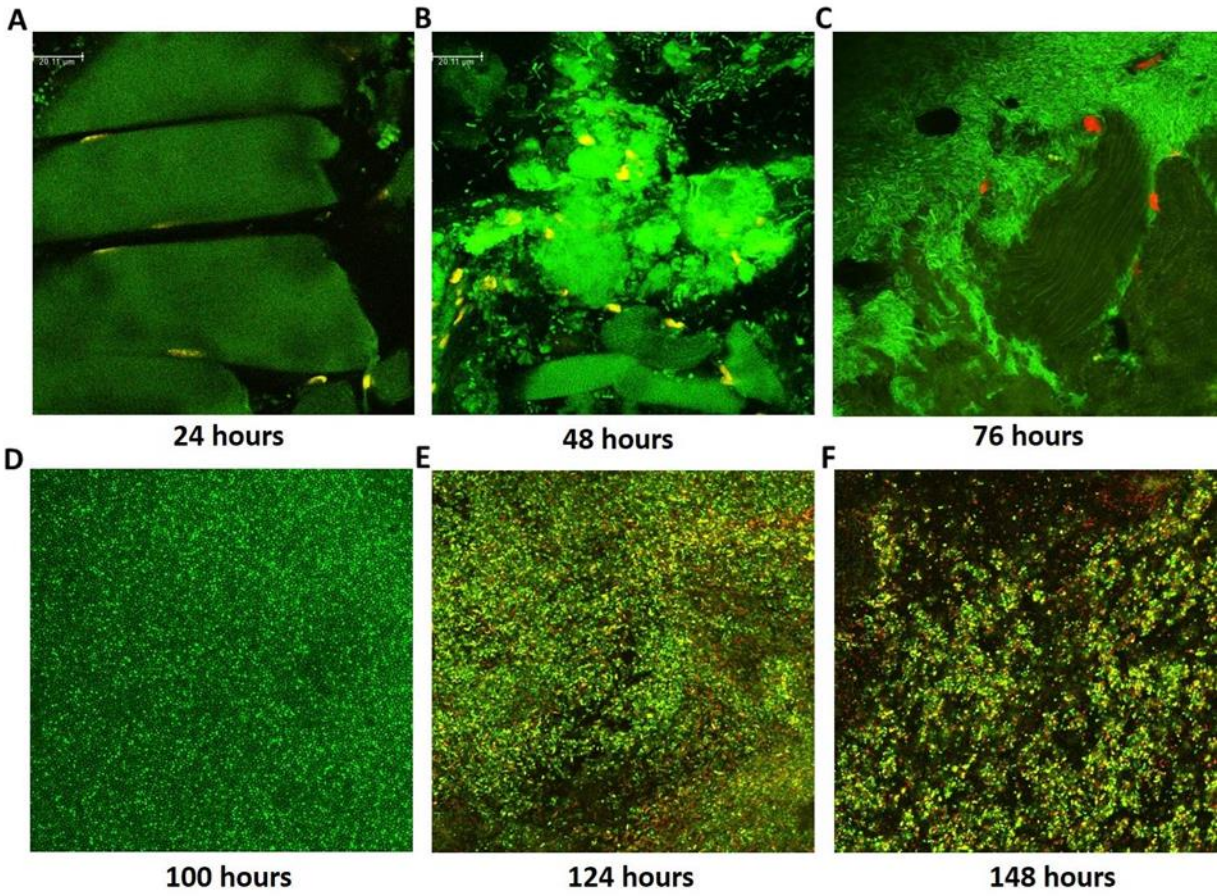


Figure 5.1 CLSM micrographs of *P. fragi* biofilm cycle on meat incubated at 10 ^{0}C from 24 hours to day 148 hours. Live cells are stained with SYTO 9 (green) while dead cells and meat nuclei are stained with PI (red).

***Analyzed sequencing data*:** RNA was extracted from biofilms grown on meat at three different time durations. The extracted RNA from the three stages of the biofilm gave high yields of good quality, non-degraded total RNA with RIN numbers ranging between 9 and 10 (Supplementary material 1). A total of 24–36 million FastQ reads per sample were generated by the high-throughput Illumina sequencing run (Appendix.3)

According to the results of volcano and MA plots, the highest number of differentially expressed genes can be seen between biofilm initiation vs dispersal stages (Figure 5.2A and 2D). The false discovery rate shows the significance of the difference in gene expression between two conditions that were tested at each stage. Compared to the other two stages, a limited number of differentially expressed genes were present, and many genes were expressed at a lower level between maturation vs dispersal (Figure 5.2C and 2F).

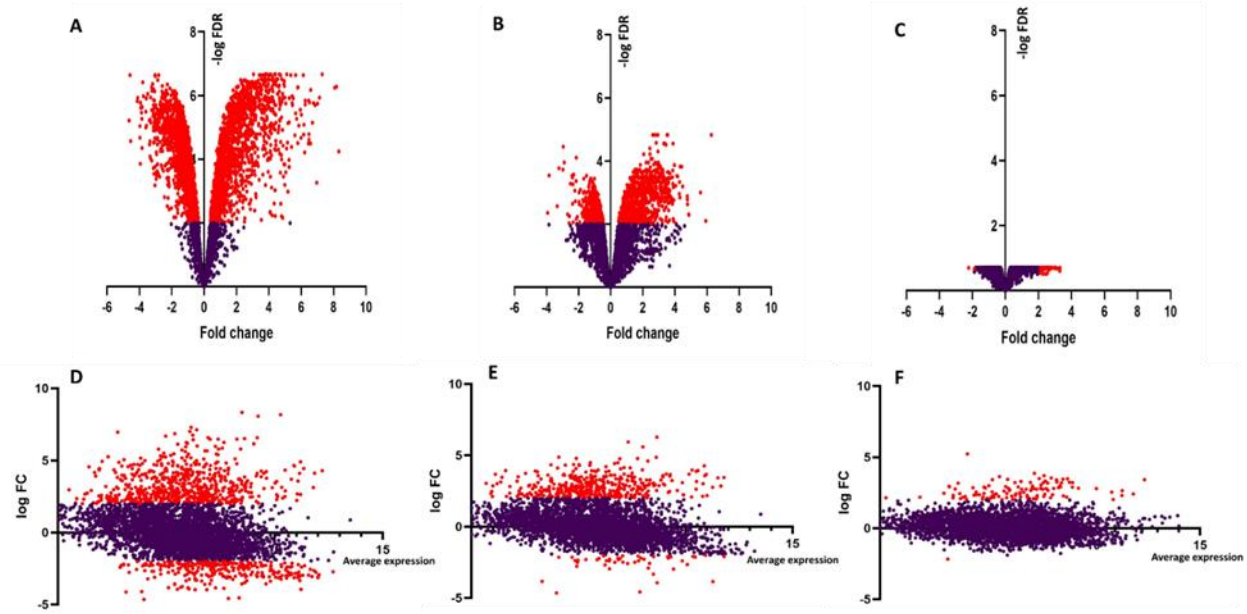


Figure 5.2 Volcano plots showing differentially expressed genes at biofilm initiation vs dispersal (A), initiation vs maturation (B) and maturation vs dispersal (C). The X axis represents the fold changes and Y axis represents the -log10 FDR. Each dot shows the change in expression in one gene in *P. fragi* genome. Significantly differentially expressed genes are highlighted in red and non-significant expressions are presented in purple. MA plots also depict differentially expressed genes at initiation vs dispersal (D), initiation vs maturation (E) and maturation vs dispersal (F). The average expression (over both condition and treatment samples) is represented on the x-axis and Y axis depicts logFC.

Compared to upregulated genes, downregulated ones had low fold changes in all three stages of the biofilm cycle (Tables 5.2-5.5). According to the Venn diagram (Figure 5.3A), 91 (12.9%) upregulated genes were common to all three main stages of the biofilm whereas no genes were common to all 3 stages in down regulation. Out of the total down regulated genes, 83.6% belonged exclusively to initiation vs dispersal stage and 334 common genes were present between initiation vs maturation and initiation vs dispersal.

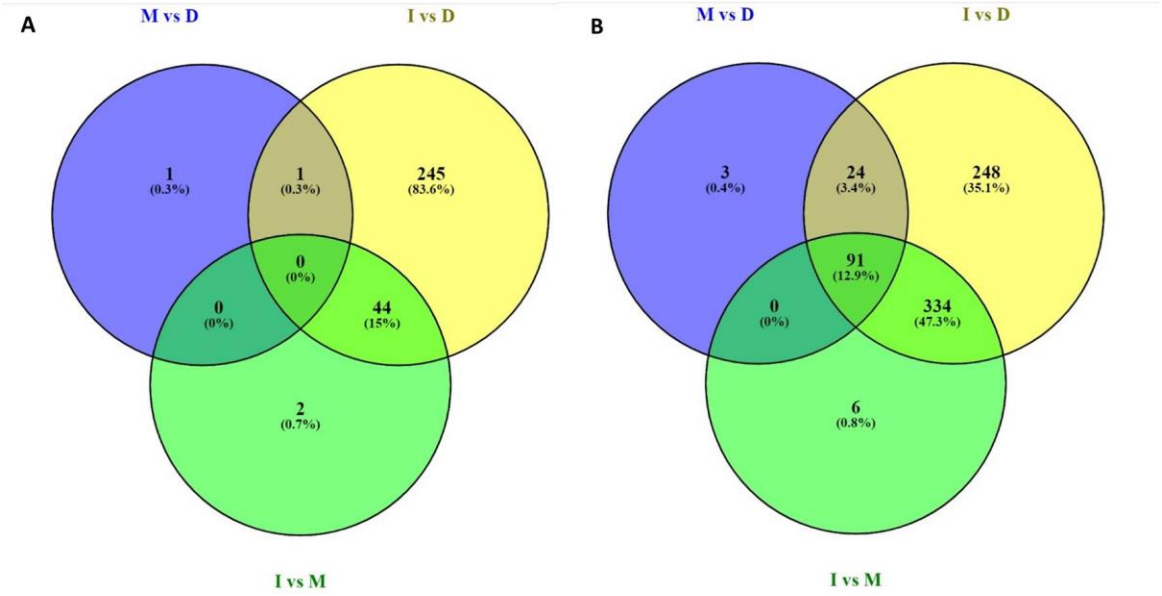


Figure 5.3 Venn diagram of significantly (P<0.01) down regulated (A) and up regulated genes (B) of initiation, maturation and dispersal stages of *P. fragi* biofilms formed on chilled beef

***COG functional categories and their main metabolic functions*:** The DGEs of each stage were classified using the known function of the gene products and with protein functions determined from the COG database (Figure 5.4, Table 5.1). The total number of genes in each COG category and the percentages of up and downregulated genes are listed in Figure 5.4. Out of 4333 genes of *P. fragi* 1793 genome, 225 were listed as not in COG where those genes could not be allocated to any COG category.

Table 5.1 The number of genes in *P. fragi* 1793 genome and allocated to each COG category

COG function	COG category	number of genes within COG
Information storage and processing		
J: Translation	J	207
K: Transcription	K	403
L: Replication, recombination and repair	L	144
Cellular processes and signaling		
D: Cell cycle control	D	57
V: Defense mechanisms	V	59
T: Signal transduction mechanism	T	163
M: Cell wall/membrane biogenesis	M	270
N: Cell motility	N	45
U: Intracellular trafficking and secretion	U	92
O: Post-translational modification, protein turnover,	O	130
Metabolism		
C: Energy production and conversion	C	260
G: Carbohydrate transport and metabolism	G	142
E: Amino acid transport and metabolism	E	329
F: Nucleotide transport and metabolism	F	121
H: Coenzyme transport and metabolism	H	146
I: Lipid transport and metabolism	I	152
P: Inorganic ion transport and metabolism	P	318
Q: Secondary metabolites biosynthesis, transport and catabolism	Q	72
Poorly characterized		
S: function unknown	S	851
Unclassified	R	0

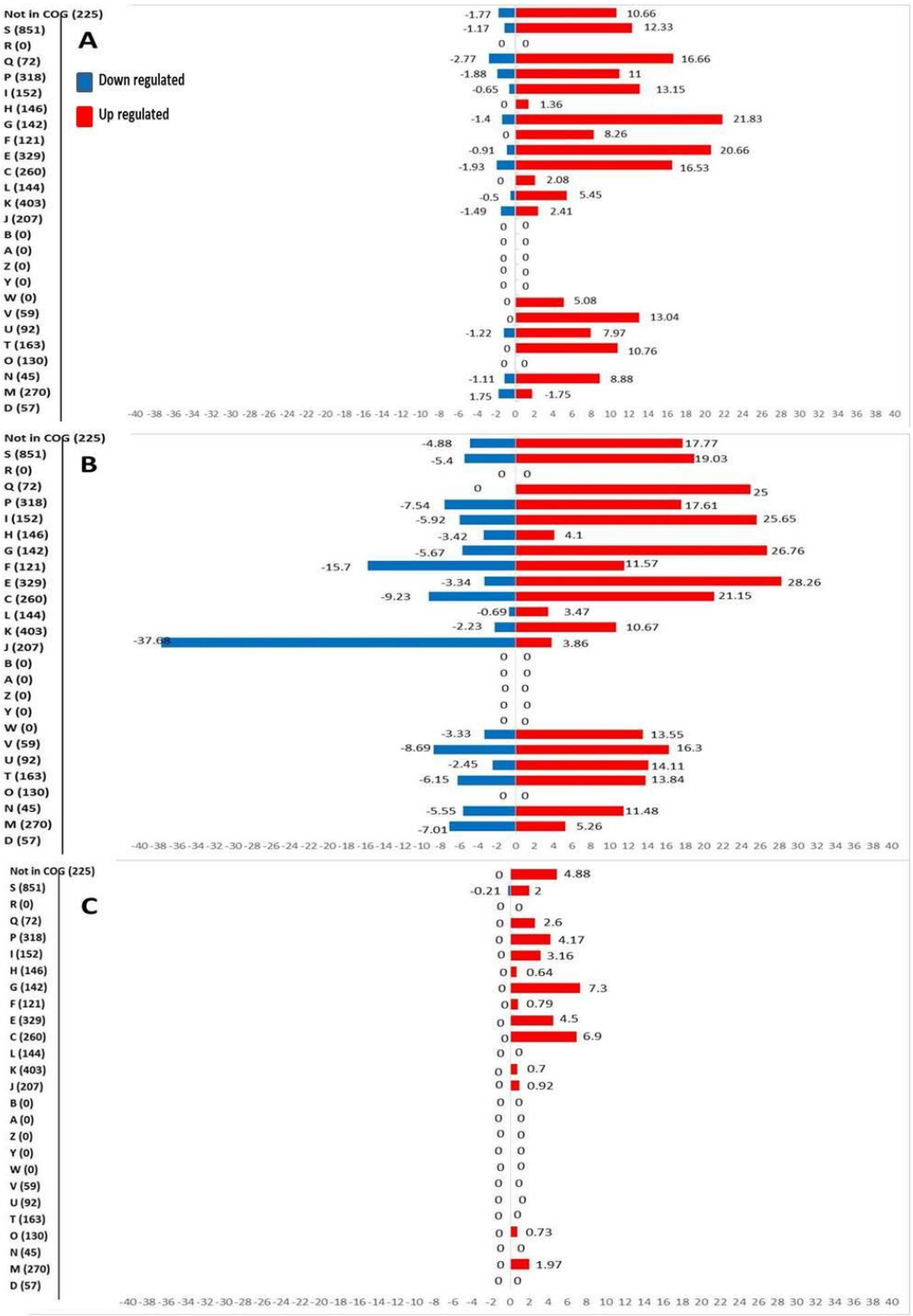


Figure 5.4 The percentages of significantly upregulated and downregulated genes of *P. fragi* 1793 biofilm formed on chilled beef at initiation vs maturation (A), initiation vs dispersal (B), maturation vs dispersal (C) according clusters of orthologous groups (COGs)

***DEGs in biofilm initiation vs maturation*:** DEGs in each of the three stages of the biofilm were assessed according to the selected criteria of (FDR) of <0.01, log2 fold change of >2 and a statistical significance of < 0.01. The total number of predicted genes in the *P. fragi* 1793 genome was 4333. A total of 419 genes were significantly differentially expressed between biofilm initiation and maturation which equates to 9.66 % of the total genome. Of those 382 genes were significantly upregulated while 37 genes were significantly downregulated. The genes encoding 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase, Flp family type IVb pilin, histidine kinases and ribosome modulation factor were the most upregulated genes with log2 fold changes of 6.72, 5.92, 4.87and 4.86 respectively (Table 5.2). The genes encoding FUSC (fusaric acid resistance) family protein, alpha-hydroxy-acid oxidizing enzyme, tonB-dependent siderophore receptors and heme utilization protein were among the most downregulated with log2 fold changes of -3.93, -3.84, -3.34, and -3.21, respectively (Table 5.3)

Table 5.2 Fifteen upregulated genes with the highest fold changes between biofilm initiation vs maturation

Locus	Query	FC	FDR	Avg expression	COG cat	Description
CJU75_06635	PAA38363.1	6.27	1.48E-05	8.66	E	catalyzes the transfer of a methyl group
CJU75_22210	PAA32247.1	5.92	0.0078	7.31	S	flp/fap pilin component
CJU75_14375	PAA34791.1	5.58	0.00098	8.00	NA	unknown function
CJU75_22215	PAA32248.1	4.87	0.00503	4.07	K	histidine kinase, response regulator
CJU75_22455	PAA32141.1	4.86	0.00488	8.62	J	ribosome modulation factor
CJU75_18560	PAA33363.1	4.78	0.00233	4.92	S	membrane protein
CJU75_05405	PAA38134.1	4.77	0.00164	7.76	E	glutamate decarboxylase
CJU75_05400	PAA38133.1	4.76	0.00128	6.82	E	Belongs to the glutaminase family
CJU75_06940	PAA37735.1	4.59	0.01133	8.24	E	creatinase
CJU75_15120	PAA34487.1	4.48	0.00063	5.55	P	haloacid dehalogenase-like hydrolase
CJU75_16620	PAA33979.1	4.42	0.00015	6.68	C	arylsulfatase A and related enzymes
CJU75_19875	PAA32825.1	4.34	0.03145	4.77	S	cytochrome c oxidase accessory protein
CJU75_16745	PAA34001.1	4.33	0.01393	5.02	E	enzyme of the cupin superfamily
CJU75_21575	PAA32308.1	4.32	0.00014	3.71	S	formation of L-homocysteine from OSHS
CJU75_15085	PAA34481.1	4.30	0.00143	4.38	S	protein conserved in bacteria

Table 5.3 Fifteen downregulated genes with the highest fold changes between biofilm initiation vs maturation

Locus	Query	FC	FDR	Avg expression	COG cat	Description
CJU75_06450	PAA38329.1	-3.93	0.00435	8.67	S	transmembrane transporter activity
CJU75_21035	PAA32410.1	-3.83	0.00028	3.30	C	catalyzes the conversion of L-lactate to pyruvate
CJU75_06445	PAA38328.1	-3.84	0.01031	11.27	S	protein of unknown function
CJU75_09520	PAA36861.1	-3.34	0.00267	9.44	P	Ton B dependent siderephore receptor
CJU75_13340	PAA35669.1	-2.97	0.00018	9.44	C	functions as a proton pump across the membrane
CJU75_13335	PAA35668.1	-2.95	3.5E-05	6.44	C	NADP transhydrogenase
CJU75_13330	PAA35667.1	-2.89	0.00011	8.26	C	functions as a proton pump across membrane
CJU75_14700	PAA34854.1	-2.68	0.00485	6.23	IQ	Acyl-CoA synthetases AMP-acid ligases II
CJU75_13125	PAA35626.1	-2.66	0.03122	10.08	EI	biotin carboxylase
CJU75_12490	PAA35503.1	-2.59	0.00920	7.29	E	choline dehydrogenase and related flavoproteins
CJU75_12960	PAA35593.1	-2.57	0.00555	8.32	K	transcriptional regulator
CJU75_08405	PAA37329.1	-2.50	0.00052	4.94	S	Cupin 2, conserved barrel domain protein
CJU75_12495	PAA35674.1	-2.50	0.01113	6.69	S	gluconate 2-dehydrogenase
CJU75_22975	PAA31039.1	-2.48	0.02555	7.62	D	protein conserved in bacteria
CJU75_13130	PAA35627.1	-2.40	0.01463	9.85	CI	AAA domain

Table 5.4 Fifteen upregulated genes with the highest fold changes between biofilm initiation vs dispersal

Locus	Query	FC	FDR	Avg expression	COG cat	Description
CJU75_22210	PAA32247.1	8.32	5.67E-05	8.49	S	flp/fap pilin component
CJU75_22455	PAA32141.1	8.17	5.24E-07	10.27	J	converts 70S ribosomes to an inactive dimeric form
CJU75_18560	PAA33363.1	7.29	2.15E-07	6.14	S	hypothetical protein in membrane
CJU75_16745	PAA34001.1	7.12	1.15E-06	6.40	E	methionine gamma-lyase
CJU75_19875	PAA32825.1	7.00	1.8E-06	6.09	S	enzyme of the cupin superfamily
CJU75_01795	PAA40425.1	6.95	0.00053	2.75	S	catalyzes the degradation of glycine
CJU75_08955	PAA37432.1	6.84	1.97E-06	5.38	I	related beta-hydroxyacid dehydrogenases
CJU75_08945	PAA37430.1	6.75	1.72E-06	6.22	K	nucleoside-diphosphate-sugar epimerases
CJU75_22215	PAA32248.1	6.68	7.06E-06	4.97	K	histidine kinase
CJU75_06940	PAA37735.1	6.57	3.19E-05	9.20	E	creatinase
CJU75_05405	PAA38134.1	6.50	1.62E-06	8.61	E	belongs to the group II decarboxylase family
CJU75_10450	PAA36312.1	6.47	2.43E-05	7.39	E	sarcosine oxidase, subunit beta
CJU75_10445	PAA36311.1	6.47	1.63E-05	8.26	E	catalyzes interconversion of serine and glycine
CJU75_08960	PAA37433.1	6.45	1.44E-06	7.02	EG	belongs to the IlvD Edd family
CJU75_09225	PAA36805.1	6.43	3.18E-05	6.23	C	destroys radicals produced within the cells
CJU75_05400	PAA38133.1	6.24	1.43E-06	7.69	E	belongs to the glutaminase family

Table 5.5 Fifteen downregulated genes with the highest fold changes between biofilm initiation vs dispersal

Locus	Query	FC	FDR	Avg expression	COG cat	Description
CJU75_09520	PAA36861.1	-4.57	6.07E-06	3.96	P	tonB dependent siderophore receptor
CJU75_06450	PAA38329.1	-4.54	2.68E-05	8.38	S	FUSC family, transmembrane transporter activity
CJU75_21035	PAA32410.1	-4.13	1.49E-06	3.14	S	protein of unknown function (DUF2790)
CJU75_22970	PAA31038.1	-3.96	9.09E-06	6.32	S	protein conserved in bacteria, ppGpp synthetase,
CJU75_06445	PAA38328.1	-3.94	0.000138	11.21	C	catalyzes the conversion of L-lactate to pyruvate
CJU75_12665	PAA35537.1	-3.94	1.66E-06	5.51	P	part of the ABC transporter complex in taurine import
CJU75_12670	PAA35538.1	-3.91	2.41E-06	6.04	P	taurine ABC transporter
CJU75_12655	PAA35593.1	-3.85	2.21E-06	6.00	Q	taurine catabolism dioxygenase
CJU75_12960	PAA35593.1	-3.86	3.42E-06	7.00	K	transcriptional regulator
CJU75_12655	PAA35535.1	-3.85	2.21E-06	6.00	Q	taurine catabolism dioxygenase
CJU75_12495	PAA35674.1	-3.84	5.32E-06	6.00	S	gluconate 2-dehydrogenase
CJU75_19920	PAA32834.1	-3.84	4.42E-05	7.27	NT	chemotaxis, protein
CJU75_12660	PAA35536.1	-3.83	2.32E-06	5.09	P	ABC-type nitrate sulfonate bicarbonate transport system
CJU75_14700	PAA34854.1	-3.79	3.82E-07	5.66	IQ	acyl-CoA synthetases AMP-acid ligases II
CJU75_20405	PAA32548.1	-3.75	1.04E-06	5.73	M	nucleoside-binding outer membrane

Differentially expressed genes between these stages were categorized into 18 COG functional categories. A total of 18 genes had more than one general category letter association in the COG database and were treated as belonging to both. The upregulated DEGS belonged to the following COG categories: carbohydrate transport and metabolism (COG G; 21.83%), amino acid transport and metabolism (COG E; 20.66%), secondary metabolites biosynthesis, transport, and catabolism (COG Q; 16.66%), energy production and conversion (COG C; 16.53%), lipid transport and metabolism (COG I; 13.15%), intracellular trafficking, secretion, and vesicular transport (COG U; 13.04%), function unknown (COG S; 12.33%), inorganic ion transport and metabolism (COG P; 11%) and post-translational modification, protein turnover, and chaperones (COG O; 10.76%) (Figure 5.4A). Significantly down regulated genes belonged to the COG categories Q (2.77%), C (1.93%), P (1.88), D (1.77), M (1.75%), J (1.49%), G (1.4%) and T (1.22%), respectively (Figure 5.4A). In all the COG categories, the numbers of upregulated genes were higher than downregulated genes.

DEGs in biofilm maturation vs dispersal: According to the selected FDR and log2 fold change, 122 genes which equates to 2.81 % of the total genome was differentially expressed between maturation vs dispersal. Out of that, 120 genes were significantly up regulated while 2 genes were down regulated. Among them, the ribosome modulation factor, glycine cleavage system protein, heme-binding protein and superoxide dismutase [Mn], were the most upregulated with log2 fold changes of 3.30, 3.28, 3.05 and 3.03 respectively. At the same time genes related to pyruvate dehydrogenase (PDH) enzyme complex have been upregulated which included, pyruvate 2-oxoglutarate dehydrogenase complex dehydrogenase, dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex, pyruvate 2-oxoglutarate dehydrogenase complex and pyruvate 2-oxoglutarate dehydrogenase complex. The fold changes of these genes were 3.45, 3.18, 3.05 and 2.96 respectively.

Genes for a hypothetical protein and DUF2292 domain-containing protein were the most downregulated with log2 fold changes of -2.67 and -2.17, respectively. The differentially expressed genes were categorized into 14 functional groups. Between maturation vs dispersal, 6 genes had more than one COG category assigned to them. The highest number of upregulated genes belonged to carbohydrate transport and metabolism (COG G; 7.3%), energy production and conversion (COG C; 6.9%), amino acid transport and metabolism (COG E; 4.5%), lipid transport and metabolism (COG I; 3.16%) and energy production and conversion (COG Q; 2.6%) (Figure 5.4C). Both the down regulated genes were categorized into COG category S.

DEGs in biofilm initiation vs dispersal: The highest number of differentially expressed genes can be seen between biofilm initiation vs dispersal. According to the selected FDR and fold change, 933 genes which equate to 21.53% of the total genome were differentially expressed. Out of that, 658 were significantly up regulated and 275 genes were significantly down regulated between biofilm initiation and dispersal. Genes encoding Flp family type IVb pilin, ribosome modulation factor, hypothetical protein, methionine gama-lyase, cupin domain containing protein and histidine kinase were the most upregulated with log2 fold changes of 8.23, 8.17, 7.29, 7.00 and 6.68, respectively (Table 5.4). Genes coding TonB-dependent siderophore receptor, FUSC family protein, DUF2292 domain-containing protein, (p) ppGpp synthetase and chromosome partitioning protein ParA were the most downregulated with log2 fold changes of -4.57, -4.54, -4.10, -3.969 and -3.698 respectively (Table 5.5).

Differentially expressed genes were categorized into 18 functional categories. A total of 25 genes had more than one general category letter association in the COG database and were treated as belonging to both. The majority of upregulated DEGS belonged to the following COG categories: Secondary metabolites biosynthesis, transport, and catabolism (COG Q; 25 %), Lipid transport and metabolism (COG I; 25.65%), Amino acid transport and metabolism (COG E; 20.66%), Carbohydrate transport and metabolism (COG G; 26.76%), Inorganic

ion transport and metabolism (COG P; 17.61%) and Energy production and conversion (COG C; 21.15%).

Compared to the other two stages, higher numbers of downregulated genes were present between biofilm initiation vs dispersal. At this stage, the highest proportion of down regulated genes belonged to COG J (37.68%) which is related to translation, ribosomal structure and biogenesis. Nucleotide transport and metabolism (COG F; 15.7%), energy production and conversion (COG C; 9.23%) and intracellular trafficking, secretion, and vesicular transport (COG U; 8.69%) were the next highest down regulated categories (Fig. 5.4B) of this stage.

***Verification of DEGs with RT-qPCR*:** Eleven genes (7 upregulated and 4 downregulated) with highest fold changes were selected from three stages of the biofilms cycle and qRT-PCR was performed. The results of qRT-PCR experiments confirmed the gene expression trend observed in RNA seq data (Table 5.6). Differences in fold changes could likely be due to differences in sensitivity and specificity between RT-qPCR and high throughput sequencing technology.

Table 6. Gene expression fold changes generated by RNA seq analysis and qRT-PCR for the selected genes.

Stage of biofilm	Locus tag	Fold change in RNA seq	Fold change in qRT-PCR
Initiation vs maturation	CJU75_22245	4.862458	8.22
	CJU_06635	6.270233	4.02
	CJU75_09520	-3.34048	-1.22
	CJU75_06450	-3.93499	-2.82
Initiation vs dispersal	CJU-22245	8.1725	5.18
	CJU_06635	5.79	9.07
	JU75_19875	7.00	10.22
Maturation vs dispersal	CJU75_03765	-1.8829113	-1.45
	CJU75_13985	-2.2248322	-2.13
	CJU75_17410	3.05179629	5.08
	CJU75_01795	3.28964006	4.89

5.5 Discussion

Biofilms are surface adhered communities of microorganisms and are a staple mode of life in many bacterial species. Biofilm bacteria have different gene expression pattern compared to planktonic bacteria (McDougald et al., 2011). Even though the duration of each stage can vary based on bacterial species and environmental conditions, all biofilms follow a cycle of bacterial irreversible attachment which initiates biofilm formation, maturation and dispersal of cells to colonise new surfaces (Flemming et al., 2016; Seckbach, 2010). Studies have shown that biofilm formation is a genetically regulated process and the gene expression profile at each stage is different (Seckbach, 2010). At the same time, transcriptomic analyses of different stages of biofilm development across different bacterial species have failed to show a consistent correlation between all species (McDougald et al., 2011). Therefore, in this research we studied the global gene expression pattern of different stages of *P. fragi* biofilms when it is formed on beef stored at low temperature under industry applicable conditions.

In this research, RNA extraction time points were selected based on previous research done on *P. fragi* biofilms formed on chilled meat and overlap of main stages was avoided (Wickramasinghe et al., 2019). According to past research, 48 hours was selected as the initiation stage as biofilm maturation does not occur during that period and signs of dispersal do not appear at 76 hours when biofilms are formed at 10 ℃. Since biofilms were grown on fresh beef muscle, it was important to specifically remove bovine rRNA along with bacterial rRNA. To obtain reliable results, it is important to sequence RNA with high integrity. The RNA isolated in our study was of good integrity and gave high quality reads. Therefore, post sequence trimming of low-quality reads was not necessary. According to the MDS plots obtained from DEGUST (supplementary material 4), there was limited variability in results between the four biological replicates.

DEG in biofilm initiation vs maturation: Biofilms are formed when planktonic bacteria attach to each other or to a surface irreversibly and develop individual microcolonies. With time these microcolonies grow as bacteria multiply and form larger structures known as macro colonies. According to our CLSM images, *P. fragi* biofilms start forming small microcolonies around 48 hours after incubation of meat at chilled/ temperature abuse.

In the pairwise comparisons used in this study, biofilm initiation was considered as the control and the gene expression fold changes of the maturation which was considered as the treatment, was assessed relative to initiation. According to our data, most genes were upregulated as opposed to being down regulated. In addition, the fold changes were higher for upregulated genes than for down regulated genes. Amino acid metabolism occurs rapidly as the biofilm increases in size and cell number when it reaches maturation. More energy is required for exopolysaccharide secretion and for cell division.

Based on the COG categorization of the significantly differentially expressed genes, the majority of upregulated genes at maturation were associated with the metabolism of the bacteria. Among them, COGs G and E which represented Carbohydrate transport and metabolism and Amino acid transport and metabolism contained the highest numbers of genes. This is most likely due to the rapid increase in the number of bacterial cells within biofilms. At this stage, it is likely that the cells are taking advantage of the nutrient dense environment. The cell numbers increase from 10^4 CFU cm^{-2} to 10^8 CFU cm^{-2} within 28 hours (Wickramasingheet al., 2019b) requires energy and nitrogen for new protein and structural development.

The genes encoding creatininase enzyme (PAA37735.1) was also significantly upregulated by 6.57 fold. One of the specific characteristics of *P. fragi* is their ability to utilize creatinine and creatine when the substrates are depleted of glucose and lactase, while other psychrotophic spoilage pseudomonads such as *Pseudomonas lundensis* and *P. fluorescence* lack this ability. When the cell numbers reach around 10^{7-8} CFU/cm^2, the meat surface becomes depleted of glucose and other simple carbohydrates (Mead,

2007; Nychas et al., 2008). At the maturation stage, the cell numbers are around 10^{7-8} $CFUcm^{-2}$ and creatininase is likely to be used for energy in *P. fragi* biofilms.

Several genes related to taurine metabolism which include taurine transporter ATP-binding subunit, taurine ABC transporter permease, taurine ABC transporter substrate-binding protein and taurine dioxygenase were significantly down regulated. To date the, effect of taurine on bacterial physiology and biofilm growth has been poorly explored. However, a study on *Acinetobacter oleivorans* have shown that taurine has biofilm inhibitory effects by decreasing total EPS mass and interfering with quorum sensing (Eom & Park, 2017). Some studies have found that in order for it to be utilized, taurine needs to be transported into the bacterial cells (Huxtable, 1992). Taurine is an effective osmoregulatory compound and is used as a compatible solute that can protect proteins, nucleic acids, and membranes from the harmful effects of heat, freezing and drying (Mosier et al., 2013). Taurine is likely to play a role in protecting the bacteria when they are growing in low temperatures. Further studies are necessary to establish the reason for downregulation of taurine transporter systems in *P. fragi* biofilms.

DEGS in biofilm maturation vs dispersal: The lowest percentages of significantly up and down regulated genes can be seen between biofilm maturation vs dispersal which means that these stages have somewhat similar levels of expression. The two significantly down regulated genes belonging to COG S where one gene with -2.67 fold change encoded for a hypothetical protein while the other with -2.17 fold change encoded for DUF 2292 containing protein which stands for domain of unknown function.

The highest percentage of upregulated genes belonged to COG G and most of the genes encoded for the major facilitator superfamily (MFS) which facilitates the movement of solutes across the cell membrane in response to chemiosmotic gradients (. Yan, 2015). The second most upregulated COG category was COG C which contained genes that

encode hydrolases, oxidoreductases and dehydrogenases. COG C contained genes that act upon radicals such as superoxide dismutase which are normally produced within the cells and are toxic to biological systems. During the latter stages of the biofilm development, toxic compounds may accumulate, and upregulation of such genes may be necessary.

In the latter stages of the meat grown biofilm, cell numbers reduce after they reaches the population maximum and according to CLSM images the biofilm structures break down. When the environmental conditions become unfavourable, a portion of the cells in biofilms disperse and leave the biofilm. For these cells which are enmeshed in a polymeric matrix of polysaccharides, proteins and eDNA to be released, matrix degrading enzymes need to be secreted. The upregulated genes encode degradative enzymes such as nucleases and hydrolases including aldehyde dehydrogenase, short chain dehydrogenase, aspartate ammonia-lyase, and ureidoglycolate which may aid in degradation of matrix material.

The results further showed that several genes related to pyruvate dehydrogenase (PDH) enzyme complex were significantly upregulated. Past studies have shown that depletion of pyruvate from growth medium impaired biofilm formation in *P. aeruginosa* (Petrova, Schurr, Schurr, & Sauer, 2012). Recent studies have shown that the use of exogenous (PDH) enzyme can cause dispersal in existing *P. aeruginosa* biofilms where sessile surface attached bacteria were released in planktonic state (Goodwine et al., 2019; Han et al., 2019). The dispersion of biofilms was triggered by PDH through the action of pyruvate depletion. Since several genes related to PDH are significantly upregulated, it can be concluded that the use of exogenous PDH to deplete pyruvate from inside the biofilm can cause dispersal in *P. fragi* biofilms as well.

DEGs in biofilm initiation vs dispersal: Expression of genes at dispersal was assessed relative to the biofilm initiation and fold changes at initiation was considered zero. The majority of genes significantly differentially expressed at initiation vs maturation stage are similar to the genes significantly differentially expressed at initiation vs dispersal

stage. However, at dispersal stage the genes have higher fold changes as the biofilm is further matured. In the list of significantly upregulated genes, the highest fold change of 8.32 was observed in genes encoding Flp family type IVb pilin related to locus tag CJU75_22210 (PAA32247.1). Another locus tag CJU75_08345 (PAA37317.1.) also encoding for Flp family type IVb pilin protein has also been significantly upregulated by a fold change of 5.77. Type IV pilins (T4P) are small structural proteins which does various functions in bacteria. Its well-characterized roles include adherence to living and non-living surfaces as well as to other bacteria (Bohn et al., 2009; Giltner, Nguyen, & Burrows, 2012). The adherence to surfaces and to each other are crucial steps in biofilm formation. It has been reported that *P .aeruginosa* mutants lacking T4P are deficient in biofilm formation and biofilm structures are impaired. (Chiang & Burrows, 2003; O' Toole & Kolter, 1998). The T4Ps are likely to be key genes that govern *P. fragi* biofilm formation.

The second highest upregulated gene with 8.17 fold change was the ribosome modulation factor (RMF) which is a ribosome-associated protein. Studies on RMF mutants and parent strains have found that survival of *Escherichia coli* under extreme environmental conditions is higher in parent strains (EL-Shroud et al, 2008). Efficient functioning of the ribosomes is essential for protein synthesis, growth and survival of the bacteria. RMF protects the ribosomes and helps to maintain its structure and function which maintains the cell viability under cold environmental conditions. My results suggest it is possible that RMF provides stability for ribosomes when the bacteria are grown under chilled temperature conditions which are stressful to the bacteria.

Genes coding for methionine gamma-lyase, were also highly upregulated with a fold change of 7.12. This enzyme degrades sulfur-containing amino acids to α-keto acids, ammonia, and thiol. Also, genes such as L-serine ammonia-lyase and aspartate ammonia-lyase are also highly upregulated at this stage which catalyze amino acids and release NH_3 (Fibriansah, Veetil, Poelarends, & Thunnissen, 2011; Qin, Yan, Ma, & Jiang, 2015). By this stage bacteria in the biofilm have utilized glucose and simple

sugars on meat and have begun to degrade proteins via proteolysis. The putrid odors released during the latter stages of spoiled, chilled meat, is due to the catabolism of amino acids from muscle protein.

Among the highly down regulated genes at the biofilm dispersal stage, genes encoding for the TonB-dependent siderophore receptor (PAA36861.1) had a fold change of -4.57. TonB dependent siderephore receptors are bacterial outer membrane proteins that bind and transport ferric chelates (Noinaj, Guillier, Barnard, & Buchanani, 2010). Bacteria use siderophores to chelate iron. Due to their molecular weight, siderophores are not able to diffuse through the porins present in the outer membrane of Gram-negative bacteria into the cytoplasm. Bacteria use TonB dependant receptors to actively transport siderophores into the periplasm (Seckbach, 2010). In the periplasm siderophores are bound by a periplasmic binding protein and delivered to an inner membrane by the ABC transporter. In this study, genes encoding the ABC transporter permease (PAA35243.1) were also significantly down regulated. Although it was previously suggested that *P. fragi* did not produce siderophores, (Brenner, 2005; Champomier-Vergès et al., 1996) the results of this study clearly indicate that *P. fragi* contain genes that encode for siderophores. Also, Stanborough et al, (2018) found that *P. fragi* produce a vibrioferrin-mediated iron acquisition system under iron starvation.

Genes that encode FUSC proteins have also been down regulated with a -3.52 fold change. Studies have found pathogenic bacteria such as *Pectobacterium* remain virulent under iron limiting environments using iron containing proteins such as ferredoxin, pirated from their hosts (Grinter et al., 2018; Mosbahi, Wojnowska, Albalat, & Walker, 2018). This import pathway is facilitated by FUSC proteins and it may play a role in iron acquisition in *P. fragi* as well. Currently no information is available on the functions of the FUSC family proteins in *P. fragi.* Our results further show that heme utilization protein (PAA32411.1) was also significantly down regulated. Certain pathogenic bacteria use heme as an alternative source of iron and when they are incapable of producing siderophores (Kaylie, Brittni, & Jeremiah, 2019).

Based on these results, it is likely that *P. fragi* uses several iron uptake systems for its function and its regulation varies as the biofilm matures. Iron is an essential micronutrient required for bacterial growth and studies have found it to be is essential for biofilm formation in *P. aeruginosa* (Zhu, Rice, & Barraud, 2019). According to these results, it is clear that as *P. fragi* biofilm matures, it down regulates the genes required for iron uptake. The exact reason for this is currently unknown. However, high concentrations of iron can be toxic to bacteria as it can lead to reactive oxygen species (Kang & Kirienko, 2018). It has been shown for *P. aeruginosa* that once the biofilms are formed, the matrix exopolysaccharides can store iron (Yu, Wei, Zhao, Guo, & Ma, 2016). A study on *P. aeruginosa* by Yang et al. (2007) showed that high concentrations of iron supress extra-cellular DNA release in biofilm which is a crucial part of its structure and it weakens the biofilm structural development. When *P. fragi* is growing in an iron rich environment like meat, they may down regulate genes that govern iron acquisition systems in order to control high concentrations of iron in the biofilm matrix.

A recent study has found that the exposure of *P. aeruginosa* biofilms to exogenous Nitric oxide (NO) inhibited the expression of iron acquisition-related genes and the production of siderophores (Zhu et al., 2019). Nitric oxide is a known dispersal agent in *P. aeruginosa* biofilms. Similarly, when *P. fragi* biofilms matures, the release of nitrogenous byproducts due to proteolysis of meat also increase and this may trigger down regulation of iron acquisition related genes. Therefore, the use of exogenous NO releasing food grade compounds with a combination of high concentrations of iron can have potential in biofilm control on meat.

Compared to other stages of the biofilm cycle, the highest number of downregulated COGs can be seen between initiation vs dispersal stage. It is interesting to see that COG J which is related to translation, ribosomal structure and biogenesis contained the highest percentage of genes. Most significantly down regulated genes of COG J coded for ribosomal proteins, ribosome binding proteins, rRNA binding proteins and accessory proteins required for ribosome assembly. It can be hypothesized at the dispersal stage of the biofilm cycle, protein production ceases or gets reduced considerably. Also, COGs F and C had the next highest down regulated percentages which are related to nucleotide

transport and metabolism and energy production and conversion, respectively. Genes listed in COG F contained genes encoding for catalytic enzymes such as adenylosuccinate lyase subfamily, dGTPase family, Nudix hydrolase family and purine pyrimidine phosphoribosyl transferase family.

A higher percentage of genes categorized in COG D which represents cell cycle control, cell division and chromosome partitioning was down regulated than up regulated. It indicates that once the biofilms reach their population maximum of around 10^{11}-10^{12} CFU^{-2}, the cell division and protein synthesis cease. The CLSM images corelates well with the RNA seq results. At around 124 hours, there are less green coloured (Live) cells in the degrading biofilm. The majority of the cells in the biofilm have taken yellow and red colours which indicates the cells are losing their viability and dying.

The 91 common upregulated genes between three stages of biofilm cycle (Figure 4) may be responsible for basic metabolic functions. Also, there were 225 genes that could not be classified to any of the current COG functional categories. Aside from the genes which have known functions, there were many hypothetical proteins significantly differentially expressed in all three stages of the biofilm indicating that they are likely important in biofilm formation. However, their exact functions remain unknown.

5.6 Conclusion

This study used an experimental model that closely mimics practical industry conditions and the data provides a molecular basis for *P. fragi* biofilm formation on chilled meat. This study helped to identify the key genes that are up and down regulated at important stages of a biofilm formed on beef muscle under chilled conditions. This study investigated global gene expression for a population of bacteria within the biofilm. However, within a dense biofilm, gene expression can sometimes vary between upper and bottom layers. Single cell RNA sequencing could be a solution for this issue but can be quite complex when the biofilms are formed in complex meat tissue. Biofilms are formed when planktonic bacteria attach irreversibly to a surface. Planktonic bacteria were not included in this project due to excessive variability as planktonic bacteria are

grown in broth media which is an environment considerably different to complex meat muscle and the comparison would not be useful. The similarity in genes expressed between maturation and dispersal is most likely due to the selected time points. More differences could have been detected if RNA were extracted from a very mature, degrading biofilm. However, it is not practical to extract RNA at late dispersal stage as the majority of cells have lost their viability and the rest are dead. RNA extraction from dead cells was avoided by selecting 115 hour for extraction.

Knowledge about the gene expression can help explain many of phenotypic changes that occur during biofilm formation on meat. Understanding the cues that can trigger gene expression related to biofilm dispersal may aid in developing effective control measures. The ribosome modulation factor and creatininase aided the survival of *P. fragi* under low-temperature conditions. We have also found that iron uptake systems get significantly down regulated as the biofilm matures. High concentrations of iron and exogenous NO can be specific targets that may be useful in controlling *P. fragi* biofilms. The use of pyruvate dehydrogenase could also be a promising approach in removal of *P. fragi* biofilms via triggering dispersal.

5.7 References

Bohn, Y. S. T., Brandes, G., Rakhimova, E., Horatzek, S., Salunkhe, P., Munder, A.,Wiehlmann, L. (2009). Multiple roles of *Pseudomonas aeruginosa* TBCF10839 PilY1 in motility, transport and infection.*Molocular Microbiology, 71*(3), 730-747. doi:10.1111/j.1365-2958.2008.06559.x

Brenner, D. J. (2005). *Bergey's Manual® of Systematic Bacteriology : Volume Two The Proteobacteria Part B The Gammaproteobacteria / edited by Don J. Brenner, Noel R. Krieg, James T. Staley, George M. Garrity, David R. Boone, Paul Vos, Michael Goodfellow, Fred A. Rainey, Karl-Heinz Schleifer* (Second Edition.. ed.). Boston, MA: Boston, MA : Springer US.

Champomier-Vergès, M. C., Stintzi, A., & Meyer, J. M. (1996). Acquisition of iron by the non-siderophore-producing *Pseudomonas fragi. Microbiology*, *142*(5), 1191-1199. doi:10.1099/13500872-142-5-1191

Chiang, P., & Burrows, L. L. (2003). Biofilm Formation by hyperpiliated mutants of *Pseudomonas aeruginosa*. *Journal of Bacteriology.,* 185(7), 2374. doi:10.1128/JB.185.7.2374-2378.2003

Diane, M., Scott, A. R., Nicolas, B., Peter, D. S., & Staffan, K. (2011). Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nature Reviews Microbioliolgy 10*(1), 39. doi:10.1038/nrmicro2695

Eom, H., & Park, W. (2017). Inhibitory Effect of Taurine on Biofilm formation during alkane degradation in *Acinetobacter oleivorans* DR1. *Microbial Ecol*ogy, *74*(4), 821-831. doi:10.1007/s00248-017-1010-2

Fibriansah, G., Veetil, V. P., Poelarends, G. J., & Thunnissen, A.-M. W. H. (2011). Structural basis for the catalytic mechanism of aspartate ammonia lyase. *Biochemistry, 50*(27), 6053-6062. doi:10.1021/bi200497y

Giltner, C., Nguyen, Y., & Burrows, L. (2012). Type IV Pilin Proteins: Versatile Molecular Modules. *Microbiology Molecular Biology Reviews, 76*(4), 740-772. doi:10.1128/MMBR.00035-12

Goodwine, J., Gil, J., Doiron, A., Valdes, J., Solis, M., Higa, A., Sauer, K. (2019). Pyruvate-depleting conditions induce biofilm dispersion and enhance the efficacy

of antibiotics in killing biofilms in vitro and *in vivo*. *Scientific Reports 9*(1), 3763. doi:10.1038/s41598-019-40378-

Grinter, R., Hay, I., Song, J., Wang, J. W., Teng, D., Dhanesakaran, V., Lithgow, T. (2018). FusC, a member of the M16 protease family acquired by bacteria for iron piracy against plants. *PLoS. Biology., 16*(8). doi:10.1371/journal.pbio.2006026

Han, C., Goodwine, J., Romero, N., Steck, K. S., Sauer, K., & Doiron, A. (2019). Enzyme-encapsulating polymeric nanoparticles: A potential adjunctive therapy in *Pseudomonas aeruginosa* biofilm-associated infection treatment. *Colloids Surf B Biointerfaces, 184*, 110512. doi:10.1016/j.colsurfb.2019.110512

Hans-Curt, F., Jost, W., Ulrich, S., Peter, S., Scott, A. R., & Staffan, K. (2016). Biofilms: an emergent form of bacterial life., *Nature Reviews Microbiology 14*(9), 563. doi:10.1038/nrmicro.2016.94

Huxtable, R. (1992). Physiological actions of taurine. *Physiology Reviews 72*(1), 101. doi:10.1152/physrev.1992.72.1.101

Kang, D., & Kirienko, N. (2018). Interdependence between iron acquisition and biofilm formation in *Pseudomonas aeruginosa. Journal of Microbiology, 56*(7), 449-457. doi:10.1007/s12275-018-8114-3

Kaylie, L. R., Brittni, R. K., & Jeremiah, G. J. (2019). Heme uptake and utilization by Gram-negative bacterial pathogens. *Frontiers Microbiology., 9.* doi:10.3389/fcimb.2019.00081

Law, C. W., Chen, Y., Shi, W., & Smyth, G. K. (2014). voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biology, 15*(2), R29-R29. doi:10.1186/gb-2014-15-2-r29

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods, 25*(4), 402-408. doi:10.1006/meth.2001.1262

Mead, G. C. (2007). *Microbiological analysis of red meat, poultry and eggs / edited by G.C. Mead*. Boca Raton, Fla. : Cambridge: Boca Raton, Fla. : CRC Press, Cambridge : Woodhead Pub.

Mosbahi, K., Wojnowska, M., Albalat, A., & Walker, D. (2018). Bacterial iron acquisition mediated by outer membrane translocation and cleavage of a host

protein. *Proceedings at National Academy of Sciences U.S.A. 115*(26), 6840-6845. doi:10.1073/pnas.1800672115

Mosier, A. C., Justice, N. B., Bowen, B. P., Baran, R., Thomas, B. C., Northen, T. R., & Banfield, J. F. (2013). Metabolites associated with adaptation of microorganisms to an acidophilic, metal-rich environment identified by stable-isotope-enabled metabolomics. *mBio, 4*(2). doi:10.1128/mBio.00484-12

Noinaj, N., Guillier, M., Barnard, T., & Buchanani, S. (2010). TonB-dependent transporters: regulation, structure, and function. *Annual Reviews Microbiology, 64*, 43.

Nychas, G.-J. E., Skandamis, P. N., Tassou, C. C., & Koutsoumanis, K. P. (2008). Meat spoilage during distribution. *Meat Science, 78*(1), 77-89. doi:10.1016/j.meatsci.2007.06.020

O' Toole, G. A., & Kolter, R. (1998). Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molecular Microbiology, 30*(2), 295-304. doi:10.1046/j.1365-2958.1998.01062.x

Petrova, O. E., Schurr, J. R., Schurr, M. J., & Sauer, K. (2012). Microcolony formation by the opportunistic pathogen *Pseudomonas aeruginosa* requires pyruvate and pyruvate fermentation. *Molecular Microbiology, 86*(4), 819-835. doi:10.1111/mmi.12018

Qin, Z., Yan, Q., Ma, Q., & Jiang, Z. (2015). Crystal structure and characterization of a novel l-serine ammonia-lyase from *Rhizomucor miehei*. *Biochemistry Biophysics. 466*(3), 431-437. doi:10.1016/j.bbrc.2015.09.043

Seckbach, J. (2010). *Microbial Mats:Modern and Ancient Microorganisms in Stratified Systems / edited by Joseph Seckbach, Aharon Oren*. Dordrecht: Dordrecht : Springer Netherlands.

Stanborough, T., Fegan, N., Powell, S. M., Tamplin, M., & Chandry, P. S. (2018). Vibrioferrin production by the food spoilage bacterium *Pseudomonas fragi. FEMS Microbiology Letters, 365*(6). doi:10.1093/femsle/fnx279

Wickramasinghe, N. N., Ravensdale, J. T., Coorey, R., Dykes, G. A., & Scott Chandry, P. (2019). In situ characterisation of biofilms formed by psychrotrophic meat

spoilage pseudomonads. *Biofouling*, *35*(8), 840-855. doi:10.1080/08927014.2019.1669021

Yan, N. (2015). Structural Biology of the Major Facilitator Superfamily Transporters. *Annuual Reviews Biophysics.*, *44*(1), 257-283. doi:10.1146/annurev-biophys-060414-033901

Yang, L., Barken, K. B., Skindersoe, M. E., Christensen, A. B., Givskov, M., & Tolker-Nielsen, T. (2007). Effects of iron on DNA release and biofilm development by *Pseudomonas aeruginosa. Microbiology (Reading, England), 153*(5), 1318-1328. doi:10.1099/mic.0.2006/004911-0

Yu, S., Wei, Q., Zhao, T., Guo, Y., & Ma, L. Z. (2016). A survival strategy for *Pseudomonas aeruginosa* that uses exopolysaccharides to sequester and store iron to stimulate Psl-dependent biofilm formation. *Applied Environmental Microbiology*, *82*(21), 6403-6413. doi:10.1128/AEM.01307-16

Zhu, X., Rice, S. A., & Barraud, N. (2019). Nitric Oxide and Iron Signaling Cues Have Opposing Effects on Biofilm Development in *Pseudomonas aeruginosa*. *Applied Environmental Microbiology 85*(3). doi:10.1128/AEM.02175-18

CHAPTER 6

Nitric Oxide mediated dispersal of biofilms formed on meat by psychrotrophic meat spoilage pseudomonads

6.1 Abstract

Previous studies done on these species showed that despite being highly proteolytic and having continued access to nutrients, biofilms formed by *P. fragi* dispersed after reaching a maximum population. During proteolysis of meat, nitrogenous by products are released. Different concentrations of oxide releasing compounds (PAPA NONOate and Sodium nitroprusside (SNP)) were added to pre-formed *P. fragi* biofilms on surface sterilized, chilled beef to assess the dispersal potential of these compounds. Most of the tested concentrations of both compounds did not cause a significant dispersal effect in preformed biofilms. SNP concentration of 40 umol l^{-1} caused two log reductions of the biofilm cell numbers compared to the untreated controls. The use of nitric oxide donor compounds under correct concentration could be effective in controlling *P. fragi* biofilm growth on meat.

6.2 Introduction

It is well known that the biofilm mode of life is a staple for most microorganisms including bacteria (McDougald, D., Rice, S., Barraud, N. Steinberg, P.D. & Kjelleberg, S. (2012).). Psychrotrophic meat spoilage pseudomonads such as *Pseudomonas fragi*, *Pseudomonas lundensis* and *Psedumonas putida* readily form biofilms under chilled conditions (Ercolini et al., 2007). These psychrotrophic *Pseudomonas* species are highly proteolytic, and secrete enzymes when the growth substrate gets depleted of glucose and simple sugars (Ercolini et al., 2007). The proteolytic nature of these bacteria is considered to be an advantage as they can utilize more complex nutrients when competing with other psychrotrophic microorganisms for ecological niches. Although different microbial species produce biofilms of different structures, under varying environmental conditions and persist for different time periods, one commonality of all biofilms is their 'life cycle' which include reversible attachment, irreversible attachment, microcolony formation, maturation and dispersal (Doyle, 2001). These dispersed cells can colonize new surfaces to initiate more biofilms.

Biofilm dispersal can be an active or a passive process. Erosion and sloughing of cells can be passive due to environmental factors whereas active dispersal is a genetically regulated process which occurs in mature biofilms (Baudin, Cinquin, Sclavi, Pareau, & Lopes, 2017). Previous research has shown that several environmental and genetic cues can trigger biofilm dispersal (Gjermansen, Nilsson, Yang, & Tolker-Nielsen, 2010). N-acyl homoserine lactone (AHL) molecules and other chemical molecules produced by bacteria themselves can trigger dispersal (Myszka et al., 2016). Physiological cues such as D-amino acids, gases such as nitric oxide (NO), changes in nutrient composition, lack of oxygen for aerobic bacteria and matrix degrading enzymes can also cause biofilm dispersal (Nicolas Barraud et al., 2009).

As discussed in chapter 03, biofilms of *P. fragi* and *P. lundensis* dispersed after reaching a population maximum of $10^{11\text{-}12}$ CFU cm^{-2}. Despite being highly proteolytic, and having access to nutrients, biofilms formed on meat by spoilage pseudomonads eventually dispersed and the majority of cells lost their viability and died. However, the cause of

dispersal is currently unknown. During proteolysis of meat by psychrotrophic pseudomonads, many nitrogenous byproducts are produced including amino acids and volatile nitrogenous compounds (Stanborough et al., 2018). It can be hypothesized that these nitrogenous byproducts trigger biofilm dispersal in these proteolytic bacteria despite them having continued access to nutrients from meat. Therefore, in this research the dispersal of biofilms formed by *P. fragi by NO doner compounds under several conditions was studied.*. In this project, several concentrations of sodium nitroprusside (SNP) and PAPA NONOate were selected as NO donor compounds to be tested against *P. fragi* 1793 biofilms formed on meat.

6.2 Materials and methods

***Beef substrate preparation*:** Beef 'eye round' cut was used throughout the series of experiments to minimize the variability that can arise from muscle's texture and composition. Fresh meat was purchased from the local butchers on the same morning of each experiment. The meat was cut in to 3 kg portions from large meats cuts at the butchers and was brought as chilled overwraps to the laboratory. The meat was kept at 3 ^{o}C in the laboratory and was processed within thirty minutes from the time of purchase.

A surface sterilization method was applied where the muscle tissue was fully immersed in boiling water for 10 minutes. The drained muscle was placed inside a sterilised bio safety cabinet (BSC) and the boiled, burnt exterior of the muscle was aseptically removed. The raw interior was used for sample preparation. The sterile meat was sliced with a sterilized stainless-steel deli slicer placed inside a (BSC). The slicer was adjusted to obtain meat slices with 3 mm thicknesses which were further sectioned into 15* 15 mm pieces with a sterile scalpel. Each slice was placed separately in wells of a six well microtiter plate. To avoid drying of the muscle, 300 µl of phosphate buffered saline (PBS) was added to each well containing meat.

An overnight culture of *P. fragi* 1793 was prepared by inoculating single colony isolates into 5 ml of tryptone soy broth (TSB, Oxoid, Basingstoke, United Kingdom) and incubating at 25 ºC for 18 hours in a shaking incubator at 180 rpm. After incubation, cultures were decimally diluted to inoculate the meat slices with approximately 10^4 CFU cm^{-2}. Surface sterilized control samples were treated with 100 µl of TSB. The microtiter plates were covered with the lids and incubated at 10 ºC for 52 hours.

***Preparation of the NO donor compounds*:** SNP and PAPA NONOate were weighed and dissolved in 1X PBS solution to obtain the required set of concentrations (320, 160, 80, 40 and 20 µmol l^{-1}). Then a 200 µl of the prepared solution was added separately on to each meat slice and 200 µl of PBS was added to the control samples without NO donor compounds. Then the plates were covered with lids and incubated at 10 ºC for 14 hours. After that, the plates were removed from the incubator and the biofilms were gently washed with 300 µl of PBS. Three biological replicates with three technical replicates for each concentration were carried out

***Preparation of meat slices to obtain CFU counts*:** Meat slices were removed from microtitre plates and placed separately in Falcon tubes containing 9 ml of maximum recovery diluent (MRD) (Oxoid, Basingstoke, UK). The corresponding wells of the microtitre plates were washed with 1 ml of MRD and transferred to 9 ml with the meat sample. The tube was vortex mixed for one minute and shaken at maximum speed using a mechanical flask shaker (Griffin and Tatlock Ltd, Birmingham, London) for five minutes. Afterwards, biofilms were disrupted with sonication by placing the tubes in an ultrasonic water bath (Ultrasonics. Pty. Australia), for five minutes. The tubes were then vortex mixed for 30 seconds. Tenfold serial dilutions were made and 100 µl of the dilutions were plated on *Pseudomonas* isolation agar (Oxoid, Basingstoke, UK). The plates were incubated at 25 ºC for 48 hours. The CFUs were determined and the cell densities of the biofilms were calculated, and the results were expressed as CFU in a logarithmic scale per square centimetre of beef. Then the average and standard deviation (SD) were calculated for each biological replicate.

***Imaging the treated biofilms with CLSM*:** The treated samples were imaged with Leica SP 5 Confocal Laser Scanning Microscope (CLSM) (Leica Microsystems, Heidelberg, Germany). Syto 9 and propidium iodide (PI) nucleic acid dyes were used to fluorescently stain the live and dead cells respectively in the biofilms.

Fluorescent stains were prepared by adding 5 µl of SYTO 9 and 5 µl of PI in 1 ml of Milli-Q water. A 100 µl of the dye mixture was added to each muscle slice. The stained samples were incubated in the dark at room temperature which is around 25 ºC for 15-20 minutes. Samples were then removed from the microtiter plates using sterile forceps and placed on clean glass slides. A cover slip was gently placed on top of each muscle slice and samples were imaged under the 100x oil immersion objective.

Stained meat samples were excited with the 488 nm laser line from Argon laser at 20% intensity. The samples were scanned at a speed of 200 Hz and imaged at 1200 * 1200 resolutions. The emission wavelength was collected between 490-590 nm for SYTO 9 (displayed green) and images were captured.

6.3 Results and discussion

When *P. fragi* and *P. lundensis* grow on meat as biofilms under aerobic conditions, the cell numbers increase rapidly. When the numbers reach around 10^7 CFU cm^{-2}, glucose and simple sugars within the muscle get depleted (Ercolini, Russo, Nasi, Ferranti, & Villani, 2009) and bacteria secrete proteolytic enzymes which breakdown proteins into amino acids, NH_3, and other volatile compounds. When the CFU counts on meat reach around 10^{11}-10^{12} CFU cm^{-2} the biofilm structures begin to degrade and cell numbers decline (Wickramasinghe, Ravensdale, Coorey, Dykes, & Scott Chandry, 2019). The increase in nitrogenous byproducts could be the cause of dispersal of these biofilms formed by proteolytic bacteria. Therefore, in this study, we tested the potential of NO which has already been shown to cause dispersal in different bacterial species (Cutruzzolà & Frankenberg-Dinkel, 2016).

In this study we selected SNP and PAPA NONOate as the NO donor compounds. The compounds were added 14 hours after incubation which is prior to initiation of dispersal. In our results, statistically significant differences in the CFU numbers could not be detected between most of the tested concentrations and untreated control samples. Based on the CFU numbers (Tables 6.1, 6.2 and 6.3), and CLSM images (Figure 1 and 2), many concentrations of PAPA NONOate (20,40, 80, 160 and 320 µmol l^{-1}) and SNP (80, 160, 320 µmol l^{-1}) do not appear to cause a significant effect in causing biofilm dispersal. The average CFU numbers were 10^{10} CFU cm^{-2}. At the same time, it is clear that these compounds do not promote biofilm growth as the CFU numbers in treated samples are similar to CFU numbers in no treatment controls.

Table 6.1 The $\log_{10}$ CFU cm^{-2} of biological replicate one of *P. fragi* biofilms formed on meat treated with nitric oxide donor compounds. T1, T2, & T3 represents technical replicates

Compound (µmol/l)	T1	T2	T3	avg	SD
PAPA 320	10.5	10.32	10.44	10.42	0.091
PAPA 160	10.81	10.92	9.77	10.5	0.634
PAPA 80	9.85	10.89	9.87	10.20333	0.594
PAPA 40	10.51	10.04	9.09	9.88	0.723
PAPA 20	10.62	10.55	10.36	10.51	0.134
SNP 320	10.79	9.95	9.94	10.22667	0.487
SNP 160	8.56	9.32	9.39	9.09	0.460
SNP 80	9.04	8.95	9.04	9.01	0.051
SNP 40	9.17	8.99	9.17	9.11	0.103
SNP 20	10.89	9.61	10.71	10.40333	0.692
No treatment control	10.77	10.57	9.77	10.37	0.529

Table 6.2 The $\log_{10}$ CFU cm^{-2} of biological replicate twoof *P. fragi* biofilms formed on meat treated with nitric oxide donor compounds.T1, T2, & T3 represents technical replicates

Compound (µmol/l)	T1	T2	T3	avg	SD
PAPA 320	10.9	10.97	10.87	10.91	0.051
PAPA 160	9.79	10.07	9.74	9.86	0.177
PAPA 80	9.61	9.96	9.9	9.82	0.187
PAPA 40	9.95	10.04	9.3	9.76	0.403
PAPA 20	8.97	10.25	10.11	9.77	0.702
SNP 320	9.25	9.8	9.67	9.57	0.287
SNP 160	9.07	9.94	9.74	9.58	0.455
SNP 80	8.94	9.17	8.95	9.02	0.13
SNP 40	8.9	9.04	8.17	8.70	0.467
SNP 20	9.92	9.49	9.71	9.70	0.215
No treatment control	11.27	11.68	10.77	11.24	0.455

Table 6.3 The $\log_{10}$ CFU cm^{-2} of biological replicate three of *P. fragi* biofilms formed on meat treated with nitric oxide donor compounds. T1, T2, & T3 represents technical replicates

Compound (µmol/l)	T1	T2	T3	avg	SD
PAPA 320	10.9	10.97	10.87	10.91	0.051
PAPA 160	9.79	10.07	9.74	9.86	0.177
PAPA 80	9.61	9.96	9.9	9.82	0.187
PAPA 40	9.95	10.04	9.3	9.76	0.403
PAPA 20	8.97	10.25	10.11	9.77	0.702
SNP 320	9.25	9.8	9.67	9.57	0.287
SNP 160	9.07	9.94	9.74	9.58	0.455
SNP 80	8.94	9.17	8.95	9.02	0.13
SNP 40	8.9	9.04	8.17	8.70	0.467
SNP 20	9.92	9.49	9.71	9.70	0.215
No treatment control	11.27	11.68	10.77	11.24	0.455

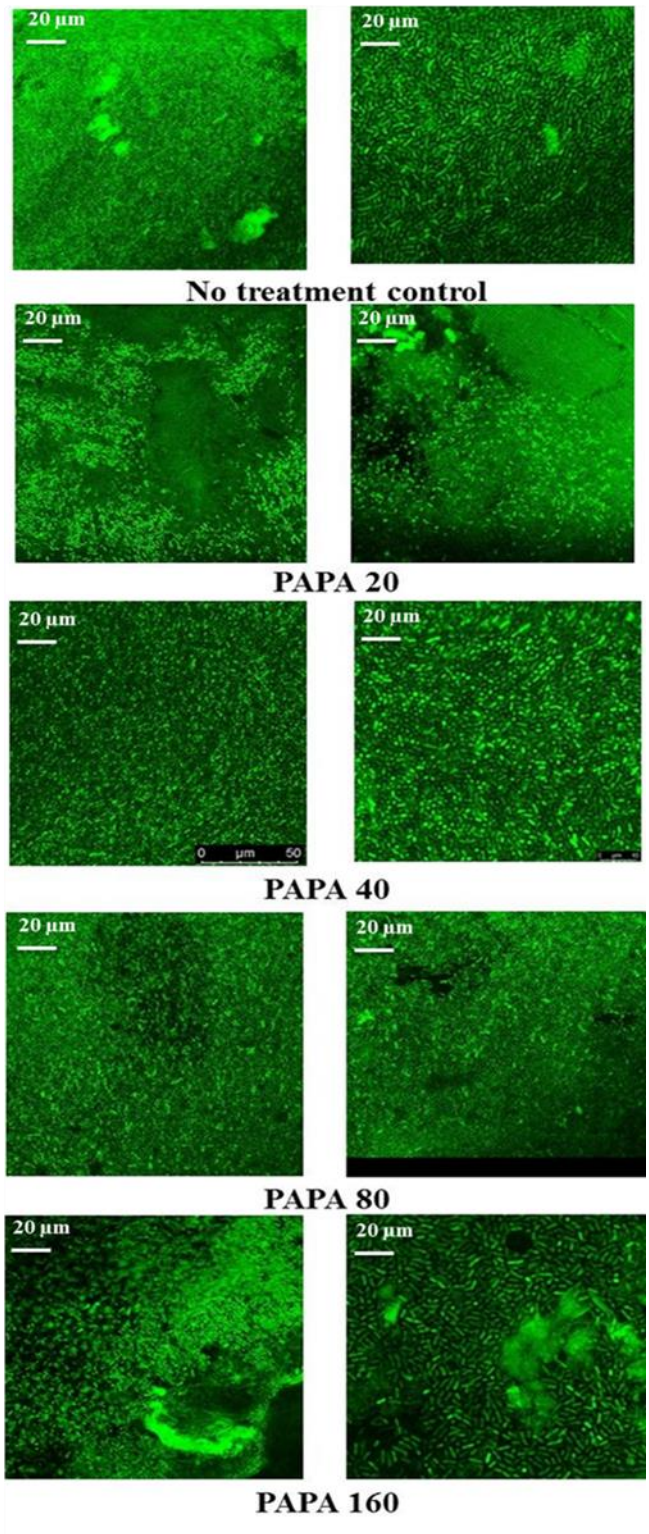


Figure 6.1 CLSM micrographs of *P. fragi* 1793 biofilm treated with different concentrations of PAPA NONOate

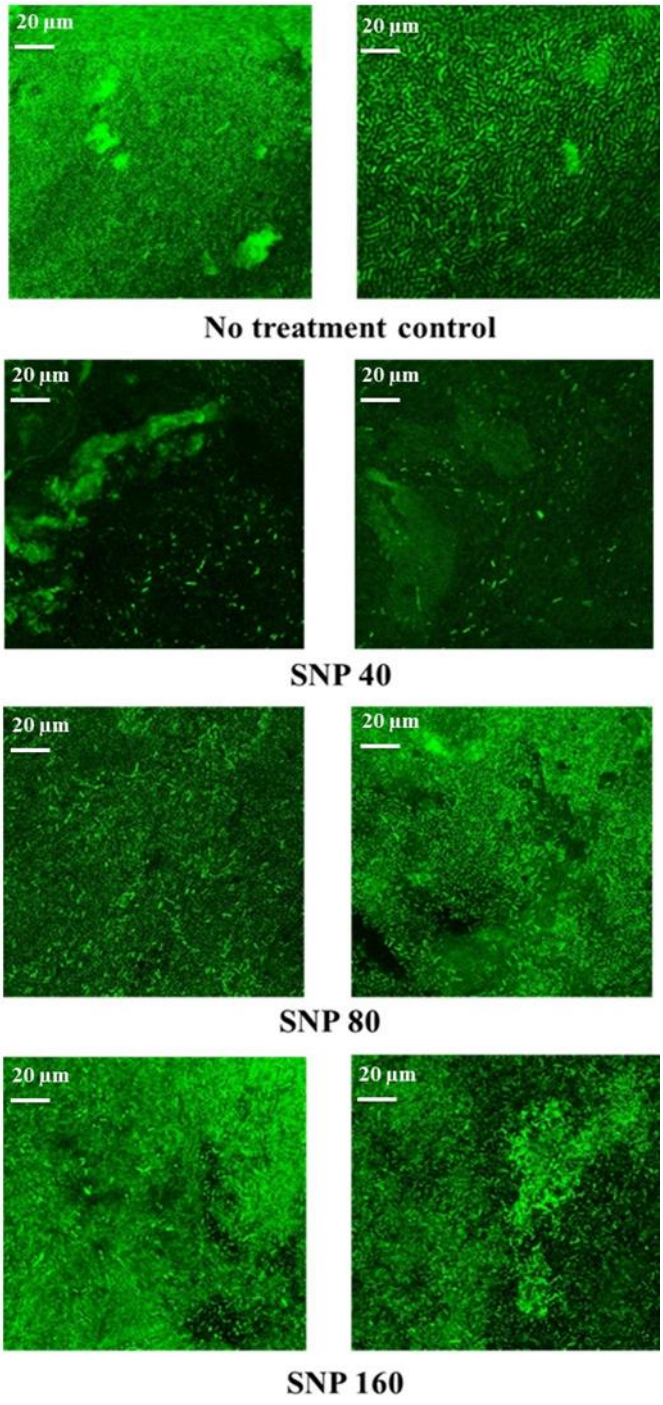


Figure 6.2. CLSM micrographs of *P. fra*gi 1793 biofilm treated with different concentrations of SNP

Based on CFU numbers, the meat slices inoculated with 40 µmol l^{-1} of SNP were significantly lower compared to other concentrations. The average CFU counts were around 10^8 CFU cm^{-2} with two $\log_{10}$ reductions compared to no treatment controls (Figure 2). The cell numbers in meat slices were 10^8-10^9 CFU cm^{-2}. Also, the low CFU numbers were repeatable across the three biological replicates and their technical replicates. That could likely be due to the correct concentration of NO was used in those samples.

The differences in cell numbers for the compounds of similar concentration could likely be due to the differences in the rates of NO release by these compounds. PAPA NONOate has NO release half-life of 77 minutes and SNP has NO release half-life of 90 minutes (. Barraud, Kelso, Rice, & Kjelleberg, 2015) which are moderate rates of NO release and both the compounds are readily soluble in water. A significant dispersal effect was not observed in any of the PAPA NONOate concentrations used in our experiment. The correct rate of NO release required for meat grown biofilm dispersal may not have been met in our experiment for this compound.

There are several NO donor compounds mentioned in literature such as sodium nitroprusside (SNP), 6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-hexanamine (MAHMA NONOate) or aminoxyl free radicals (nitroxides) with different rates of NO release. Very rapid or very slow rates on NO release are known to be ineffective in causing dispersal effect (Barraud et al., 2006).

Previous research that tested NO donor compounds against *Pseudomonas aeruginosa* biofilms has shown that addition of 500 nM SNP to biofilms formed on agar plates resulted in a 25% increase in swimming motility and a 77% increase in swarming motility compared to no treatment controls (Barraud et al., 2006) These effects could not be achieved at higher concentrations of SNP (12.5 µM and above).

High concentrations of NO can be lethal for biofilms and dispersal is induced with low sublethal concentrations (Barraud et al., 2006). Based on their studies, it was suggested that low concentrations of NO induces the transition of sessile biofilm cells to free-swimming planktonic cells. This may explain why dispersal was only observed in our study at 40 µmol l^{-1}. In order for dispersal to be triggered, NO should be released within a sublethal concentration range (Barraud et al., 2015).

Past studies have shown that NO is able to induce biofilm dispersal in *P.aeruginosa, Escherichia coli, Vibrio cholerae, Staphylococcus epidermidis, Bacillus licheniformis, Serratia marcescens, Legionella pneumophila, Nitrosomonas europaea, and Neisseria gonorrhoeae* (Barraud et al., 2009; Carlson, Vance, & Marletta, 2010). Nitric oxide is a diatomic gas, a known signaling molecule in prokaryotes, and is able to induce the dispersal of biofilms of many bacterial species by lowering the c-di-GMP levels (Nicolas Barraud et al., 2012). It has been shown that high levels of c-di-GMP levels induce biofilm formation while low c-di-GMP levels induce biofilm dispersal (Diane et al., 2011). In several bacterial systems NO has been shown to be produced directly from the microorganism itself, as an intermediate or byproduct of specific metabolic pathways, such as denitrification which is a form anaerobic respiration (Cutruzzolà & Frankenberg-Dinkel, 2016).

To date, the effects of NO have not been tested for psychrotrophic meat spoilage pseudomonads. Time limitation of our PhD prevented further detailed analysis of biofilm dispersal agents. Extensive studies are necessary with different NO donor compounds to find food compatible compounds and concentrations that can effectively cause dispersal of biofilms formed on meat.

Apart from being a potential dispersal agent, the use of NO has several beneficial aspects in terms of preservation of meat quality and sensory characteristics. Nitrite or nitrate are popular means of preservation of the meat in food industry (Skibsted, 2011). During meat curing, the nitric oxide formed from nitrite reacts with various components present in the muscle. Nitrate salts are reduced to nitrite and then to gaseous NO. Therefore, nitrite may also have potential in controlling biofilm and slime formation on meat. In the current food industry, Nitric oxide is recognized for its role in improving color and stability of

meat products (Parthasarathy & Bryan, 2012). The pigment causing the bright reddish pink color of cured, uncooked meat is mainly nitric oxide myoglobin (NOMb) with some nitric oxide haemoglobin (Macdougall, Mottram, & Rhodes, 1975). Nitric oxide is also known for its negative effects in microbial growth on meat (Shank, Silliker, & Harper, 1962). Nitric oxide also acts as an antioxidant in processed meat by scavenging lipid derived radicals and by deactivation of peroxides (Kanner, Harel, & Rina, 1991). Recent studies have also found that maintaining NO homeostasis is crucial for optimal health and disease prevention in the human body (Parthasarathy & Bryan, 2012). Therefore, it is clear that the use of food-grade NO releasing compounds can have many beneficial outcomes and detailed future research on its biofilm control ability is highly recommended.

Also, apart from using a single eradication method, it can be more effective to use a hurdle technique where several control methods are applied on biofilms. The use of correct dispersal agents and biofilm matrix degrading enzymes (Di Martino, 2018) can be used in combination to degrade biofilms based on the matrix composition.

6.4 References

Barraud, N., Hassett, D. J., Hwang, S.-H., Rice, S. A., Kjelleberg, S., & Webb, J. S. (2006). Involvement of Nitric Oxide in Biofilm Dispersal of *Pseudomonas aeruginosa*. *The Journal of Bacteriology*, *188*(21), 7344. doi:10.1128/JB.00779-06

Barraud, N., Kardak, B. G., Yepuri, N. R., Howlin, R. P., Webb, J. S., Faust, S. N., Kelso, M. J. (2012). Cephalosporin-3′-diazeniumdiolates: Targeted NO-Donor Prodrugs for Dispersing Bacterial Biofilms. *Angewandte Chemie International Edition, 51*(36), 9057-9060. doi:10.1002/anie.201202414

Barraud, N., Kelso, M. J., Rice, S. A., & Kjelleberg, S. (2015). Nitric oxide: a key mediator of biofilm dispersal with applications in infectious diseases. *Current Pharmaceutical Design, 21*(1), 31-42. doi:10.2174/1381612820666140905112822

Barraud, N., Storey, M. V., Moore, Z. P., Webb, J. S., Rice, S. A., & Kjelleberg, S. (2009). Nitric oxide-mediated dispersal in single- and multi-species biofilms of clinically and industrially relevant microorganisms. *Microbial Biotechnology, 2*(3), 370-378. doi:10.1111/j.1751-7915.2009.00098.x

Baudin, M., Cinquin, B., Sclavi, B., Pareau, D., & Lopes, F. (2017). Understanding the fundamental mechanisms of biofilms development and dispersal: BIAM (Biofilm Intensity and Architecture Measurement), a new tool for studying biofilms as a function of their architecture and fluorescence intensity. *Journal of Microbiological Methods, 140,* 47-57. doi:10.1016/j.mimet.2017.06.021

Carlson, H. K., Vance, R. E., & Marletta, M. A. (2010). H-NOX regulation of c-di-GMP metabolism and biofilm formation in *Legionella pneumophila*. *Molecular Microbiology. 77*(4), 930-942.

Cutruzzolà, F., & Frankenberg-Dinkel, N. (2016). Origin and Impact of Nitric Oxide in *Pseudomonas aeruginosa* Biofilms. *Journal of bacteriology, 198*(1), 55-65. doi:10.1128/JB.00371-15

Di Martino, P. (2018). Extracellular polymeric substances, a key element in understanding biofilm phenotype. *AIMS Microbiology, 4*(2), 274-288. doi:10.3934/microbiol.2018.2.274

Diane, M., Scott, A. R., Nicolas, B., Peter, D. S., & Staffan, K. (2011). Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nature Reviews Microbiology, 10*(1), 39. doi:10.1038/nrmicro2695

Doyle, R. J. (2001). Microbial growth in biofilms *Methods in enzymology.* 336, 337, San Diego, Calif : Academic Press.

Ercolini, D., Russo, F., Blaiotta, G., Pepe, O., Mauriello, G., & Villani, F. (2007). Simultaneous detection of *Pseudomonas fragi, P. lundensis,* and *P. putida* from meat by use of a multiplex PCR sssay targeting the carA Gene. *Applied and Environmental Microbiology, 73*(7), 2354.

Ercolini, D., Russo, F., Nasi, A., Ferranti, P., & Villani, F. (2009). Mesophilic and psychrotrophic bacteria from meat and their spoilage potential *in Vitro* and in beef. *Applied and Environmental Microbiology, 75*(7), 1990. doi:10.1128/AEM.02762-08

Gjermansen, M., Nilsson, M., Yang, L., & Tolker-Nielsen, T. (2010). Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms: genetic elements and molecular mechanisms. *Molecular Microbiology, 75*(4), 815-826. doi:10.1111/j.1365-2958.2009.06793.x

Kanner, J., Harel, S., & Rina, G. (1991). Nitric oxide as an antioxidant. *Archives of Biochemistry Biophysics, 289*(1), 130-136. doi:10.1016/0003-9861(91)90452-O

Macdougall, D. B., Mottram, D. S., & Rhodes, D. N. (1975). Contribution of nitrite and nitrate to the colour and flavour of cured meats. *Journal of the Science of Food and Agriculture, 26*(11), 1743-1754. doi:10.1002/jsfa.2740261117

Myszka, K., Schmidt, M. T., Majcher, M., Juzwa, W., Olkowicz, M., & Czaczyk, K. (2016). Inhibition of quorum sensing-related biofilm of *Pseudomonas fluorescens* KM121 by Thymus vulgare essential oil and its major bioactive compounds. *International Biodeterioration & Biodegradation, 114*, 252-259. doi:10.1016/j.ibiod.2016.07.006

Parthasarathy, D. K., & Bryan, N. S. (2012). Sodium nitrite: The “cure” for nitric oxide insufficiency. *Meat Science, 92*(3), 274-279. doi:10.1016/j.meatsci.2012.03.001

Shank, J. L., Silliker, J. H., & Harper, R. H. (1962). The effect of nitric oxide on bacteria. *Applied Microbiology, 10*(3), 185-189. doi:10.1128/AEM.10.3.185-189.1962

Skibsted, L. H. (2011). Nitric oxide and quality and safety of muscle based foods. *Nitric Oxide, 24*(4), 176-183. doi:10.1016/j.niox.2011.03.307

Stanborough, T., Fegan, N., Powell, S. M., Singh, T., Tamplin, M., & Chandry, P. S. (2018). Genomic and metabolic characterization of spoilage-associated Pseudomonas species. *International Journal of Food Microbiology, 268*, 61-72. doi:10.1016/j.ijfoodmicro.2018.01.005

Wickramasinghe, N. N., Ravensdale, J. T., Coorey, R., Dykes, G. A., & Scott Chandry, P. (2019). In situ characterisation of biofilms formed by psychrotrophic meat spoilage pseudomonads. *Biofouling, 35*(8), 840-855. doi:10.1080/08927014.2019.1669021

CHAPTER 7

General Conclusion

7.1 Introduction

The aims of this research were to understand how biofilm formation can aid in the dominance of psychrotrophic pseudomonads on chilled meat and to study the cellular and structural arrangement of biofilms formed on raw beef. The study of biofilms is a fast-growing field as biofilm formation is a staple mode of life for most bacterial species. However, the majority of research conducted to date has used controlled laboratory environments and abiotic surfaces such as petri dishes, microtiter plates and flow cells to study biofilms. Conducting biofilm research in their natural ecosystems is often difficult as the growth matrix of many biological systems are not conducive to the laboratory setting due to many variables. The novel component of this research was the use of raw beef as the substrate for biofilm growth.

The applicability of results obtained from our research to the meat industry is likely to be more relevant than many other biofilm studies done on these species. This research was mostly focused on *P. fragi* which is the main spoilage bacterium of chilled meat worldwide, and on *P. lundensis* which is another key psychrotrophic spoilage bacterium that has yet to be extensively characterized.

7.2 Key findings, limitations and recommendations for future research

The findings of this research presented in chapters 3-7, helped us to comprehend some of the previously unknown mechanisms which aid *P. fragi* to be dominant on chilled meat. The following is a summary of the most significant findings reported in the previous chapters with recommendations for future research where new and insightful findings might be uncovered.

Chapter 02 provided an in-depth insight into the existing research done on food spoilage pseudomonads as well as revealing gaps in our current knowledge of biofilms related to food industry. This extensive literature review describes the currently known reasons for the dominance of psychrotrophic pseudomonads on meat. It emphasizes the metabolic versatility of spoilage *Pseudomonas* species and highlights the variability in results when conducting experiments under different model systems. The chapter compiles information about *P. fragi, P. lundensis, P. fluorescence* and *P. putida* from many past research studies. Thus, it aids us to comprehend which sections about these organisms require further research to be done. Areas including biofilm formation, mode of communication, matrix composition and gene expression patterns are key sections that lack information.

In chapter 03, the structural characteristics of biofilms and meat degradation pattern when *P. fragi* and *P. lundensis* biofilms were formed on meat were assessed. In that chapter, many new, intriguing and reproducible results were obtained which can aid to explain the dominance of *P. fragi* on long-term stored chilled meat. The model system which was developed using fluorescent staining of meat grown biofilms, imaging with CLSM, quantification of biofilm biomass with COMSTAT and 3D reconstructions using AVIZO has not been used in any model system to date. This model system can also be used to study mixed species biofilms using fluorescently tagged bacterial species prior to inoculation.

Our results show that highly dense, compact, lawn-like, uniform biofilms are a characteristics of *P. fragi* strains, while *P. lundensis* formed disorganized biofilms with many intercellular gaps and voids. In chapter 03, we also found the interesting phenomenon of *P. fragi* bacterial cells orientated vertically on the longitudinal axis within the biofilm due to its compact arrangement. It is likely that this dense cellular arrangement provides *P. fragi* biofilms with superior mechanical properties which aid them to withstand shear forces and harsh environmental conditions. At the same time, the specific cellular arrangement can prevent foreign bacterial species breaching the biofilm interior and utilizing expensive resources such as matrix material. Therefore, our results show that cellular arrangement and biofilm structure can aid *P. fragi* to become dominant on meat.

A main objective of this PhD was to assess if there are structural differences in *P. fragi* and *P. lundensis* biofilms when they are formed under chilled and ambient temperature conditions. Based on our results, no structural differences can be detected between biofilms formed under different temperatures. Also, we found that the maximum *Pseudomonas* cell concentration on a meat slice of any thickness, before dispersal is initiated is around 10^{11} CFU cm^{-2}. At the same time, the population maximum (μmax) of *P. fragi* and *P. lundensis* biofilms are similar, and it did not vary based on temperature conditions.

This research has found that despite being highly proteolytic and having continued access to nutrients, biofilms of *P. fragi* and *P. lundensis* strains degraded after reaching the μmax. In chapter 03, it was hypothesized that the metabolic byproducts of muscle degradation could play a role in biofilm dispersal. This observation led us to study the biofilm dispersal ability of NO donor compounds in chapter 06 which will be discussed later in this chapter.

In chapter 03, we also studied eDNA production and its network pattern in *P. fragi* and *P. lundensis* strains grown under refrigerated and ambient temperature conditions in order to detect significant differences in eDNA production. A strong correlation or a pattern could not be detected between eDNA production and temperature levels or between eDNA production and bacterial species. We concluded that eDNA production does not vary

significantly based on temperature nor has a considerable effect on biofilm structure. Some studies have used DNases to degrade and control biofilm formation. However, based on our results, targeting eDNA to control biofilm formation on chilled meat may not hold much potential as eDNA production does not increase at low temperature.

Also, we observed that despite having the ability to efficiently degrade muscle fibers, *P. fragi* and *P. lundensis* appeared unable to degrade the membranes of the bovine nuclei. The large, hollow, elliptical voids in meat slices with mature biofilms clearly confirm this observation. This observation sheds new light into muscle degradation patterns of *P. fragi* and *P. lundensis* species.

The limitations of these findings are that our model system tested only mono-species biofilms formed on meat. In most biofilms formed in natural environments, there are multiple species and strains of bacteria residing in them. It is important to study their interactions as it can also have an effect on the spoilage process. However, since at the start of the project there was no detailed information available on any sort of biofilm formation on actual meat muscle under chilled temperatures. Thus the initial studies and experiments had to be conducted using mono-species prior to testing more complex multi-species biofilms. It would be interesting to study the structural arrangement, commensalism or competition among species using a multi-species model. Future studies on multi-species biofilms can be studied with some modifications to the model system used in this research. It can be recommended that bacterial species or strains to be separately tagged fluorescently prior to inoculation on the meat for multi species models.

The matrix of a biofilm plays a key role in its robustness by providing protection from various environmental hazards (Flemming & Jost, 2010). In chapter 04, we focused on assessing the extra-cellular polymeric substances (EPS) which are key components of the biofilms matrix (Hobley et al., 2015).

A highlight of this chapter is that the biofilms were grown on sterilized, porous nitro-cellulose membranes placed on surface sterilized raw beef. By using this mechanism, the bacteria were able to gain adequate access to water and nutrients through membrane pores which were of 0.2 µm size but were unable to contact the meat surface. Therefore, during biofilm extraction, the substances from meat cannot contaminate the extract and interfere with the results.

In Chapter 4, it was shown that EPS production significantly increased when the biofilms are formed under chilled conditions. This included total carbohydrates and total proteins in the biofilm matrix. When the extracted matrix was chemically analyzed, we have found that *P. fragi* and *P. lundensis* biofilms responded to chilled conditions by increasing the matrix carbohydrate and protein content in statistically significant levels compared to biofilms grown under ambient temperature. It is clear, that increasing the EPS content when formed under low temperature is important for the survival of these biofilms. This information shows that the use of targeted matrix degrading enzymes such as proteases and glycosidases could be effective in controlling biofilm formation under low temperature. At the same time, this chapter further verifies the observation in chapter 03 about eDNA in biofilm matrix where no clear correlation could be detected in temperature levels and eDNA production. Another interesting observation is that Raman spectra showed that the planktonic bacteria contained high concentrations of guanine compared to biofilm samples. We hypothesized that this can be due to the difference in cyclic-di-GMP levels between biofilm and planktonic bacteria. Further research is recommended to confirm this hypothesis.

A limitation of this model system is that the use of nitro-cellulose membranes may have reduced the bacterial population on meat compared to biofilms formed directly on meat. In this experiment the overall quantity of carbohydrates and proteins in the matrix were determined. The exact composition of what these carbohydrates and proteins are made of is still unknown. It is important to further characterize their composition using spectroscopic methods. With such knowledge more targeted matrix degrading compounds can be selected to control slime formation on meat.

All biofilms undergo stages of transition in their life cycle which includes planktonic mode, reversible attachment, irreversible attachment, maturation and dispersal (Flemming et al., 2011). All these stages are genetically regulated but according to previous studies, common gene expression profiles related to each stage could not be found among different species (Diane et al., 2011). Since no information is currently available about genetic regulation of the biofilm cycle of spoilage pseudomonads, in chapter 05 we carried out a transcriptomic profile of *P. fragi* strain 1793 when it was grown on raw, chilled beef. To the best of our knowledge this is the first study that established a transcriptomic profile of *P. fragi* biofilm formed on raw beef.

When conducting a RNA sequencing experiment, it is important to increase the number of biological replicates and we have used four biological replicates for each stage of the biofilm. In our research, high yields of good quality RNA were extracted (appendix 4), which gave good sequencing reads. Our results clearly showed that genes encoding Flp family type IVb pilin were significantly upregulated when *P. fragi* biofilms are formed on meat. Studies have shown that type IVb pilin plays a key role in some bacterial species by promoting attachment to surfaces and to each other which aids to initiate biofilm formation (Giltner et al., 2012). Also, the expression of genes encoding ribosome modulation factor increased. This gene plays an important role in stabilizing ribosomes under chilled conditions. Efficient functioning of the ribosomes is essential for protein synthesis, growth and survival of the bacteria (El-Sharoud, 2008).Therefore, it is likely that ribosome modulation factor may aid the survival of *P. fragi*'s under chilled temperature conditions.

The results also showed that genes encoding creatininase enzyme (PAA37735.1) were significantly upregulated. One of the specific characteristics of *P. fragi* is their ability to utilize creatinine and creatine when the substrates are depleted of glucose and simple sugars, while other psychrotophic spoilage pseudomonads such as *P. lundensis*, *P. putida* and *P. fluorescence* lack this ability (Drosinos & Board, 1994). Our results showed that *P. fragi* utilize creatinine under the biofilm mode of growth which aids its longevity on meat.

RNA seq results further showed that several genes related to pyruvate dehydrogenase (PDH) enzyme complex were significantly upregulated. Pyruvate has been shown to be necessary for biofilm growth in *P. aeruginosa* (Petrova et al., 2012). The use of exogenous PDH enzyme caused dispersal in existing *P. aeruginosa* biofilms where sessile surface attached bacteria were released in planktonic state (Goodwine et al., 2019; Han et al., 2019). Thus, our RNA seq results show that PDH enzyme may also act as a dispersing agent of *P. fragi* biofilms. Further studies on PDH enzyme on *P. fragi* biofilms are recommended for future research.

A one of the key highlights of our results is that the iron uptake systems appear to be significantly down regulated in mature biofilms. Past studies have concluded that *P. fragi* do not produce siderophores (Champomier-Vergès et al., 1996). Recent research has shown that *P. fragi* does indeed produce siderophores when grown in liquid cultures, under iron limiting conditions, in planktonic state. (Stanborough, Fegan, Powell, Tamplin, et al., 2018) but no information was available of their siderophore production ability on actual meat. Our results clearly show that genes coding for siderophores have been down regulated in mature biofilms which indicates that siderophores are being used at some stage when *P. fragi* grows on meat.

Our results further showed that genes coding for FUSC proteins and heme utilization protein were down regulated. These genes are also involved in iron uptake (Grinter et al., 2018; Kaylie et al., 2019; Mosbahi et al., 2018) which indicates that *P. fragi* can utilize several mechanisms for iron uptake. According to studies of Yang et al. (2007), on *P. aeruginosa*, high concentrations of iron can weaken the biofilm structures. Since iron uptake systems are significantly down regulated, similar effects could apply for *P. fragi* biofilms as well.

At the same time, the release of exogenous NO has been shown to suppress iron uptake systems in *P. aeruginosa* biofilms (Zhu et al., 2019). Similarly, when *P. fragi* biofilms mature, the release of nitrogenous byproducts due to proteolysis of meat increase and this may also trigger down regulation of iron uptake related genes. Since it has been demonstrated previously that *P. fragi* releases volatile nitrogenous byproducts during proteolysis (Stanborough, Fegan, Powell, Singh, et al., 2018), it can be hypothesized that these byproducts trigger down regulation of iron uptake systems.

A limitation of this experiment is the use of a global gene expression analysis for the whole extracted biofilm. Most of the time, bacterial cells are arranged in many layers within a biofilm and there can be differences in gene expression between upper and lower layers and those differences cannot be taken into account in this type of an approach.

In our study, many significantly up and downregulated genes were found with currently unknown functions in COG database. These genes likely regulate metabolic functions that are important in surviving on chilled meat. Further studies are necessary on these genes to discover their role in growth as biofilms on chilled meat.

In chapter o6, the dispersal of *P. fragi* biofilms using exogenous NO donor compounds were assessed. While many of the tested concentration showed no effect in causing any dispersal effect, 40 µmol l^{-1} of SNP gave two log reductions in CFU counts compared with no treatment controls. Studies conducted on *P. aeruginosa* have shown that the rate of NO release and the concentration of NO play key roles in the effectiveness of biofilm dispersal. Higher concentrations does not necessarily cause effective dispersal (Nicolas Barraud et al., 2006). Therefore, when used in correct concentrations, NO donor compounds may act as potential dispersal agents of *P. fragi* biofilms formed on meat. Further studies with different NO release agents with different concentrations on *P. fragi*, *P. lundensis* and other psychrotrophic spoilage pseudomonads are recommended for future research.

It is also important to study food grade compounds that can release NO and cause biofilm dispersal. Nitrite and nitrates are already used in the meat industry which release NO at different rates and concentrations (Parthasarathy & Bryan, 2012). Further studies on compounds that can act as curing agents as well as biofilm controlling agents can be quite beneficial to the meat industry.

Our research developed a transcriptomic profile of *P. fragi* biofilms when formed on chilled meat. For future research, studying transcriptomic profile of biofilms treated with different dispersing agents could reveal much information on their gene expression when subjected to these compounds.

The findings of this research can be used as a platform for future biofilm studies on food materials. Biofilms are highly robust and extremely difficult to eradicated once they are formed. Once slime formation been has initiated, food products are deemed unacceptable. Based on the results of our final experiments, we have found that high concentrations of iron and pyruvate dehydrogenase enzyme and specific concentrations of NO are not suitable for biofilm formation. In order to further understand their modes of action, these compounds should be tested on *in-situ* models under industry applicable conditions. At the same time, a higher dispersal effect could be achieved if these compounds are used in combination. The use of the hurdle concept can be applied to determine if better control of slime formation can be achieved. The use of appropriate matrix degrading enzymes with correct NO donor compounds or high concentrations of iron and/or PDH may achieve better results than using any single method of control.

7.3 References

Barraud, N., Hassett, D. J., Hwang, S.-H., Rice, S. A., Kjelleberg, S., & Webb, J. S. (2006). Involvement of Nitric Oxide in Biofilm Dispersal of *Pseudomonas aeruginosa. The Journal of Bacteriology, 188*(21), 7344. doi:10.1128/JB.00779-06

Champomier-Vergès, M. C., Stintzi, A., & Meyer, J. M. (1996). Acquisition of iron by the non-siderophore-producing *Pseudomonas fragi*. *Microbiology, 142*(5), 1191-1199. doi:10.1099/13500872-142-5-1191

Diane, M., Scott, A. R., Nicolas, B., Peter, D. S., & Staffan, K. (2011). Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nature Reviews Microbiology, 10*(1), 39. doi:10.1038/nrmicro2695

Drosinos, E. H., & Board, R. G. (1994a). Metabolic activities of pseudomonads in batch cultures in extract of minced lamb. *Journal of Applied Bacteriology, 77*(6), 613-620. doi:10.1111/j.1365-2672.1994.tb02809.x

El-Sharoud, W. (2008). *Bacterial Physiology : A Molecular Approach / edited by Walid El-Sharoud*. Berlin, Heidelberg: Berlin, Heidelberg : Springer Berlin Heidelberg.

Flemming, H.-C., Neu, T. R., & Wozniak, D. J. (2007). The EPS Matrix: The "House of Biofilm Cells". *The Journal of Bacteriology, 189*(22), 7945. doi:10.1128/JB.00858-07

Flemming, H.-C., Wingender, J., Szewzyk, U., & SpringerLink (Online service). (2011). *Biofilm Highlights Springer Series on Biofilms,* 243-273.

Giltner, C., Nguyen, Y., & Burrows, L. (2012). Type IV pilin proteins: Versatile molecular modules. *Microbiology Reviews., 76*(4), 740-772. doi:10.1128/MMBR.00035-12

Goodwine, J., Gil, J., Doiron, A., Valdes, J., Solis, M., Higa, A.,Sauer, K. (2019). Pyruvate-depleting conditions induce biofilm dispersion and enhance the efficacy of antibiotics in killing biofilms *in vitro* and *in vivo*. *Scientific Reports, 9*(1), 3763. doi:10.1038/s41598-019-40378-z

Grinter, R., Hay, I., Song, J., Wang, J. W., Teng, D., Dhanesakaran, V., Lithgow, T. (2018). FusC, a member of the M16 protease family acquired by bacteria for iron piracy against plants. *PLoS. Biology., 16*(8). doi:10.1371/journal.pbio.2006026

Growth of spoilage bacteria during storage and transport of meat. (2016). *EFSA Journal, 14*(6), n/a-n/a. doi:10.2903/j.efsa.2016.4523

Han, C., Goodwine, J., Romero, N., Steck, K. S., Sauer, K., & Doiron, A.(2019) Enzyme encapsulating polymeric nanoparticles: A potential adjunctive therapy in *Pseudomonas aeruginosa* biofilm-associated infection treatment. *Colloids Surf B Biointerfaces, 184*, 110512. doi:10.1016/j.colsurfb.2019.110512

Hans-Curt, F., & Jost, W. (2010). The biofilm matrix. *Nature Reviews Microbiology, 8*(9), 623. doi:10.1038/nrmicro2415

Hans-Curt, F., Jost, W., Ulrich, S., Peter, S., Scott, A. R., & Staffan, K. (2016). Biofilms: an emergent form of bacterial life. *Nature Reviews Microbiology, 14*(9), 563. doi:10.1038/nrmicro.2016.94

Hobley, L., Harkins, C., MacPhee, C. E., Stanley-Wall, N. R., & Albers, S.-V. (2015). Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes. *FEMS Microbiology Reviews, 39*(5), 649-669. doi:10.1093/femsre/fuv015

Kaylie, L. R., Brittni, R. K., & Jeremiah, G. J. (2019). Heme uptake and utilization by Gram-negative bacterial pathogens. *Frontiers in Cellular and Infection Microbiology, 9*. doi:10.3389/fcimb.2019.00081

Mosbahi, K., Wojnowska, M., Albalat, A., & Walker, D. (2018). Bacterial iron acquisition mediated by outer membrane translocation and cleavage of a host protein. *Proceedings of the National Academy of Sciences of the United States of America, 115*(26), 6840-6845. doi:10.1073/pnas.1800672115

Parthasarathy, D. K., & Bryan, N. S. (2012). Sodium nitrite: The “cure” for nitric oxide insufficiency. *Meat Science, 92*(3), 274-279. doi:10.1016/j.meatsci.2012.03.001

Petrova, O. E., Schurr, J. R., Schurr, M. J., & Sauer, K. (2012). Microcolony formation by the opportunistic pathogen *Pseudomonas aeruginosa* requires pyruvate and pyruvate fermentation. *Molecular Microbiology, 86*(4), 819-835. doi:10.1111/mmi.12018

Yang, L., Barken, K. B., Skindersoe, M. E., Christensen, A. B., Givskov, M., & Tolker-Nielsen, T. (2007). Effects of iron on DNA release and biofilm development by

Pseudomonas aeruginosa. *Microbiology (Reading, England), 153*(5), 1318-1328. doi:10.1099/mic.0.2006/004911-0

Zhu, X., Rice, S. A., & Barraud, N. (2019). Nitric Oxide and iron signaling cues have opposing effects on biofilm development in *Pseudomonas aeruginosa. Applied and Environmental Microbiology, 85*(3). doi:10.1128/AEM.02175-18

APPENDIX 01

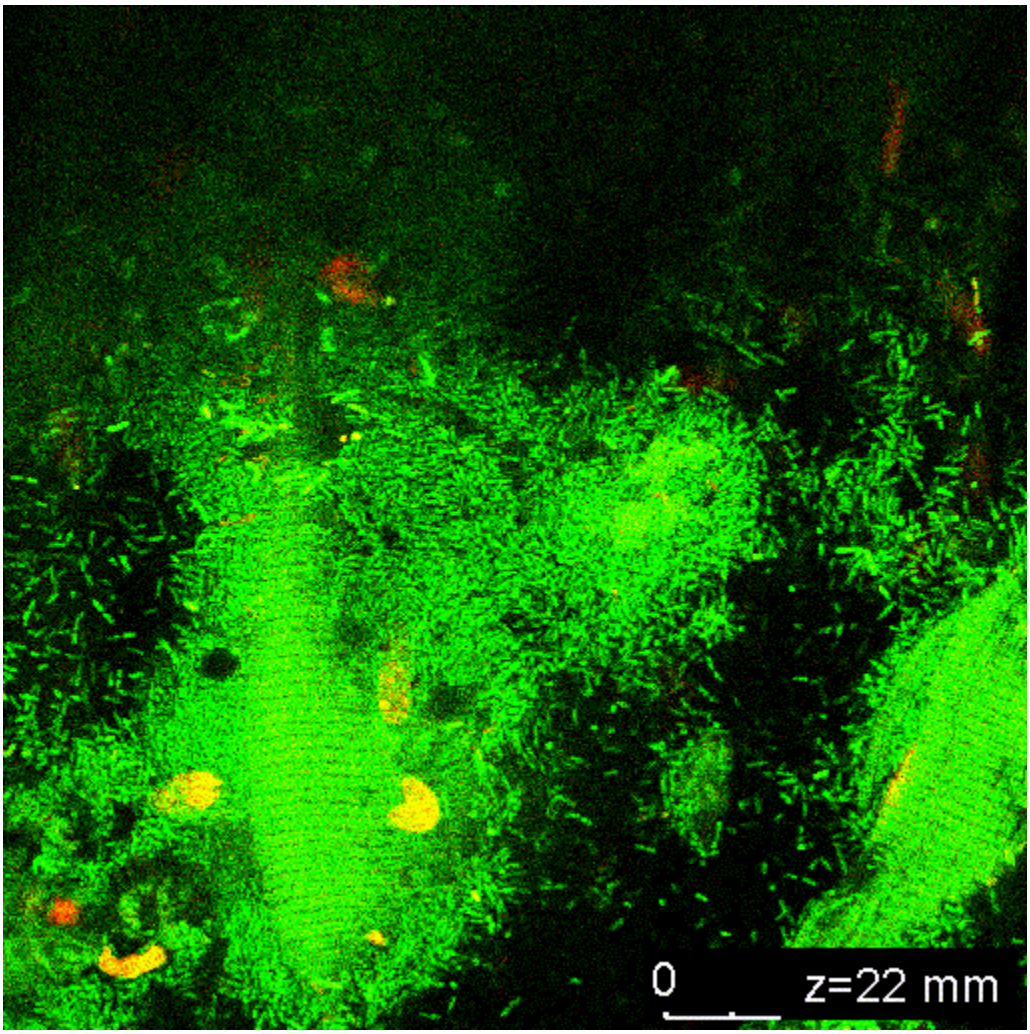


Figure S1 Moving planktonic bacteria during biofilm growth on chilled beef

APPENDIX 02

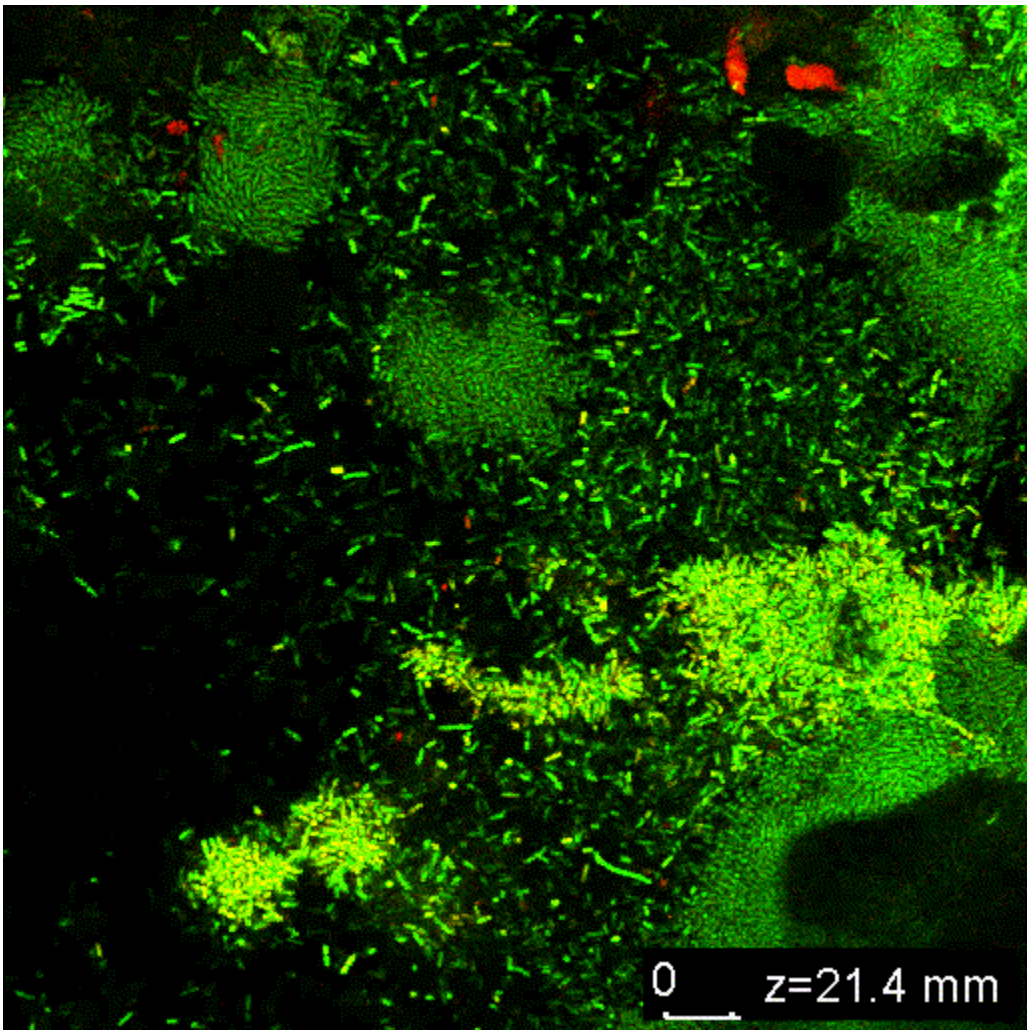


Figure S2 Moving planktonic bacteria during biofilm growth on chilled beef

APPENDIX 03

Illumina HiSeq 2500 sequencing results

HiSeq 2500 Run Results

Next-Generation Sequencing Service

Contact: Marisa Carta
Phone: 02 4570 1784
Email: M.Carta@westernsydney.edu.au

WESTERN SYDNEY UNIVERSITY

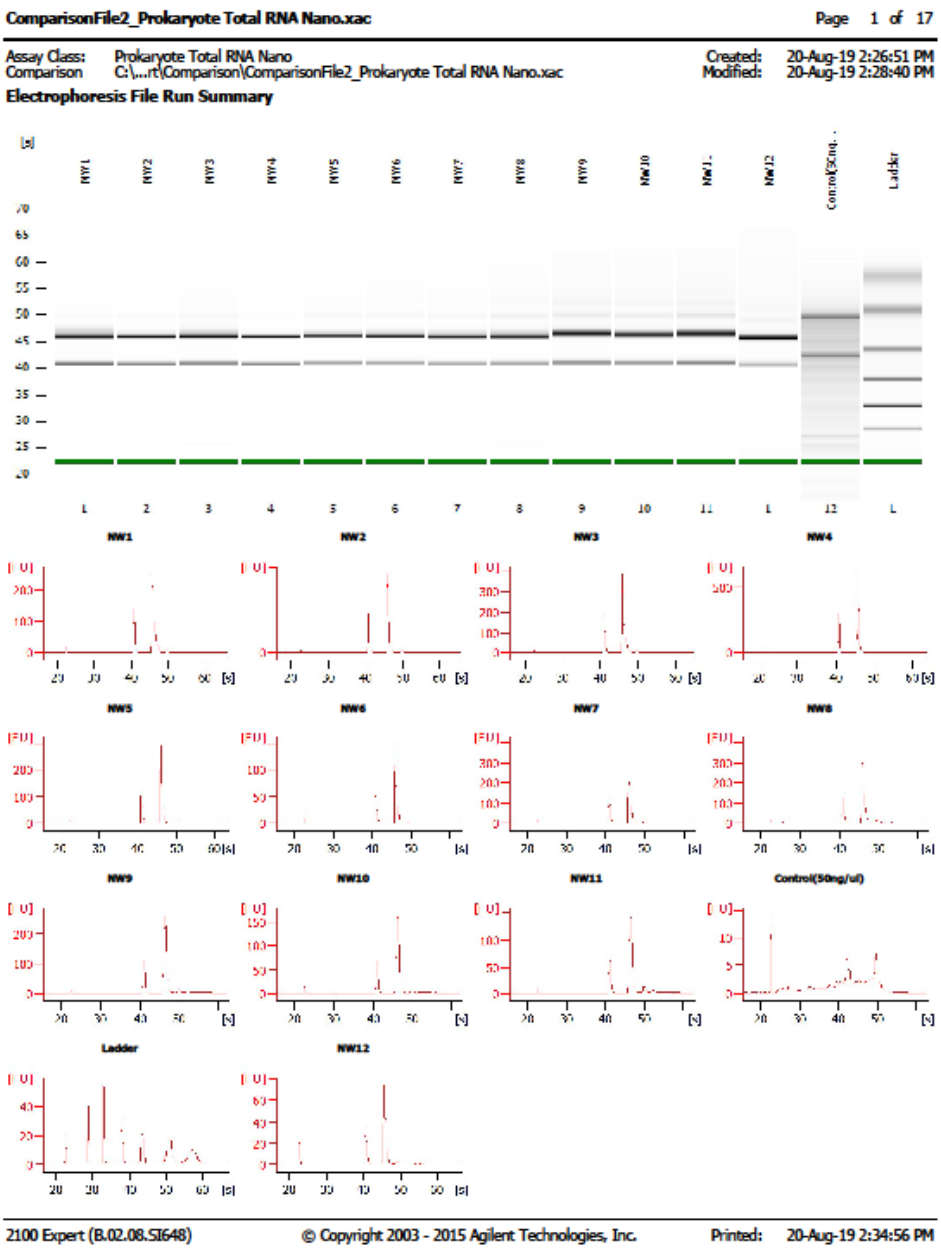
Date: 4th November, 2019

Sequencing Information	
Sequencing date	31/10/2019
Customer Name	Nirmani Wickramasinghe
Library type	Zymo-Seq Ribofree Total RNA
Read length	2 x 126 bp
Run type	Rapid Run, v2 chemistry
PhiX spike-in	5 %
Total PF reads for sequencing run	825,049,096
Total sequence yield for project (Gb)	108.91

Lane	Sample ID	Customer ID	Index 1 (i7)	Index 2 (i5)	% Read Identified (PF)	Number of reads (PF)	Project
1	NW1	NW 1	CGATGTAT	TCTTTCCC	6.9577	28,633,695	20191031_NW12
1	NW2	NW 2	TGACCAAT	TCTTTCCC	7.3644	30,307,427	20191031_NW12
1	NW3	NW 3	ACAGTGAT	TCTTTCCC	6.9376	28,550,975	20191031_NW12
1	NW4	NW 4	GCCAATAT	TCTTTCCC	7.4902	30,825,144	20191031_NW12
1	NW5	NW 5	CAGATCAT	TCTTTCCC	8.0086	32,958,565	20191031_NW12
1	NW6	NW 6	CTTGTAAT	TCTTTCCC	7.0317	28,938,234	20191031_NW12
1	NW7	NW 7	CCGCGGTT	AGCGCTAG	6.8539	28,206,517	20191031_NW12
1	NW8	NW 8	TTATAACC	GATATCGA	8.8896	36,584,230	20191031_NW12
1	NW9	NW 9	GGACTTGG	CGCAGACG	8.3915	34,534,351	20191031_NW12
1	NW10	NW 10	AAGTCCAA	TATGAGTA	7.2989	30,037,868	20191031_NW12
1	NW11	NW 11	ATCCACTG	AGGTGCGT	6.034	24,832,303	20191031_NW12
1	NW12	NW 12	GCTTGTCA	GAACATAC	8.1518	33,547,890	20191031_NW12
2	NW1	NW 1	CGATGTAT	TCTTTCCC	6.9592	28,776,949	20191031_NW12
2	NW2	NW 2	TGACCAAT	TCTTTCCC	7.3628	30,445,873	20191031_NW12
2	NW3	NW 3	ACAGTGAT	TCTTTCCC	6.9443	28,715,336	20191031_NW12
2	NW4	NW 4	GCCAATAT	TCTTTCCC	7.4869	30,959,038	20191031_NW12
2	NW5	NW 5	CAGATCAT	TCTTTCCC	8.0096	33,120,452	20191031_NW12
2	NW6	NW 6	CTTGTAAT	TCTTTCCC	7.0339	29,085,840	20191031_NW12
2	NW7	NW 7	CCGCGGTT	AGCGCTAG	6.8686	28,402,309	20191031_NW12
2	NW8	NW 8	TTATAACC	GATATCGA	8.8767	36,705,992	20191031_NW12

APPENDIX 04

Quality reports of extracted total RNA from biofilms formed on chilled beef



APPENDIX 05

Table S1. Primers used for selected genes in rt-QPCR

Locus tag	Forward primer	Reverse primer
CJU75_19875	CCTCTGGGGTGATGATGCAATA	ACTCGAAGGCGACCCCCATA
CJU75_22245	GCGTTCCTTCGCGGATATCA	TCGTTGAGTCTGTGGATGCC
CJU75_06635	TCTGGAACAGGCAGGGATCA	TTTCATCCCCCACACCCGAG
CJU75_06635	TCTGGAACAGGCAGGGATCA	TTTCATCCCCCACACCCGAG
CJU75_22245	CCTGACTGAAGAAACCCGCA	CGCTCTTCGTCTTCGCTGAT
CJU75_06450	TATAGCACTGTCGGCCCTCT	GCATAAGCAAATCCCTGGGC
CJU75_09520	GTCCGAATTGGTTGAGAGCGT	TGCCAGTTTGAGTTTCAGCGT
CJU75_01795	GGGTGATGTGGTGTTTGTGC	ATGGGTGAGGCATTGACCAG
CJU75_17410	ATGCAACTCAGGGTGCCAAT	CCAACTGCACGTTATCCAGG
CJU75_13985	AGGACGAAGCAACCATCCTG	GGGTTTTGCAGGCGGAATTT
CJU75_03765	GTGCCATAGAAAACCATGTCCG	CAAAGGTGTTTTCGGAGTCGC
16S rRNA gene	GTCACCGGCAGTCTCCTTAG	ATTGGTGCCTTCGGGAACAT